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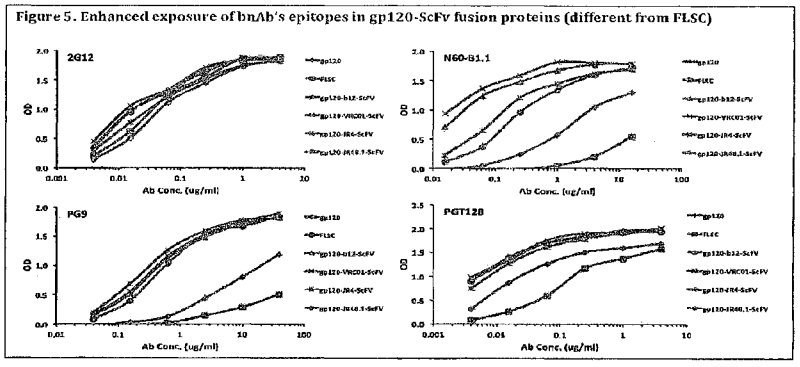
(54) **Title**: HIV-1 ENV-BINDING ANTIBODIES, FUSION PROTEINS, AND METHODS OF USE



(57) **Abstract**: Fusion proteins comprising a portion of the HIV-1 Env protein and ScFv of an enhancing antibody are disclosed that may serve in immunogenic formulations for vaccination against HIV- 1 infection. A broadly neutralizing antibody and engineered bi-/tri- specific anti-HIV-1 antibodies that may serve in the prevention and/or treatment of HIV-1 infections in a subject are also disclosed, as well as methods of using the fusions proteins and antibodies.

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HIV-1 ENV-BINDING ANTIBODIES, FUSION PROTEINS, AND METHODS OF USE

STATEMENT OF FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0001] This invention was made with government support under Grant Numbers AI087181, AI084830, CA149196 and AI084580 awarded by the NIH. The government has certain rights in the invention.

TECHNICAL FIELD

[0002] The present invention is directed to the fields of immunology and virology. More particularly, the invention is directed to novel fusion proteins that may serve in immunogenic formulations against HIV-1 infection and to a novel broadly neutralizing antibody that may serve in the prevention and/or treatment of HIV-1 infections in a subject.

BACKGROUND OF INVENTION

[0003] It is well established that neutralizing antibodies (nAbs) against the HIV-1 envelope protein (Env) can be protective, as was demonstrated by passive immunization studies in non-human primates [1-3]. Therefore, the isolation of broadly nAbs (bnAbs) that can neutralize diversified HIV-1 strains is being pursued as a major path to the rational design of an effective HIV-1 vaccine [4].

[0004] Large numbers of potent new bnAbs have been recently isolated from multiple donors [5-11], mainly due to new technologies for isolating human monoclonal antibodies (mAbs) and the employment of a high-throughput neutralization screen system [5, 12-15]. Four types of new potent HIV-1 bnAbs have been identified. 1) The newly isolated 'VRC01-like' bnAbs [7, 8, 10] can block Env binding to primary receptor CD4 and they are superior to the previous CD4 binding site (CD4bs) nAb b12 [16]. 2) The newly isolated PGT series bnAbs [9] are glycan dependent and much more potent and broadly active than the old glycan-dependent nAb 2G12 [17], which can block Env binding to co-receptor CCR5. 3) The so-called 'PG9-like' bnAbs bind conserved conformational epitopes on the Env trimer of HIV-1 involving the V1/V2 and V3 regions and glycan, including PG9, PG16 [5] and CH01~04 [11]. They are superior in breadth compared to previously identified strain-specific potent nAbs that recognize quaternary

neutralizing epitopes (QNE) of HIV-1 Env [18, 19]. 4) The new isolated bnAb 10E8 targets the membrane proximal external region (MPER) of gp41 and is much more potent and broadly active than the old MPER nAbs 2F5 and 4E10 (J. Huang et al., *Nature* (2012) 491: 406–412).

[0005] These new bnAbs collectively neutralize the majority of the highly diversified HIV-1 strains [9]. However, these bnAbs also demonstrate some unusual features that may be major roadblocks for HIV-1 vaccine development. These highly mutated bnAbs were isolated from rare (<3%) "elite neutralizers" that were selected from more than a thousand HIV-1-infected persons [5, 11, 15]. Compared with the degree of somatic mutation in other Ab responses [13, 20], a common feature of these HIV-1 bnAbs is their apparent unusually high levels of mutation, especially in the heavy chain V (VH) genes [5-11]. This high level of mutation raises a high bar for a vaccine to generate this type of Ab response, which may be a major hurdle for HIV-1 vaccine development. In addition, some of these bnAbs showed auto/poly-reactivity [7, 8, 10], which is detrimental for B cell response. Currently, no HIV-1 vaccine candidates can elicit bnAb responses [4].

[0006] Thus, there continues to be a great need for HIV-1 vaccine candidates that can elicit bnAb responses, as well as potent HIV-1 bnAbs with improved characteristics.

BRIEF SUMMARY OF INVENTION

[0007] The present invention provides (i) fusion protein immunogens comprising a common transitional conformation structure of HIV-1 Env that may be used in vaccine formulations, and (ii) an HIV-1 broadly neutralizing human monoclonal antibody (N60-B1.1). In additional, the invention provides vaccine formulations, and methods of utilizing the Env immunogens and the antibody in a variety of manners.

[0008] In a first embodiment, the present invention is directed to fusion proteins comprising a portion of the HIV-1 Env polypeptide and single-chain fragment V regions of enhancing antibodies. These Env fusion proteins include: gp120-b12-ScFV, gp120-VRC01-ScFV, gp120-JR4-ScFV and gp120-JR48.1-ScFV. Thus, in aspects of this embodiment, the invention is directed to: the gp120-b12-ScFV polypeptide comprising the amino acid sequence set forth in SEQ ID NO:8; the gp120-VRC01-ScFV polypeptide comprising the amino acid sequence set forth in SEQ ID NO:10; the gp120-JR4-ScFV polypeptide comprising the amino acid sequence

set forth in SEQ ID NO:12; and the gp120-JR48.1-ScFV polypeptide comprising the amino acid sequence set forth in SEQ ID NO:14.

[0009] In related aspects, the invention is directed to: the polynucleotide sequence encoding the gp120-b12-ScFV polypeptide set forth in SEQ ID NO:7; the polynucleotide sequence encoding the gp120-VRC01-ScFV polypeptide set forth in SEQ ID NO:9; the polynucleotide sequence encoding the gp120-JR4-ScFV polypeptide set forth in SEQ ID NO:11; and the polynucleotide sequence encoding the gp120-JR48.1-ScFV polypeptide set forth in SEQ ID NO:13.

[0010] In further related aspects, the invention is directed to variants of gp120-b12-ScFV, gp120-VRC01-ScFV, gp120-JR4-ScFV and gp120-JR48.1-ScFV. For example, the present invention encompasses: a variant having at least about 95% sequence identity over the full length of the gp120-b12-ScFV polypeptide as set forth in SEQ ID NO:8; a variant having at least about 95% sequence identity over the full length of the gp120-VRC01-ScFV polypeptide as set forth in SEQ ID NO:10; a variant having at least about 95% sequence identity over the full length of the gp120-JR4-ScFV polypeptide as set forth in SEQ ID NO:12; and a variant having at least about 95% sequence identity over the full length of the gp120-JR48.1-ScFV polypeptide as set forth in SEQ ID NO:14. As another example, the invention encompasses variants having at least about 95% sequence identity within only one or two of the domains of the gp120-b12-ScFV, gp120-VRC01-ScFV, gp120-JR4-ScFV and gp120-JR48.1-ScFV polypeptides. For example, the amino acid changes may be limited to one or more of: (i) the HIV-1 Env peptide domain of the polypeptide, (ii) the linker domain of the polypeptide, and (iii) the ScFV domain of the polypeptide. In each of the variants, the amino acid changes may be one or more of additions, substitutions or deletions, and the type of changes may vary by the domain of a particular variant polypeptide.

[0011] In a second embodiment, the present invention is directed to immunogenic formulations comprising one or more of the Env fusion protein immunogens gp120-b12-ScFV, gp120-VRC01-ScFV, gp120-JR4-ScFV and gp120-JR48.1-ScFV, one or more variants thereof, and a pharmaceutically acceptable carrier and/or adjuvant. Thus, in one aspect, the invention is directed to an immunogenic formulation comprising: (a) one or more of the polypeptides gp120-b12-ScFV (SEQ ID NO:8), gp120-VRC01-ScFV (SEQ ID NO:10), gp120-JR4-ScFV (SEQ ID NO:12) and gp120-JR48.1-ScFV (SEQ ID NO:14) and a pharmaceutically acceptable carrier

and/or adjuvant, or (b) the formulation of (a) wherein at least one of the polypeptides is a variant and wherein the variant has at least about 95% sequence identity with the full length reference sequence. In related aspects, the invention is directed to an immunogenic formulation comprising two or more of the polypeptides or variants thereof, three or more of the polypeptides or variants thereof, and each of the polypeptides or variants thereof. In an exemplary, but not limiting aspect, the invention is directed to an immunogenic formulation comprising each of the polypeptides gp120-b12-ScFV (SEQ ID NO:8), gp120-VRC01-ScFV (SEQ ID NO:10), gp120-JR4-ScFV (SEQ ID NO:12) and gp120-JR48.1-ScFV (SEQ ID NO:14) and a pharmaceutically acceptable carrier and/or adjuvant.

[0012] In a third embodiment, the invention is directed to methods of using the Env fusion protein immunogens gp120-b12-ScFV, gp120-VRC01-ScFV, gp120-JR4-ScFV and gp120-JR48.1-ScFV, variants thereof, and immunogenic formulations comprising the polypeptides and variants in all possible combinations in the generation of an immune response in a subject. In one aspect of this embodiment, the invention is directed to a method of generating an immune response in a subject comprising administering an immunologically effective amount of an immunogenic formulation as defined herein to a subject, thereby generating an immune response in a subject. In a related aspect, the invention is directed to a method of generating a protective immune response in a subject comprising administering an immunologically effective amount of an immunogenic formulation as defined herein to a subject, thereby generating a protective immune response in a subject.

[0013] In a fourth embodiment, the present invention is directed to a novel antibody that exhibits binding specificity for the HIV-1 Env polypeptide. This antibody, termed N60-B1.1 herein, comprises a VK domain having the amino acid sequence set forth in SEQ ID NO:2 and a VH domain having the amino acid sequence set forth in SEQ ID NO:4. The invention is also directed to fragments of N60-B1.1 that maintain the ability to bind the HIV-1 Env polypeptide.

[0014] In one aspect of this embodiment, the invention is directed to an isolated antibody or an HIV-1 Env protein binding fragment thereof, wherein the antibody comprises: (a) a VK domain comprising the amino acid sequence of SEQ ID NO:2, (b) a VH domain comprising the amino acid sequence of SEQ ID NO:4, (c) a VK domain comprising an amino acid sequence with at least about 95% sequence identity to SEQ ID NO:2, or (d) a VH domain comprising an amino acid sequence with at least about 95% sequence identity to SEQ ID NO:4. In particular

aspects, the invention is directed to (i) an isolated antibody or an HIV-1 Env protein binding fragment thereof wherein the antibody comprises a VK domain having the amino acid sequence of SEQ ID NO:2 and a VH domain having the amino acid sequence of SEQ ID NO:4; (ii) an isolated antibody or an HIV-1 Env protein binding fragment thereof wherein the antibody comprises a VK domain comprising the amino acid sequence of SEQ ID NO:2 and a VH domain comprising an amino acid sequence with at least about 95% sequence identity to SEQ ID NO:4; (iii) an isolated antibody or an HIV-1 Env protein binding fragment thereof wherein the antibody comprises a VK domain comprising an amino acid sequence with at least about 95% sequence identity to SEQ ID NO:2 and a VH domain comprising the amino acid sequence of SEQ ID NO:4; and (iv) an isolated antibody or an HIV-1 Env protein binding fragment thereof wherein the antibody comprises a VK domain comprising an amino acid sequence with at least about 95% sequence identity to SEQ ID NO:2 and a VH domain comprising an amino acid sequence with at least about 95% sequence identity to SEQ ID NO:4.

[0015] This embodiment of the invention includes an isolated antibody or an HIV-1 Env protein binding fragment thereof wherein the VK domain comprises amino acids 1-95 of SEQ ID NO:2 or the VH domain comprises amino acids 1-102 of SEQ ID NO:4, as well as an isolated antibody or an HIV-1 Env protein binding fragment thereof wherein the VK domain comprises amino acids 1-95 of SEQ ID NO:2 and the VH domain comprises amino acids 1-102 of SEQ ID NO:4.

[0016] In exemplary, but not limiting aspects of this embodiment, the antibodies have binding specificity for HIV-1 Env protein; the antibody is a fully human antibody or a chimeric antibody; the antibody is a monoclonal antibody; the antibody is a recombinant antibody.

[0017] In aspects of this embodiment directed to the HIV-1 Env protein binding fragments of the antibodies, the fragment may be, but is not limited to, a Fab fragment, $F(ab')_2$ fragment, and single chain Fv (scFv).

[0018] This embodiment includes pharmaceutical formulations comprising the antibodies and/or HIV-1 Env protein binding fragments thereof and a pharmaceutically acceptable carrier.

[0019] In a fifth embodiment, the present invention is directed to engineered bi- and tri-specific antibodies. Such engineered antibodies have binding affinity for two or even three different epitopes. The antigen binding domains of two or three different antibodies can be combined on a constant region frame to create bi- and tri-specific antibodies. As an example, the

antigen binding domains of the following antibodies can be used: (i) bnAbs against glycan-dependent V1V2 and V3 related regions, e.g., N60-B1.1, PG9 and PGT128; (ii) bnAbs against CD4bs, e.g., b12 and VRC01; (iii) ADCC antibodies against the cluster A region of gp120, e.g., JR4 and JR48.1. The creation and use of such bi- and tri-specific antibodies permit simultaneous and synergetic binding to gp120, for example.

[0020] In a sixth embodiment, the invention is directed to methods of using antibodies and fragments thereof in the treatment or prevention of HIV-1 infection in a subject. Thus, in one aspect the invention is directed to a method of preventing an HIV-1 infection in a subject, comprising administering a therapeutically effective amount of a pharmaceutical formulation comprising one or more of the antibodies and antibodies fragments as defined herein to a subject at risk of developing an HIV-1 infection, thereby inhibiting an HIV-1 infection in a subject. In a related aspect, the invention is directed to a method of treating an HIV-1 infection in a subject, comprising administering a therapeutically effective amount of a pharmaceutical formulation comprising one or more of the antibodies and antibodies fragments as defined herein to a subject having an HIV-1 infection, thereby treating an HIV-1 infection in a subject.

[0021] The foregoing has outlined rather broadly the features and technical advantages of the present invention in order that the detailed description of the invention that follows may be better understood. Additional features and advantages of the invention will be described herein, which form the subject matter of the claims of the invention.

BRIEF DESCRIPTION OF DRAWINGS

[0022] Figure 1. A sequence alignment of the light (VK) and heavy (VH) chain variable regions of clones N60-B1.1, N60-B1.2 (HGKV3-15*01) and IGHV4-39*01 is provided. The VK chain of N60-B1.1 comprises amino acids 1-95 of SEQ ID NO:2. The amino acid sequence of the N60-B1.2 (HGKV3-15*01) VK chain (un-mutated germline VK) is provided in SEQ ID NO:5. The VH chain of N60-B1.1 comprises amino acids 1-102 of SEQ ID NO:4. The amino acid sequence of the IGHV4-39*01 VH chain (un-mutated germline VH) is provided in SEQ ID NO:6. Non-matching residues are shown in bold below the lower sequences of the alignments. The framework (FR) and cluster complementarity determining regions (CDRs) are indicated above the alignments.

[0023] Figure 2. The results of a comparison of the binding abilities of several different antibodies to monomeric gp120 and variants thereof are provided. The variants include those having mutations in the V1/V2 loop (dV1V2), those having mutations in the co-receptor binding site (CoRbs, I420R), and those having mutations in the V3 loop (dV3). In addition to N60-B1.1, antibodies of the clones N60-B1.2, 697-3D, b12, 17b, and N12-O3.1.

[0024] Figures 3A-C. The four panels of Figure 3A demonstrate the lack of N60-B1.1 auto-reactivity where the antibody was cultured with dsDNA, insulin, ssDNA and lipopolysaccharide (LPS) and compared to the bnAbs 4E10, 2F5, PG9 and PG16. Figure 3B illustrates the *in vivo* protective effect of N60-B1.1 in a rhesus macaque model. Figure 3C provides results demonstrating that sterilizing immunity against the intra-rectal challenge of SHIV162P3 can be achieved in the rhesus macaque model.

[0025] Figure 4A-C. The three panels in the figure show the results of competition binding studies of N60-B1.1 and other antibodies against gp120.

[0026] Figure 5. The four panels illustrate the enhanced exposure of bnAb epitopes in gp120-ScFv fusion proteins.

[0027] Figure 6. The four panels illustrate the enhanced exposure of ADCC Ab epitopes in gp120-b12/VRC01 ScFv fusion proteins.

[0028] Figure 7. This figure provides examples of bi- and tri-specific HIV-1 protective Abs.

DETAILED DESCRIPTION OF THE INVENTION

[0029] Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found, for example, in Benjamin Lewin, Genes VII, published by Oxford University Press, 2000 (ISBN 019879276X); Kendrew et al. (eds.); The Encyclopedia of Molecular Biology, published by Blackwell Publishers, 1994 (ISBN 0632021829); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by Wiley, John & Sons, Inc., 1995 (ISBN 0471186341); and other similar technical references.

[0030] As used herein, "a" or "an" may mean one or more. As used herein when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more. Furthermore, unless otherwise required by context, singular terms include pluralities and plural terms include the singular.

[0031] As used herein, "about" refers to a numeric value, including, for example, whole numbers, fractions, and percentages, whether or not explicitly indicated. The term "about" generally refers to a range of numerical values (e.g., +/- 5-10% of the recited value) that one of ordinary skill in the art would consider equivalent to the recited value (e.g., having the same function or result). In some instances, the term "about" may include numerical values that are rounded to the nearest significant figure.

[0032] As suggested above, the present invention is based on the discovery by the inventors of the broadly neutralizing antibody (nAb) N60-B1.1 that exhibits binding specificity to the HIV-1 envelope protein (Env). Through the diligent efforts of the inventors to characterize this antibody, it was found, as shown below, that the antibody has protective activity in a passive immunization study in non-human primates. Characterization of the antibody further led the inventors to prepare four different fusion proteins that comprising portions of the Env protein and that are likely to serve as a potent antigens in immunogenic formulations against HIV-1 infection. The present invention is directed to the N60-B1.1 antibody, bi-/tri-specific engineered antibodies, Env fusion protein immunogens, and formulations comprising the same, and methods for using the same.

Antibodies

[0033] Details regarding the discovery and characterization of the N60-B1.1 antibody are provided below. However, the antibody can be generally characterized as comprising a VK domain having the amino acid sequence set forth in SEQ ID NO:2 and a VH domain having the amino acid sequence set forth in SEQ ID NO:4, wherein the antibody exhibits binding specificity for HIV-1 Env protein. Thus, the invention is directed, in part, to an isolated antibody comprising: (a) a VK domain comprising the amino acid sequence of SEQ ID NO:2, (b) a VH domain comprising the amino acid sequence of SEQ ID NO:4, or (c) both a VK domain comprising the amino acid sequence of SEQ ID NO:2 and a VH domain comprising the amino acid sequence of SEQ ID NO:4. The antibodies are not limited with respect to other characteristics. For example, the antibodies may be of any class, such as IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgD or IgE. The antibodies may be polyclonal, monoclonal, humanized or chimeric antibodies, and the antibodies may be in the form of an antiserum comprising the

antibodies. The antibodies may be isolated antibodies, purified antibodies, exogenous antibodies, endogenous antibodies, or a combination thereof.

[0034] It will be readily understood by the skilled artisan that variations can be made to the antibodies of the present invention, for example, alterations can be made to either or both the VK and VH domains, while maintaining the binding activity and/or binding specificity of the antibody. Thus, the present invention includes variants of the antibodies defined herein that include one or more of amino acid insertions, deletions and substitutions and yet retain binding specificity for HIV-1 Env protein. In particular, the invention includes antibody variants where the VK domain has at least about 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity over the entire length of the amino acid sequence set forth in SEQ ID NO:2 and the VH domain comprises the amino acid sequence of SEQ ID NO:4. The invention also includes antibody variants where the VK domain comprises the amino acid sequence of SEQ ID NO:2 and the VH domain has at least about 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity over the entire length of the amino acid sequence set forth in SEQ ID NO:4. The invention further includes antibody variants where the VK domain has at least about 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity over the entire length of the amino acid sequence set forth in SEQ ID NO:2 and the VH domain has at least about 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity over the entire length of the amino acid sequence set forth in SEQ ID NO:4. In a non-limiting example, the invention includes an antibody variant where the VK domain has at least about 95% sequence identity over the entire length of the amino acid sequence set forth in SEQ ID NO:2 and the VH domain has at least about 95% sequence identity over the entire length of the amino acid sequence set forth in SEQ ID NO:4.

[0035] The invention also includes isolated antibodies having binding specificity for HIV-1 Env protein where the VK domain comprises amino acids 1-95 of SEQ ID NO:2 or the VH domain comprises amino acids 1-102 of SEQ ID NO:4, as well as an isolated antibody where the VK domain comprises amino acids 1-95 of SEQ ID NO:2 and the VH domain comprises amino acids 1-102 of SEQ ID NO:4.

[0036] The antibodies encompassed within the scope of the invention include fully human antibodies, humanized antibodies, as well as chimeric antibodies. The antibodies may be monoclonal or polyclonal. Further, the antibody may be a recombinant antibody.

[0037] The present invention also encompasses fragments of the antibodies defined herein that retain the ability to bind HIV-1 Env protein. The fragments included, but are not limited to, Fab fragments, $F(ab')_2$ fragments, single chain Fv (scFv) antibodies, and fragments produced by an Fab expression library, as well as bi-specific antibody and triple-specific antibodies. It will thus be clear to the skilled artisan that all references to "antibodies" herein include both full-size antibodies as well as antibody fragments, as defined herein.

[0038] The antibodies may be produced in any species of animal, though preferably from a mammal such as a human, simian, mouse, rat, rabbit, guinea pig, horse, cow, sheep, goat, pig, dog or cat. For example, the antibodies can be human antibodies or humanized antibodies, or any antibody preparation suitable for administration to a human. For the production of the antibodies, the selected species of animal can be immunized by injection with one or more antigens, e.g., the Env fusion proteins or variants discussed herein. The antigens may be administered in conjunction with one or more pharmaceutically acceptable adjuvants to increase the immunological response. Suitable adjuvants include, but are not limited to, Freund's Complete and Incomplete Adjuvant, Titermax, Oil in Water adjuvants, as well as aluminum compounds where antigens, normally peptides, are physically precipitated with hydrated insoluble salts of aluminum hydroxide or aluminum phosphate. Other adjuvants include liposome-type adjuvants comprising spheres having phospholipid bilayers that form an aqueous compartment containing the antigen and protect it from rapid degradation, and that provide a depot effect for sustained release. Surface active agents may also be used as adjuvants and include lipoteichoic acid of gram-positive organisms, lipid A, and TDM. Quil A and QS-21 (saponin-type adjuvants), monophosphoryl lipid A, and lipophilic MDP derivatives are suitable adjuvants that have hydrophilic and hydrophobic domains from which their surface-active properties arise. Compounds normally found in the body such as vitamin A and E, and lysolecithin may also be used as surface-active agents. Other classes of adjuvants include glycan analog, coenzyme Q, amphotericin B, dimethyldioctadecylammonium bromide (DDA), levamisole, and benzimidazole compounds. The immunostimulation provided by a surface active agent may also be accomplished by either developing a fusion protein with non-active portions of the cholera toxin,

exotoxin A, or the heat labile toxin from *E. coli*. Immunomodulation through the use of anti-IL-17, anti IFN-γ, anti-IL-12, IL-2, IL-10, or IL-4 may also be used to promote a strong Th2 or antibody mediated response to the immunogenic formulation.

[0039] Means for preparing antibodies are very well known in the art. The antibodies of the invention can be prepared using any known technique that provides for the production of antibody molecules. Suitable techniques include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (Nature 256:495-497 (1975)), the human B-cell hybridoma technique (Kosbor et al., Immunol Today 4:72 (1983); Cote et al., Proc Natl. Acad. Sci 80:2026-2030 (1983)), and the EBV-hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss Inc, New York N.Y., pp 77-96 (1985)). Each of these publications is herein incorporated by reference in its entirety. Additionally, antibodies can be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi et al., *Proc Natl. Acad. Sci. USA* 86: 3833-3837 (1989), and in Winter G. and Milstein C., *Nature* 349:293-299 (1991), both of which is herein incorporated by reference in its entirety.

[0040] Humanized antibodies are those where a human antibody has been engineered to contain non-human complementarity-determining regions (CDRs) derived from an antibody produced in a non-human host against a selected antigen. Means for producing humanized antibodies are well-known in the art and include Vaswani SK, and Hamilton RG, *Ann Allergy Asthma Immunol.* 81(2):105-15 (1998) and Kashmiri SV et al., *Methods* 36 (1):25–34 (2005), each of which is herein incorporated by reference in its entirety.

[0041] Chimeric antibodies are those where an antigen binding region (e.g., $F(ab')_2$ or hypervariable region) of a non-human antibody is transferred into the framework of a human antibody by recombinant DNA techniques. Techniques developed for the production of such antibodies include the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity. Such techniques are also well known and include: Morrison et al., *Proc Natl. Acad. Sci* 81:6851-6855 (1984); Neuberger et al., *Nature* 312:604-608(1984); Takeda et al., *Nature* 314:452-454(1985), each of which is herein incorporated by reference in its entirety.

[0042] Techniques for the production of single chain antibodies are described in in U.S. Patent No. 4,946,778, incorporated herein by reference in its entirety.

[0043] Antibody fragments such as $F(ab')_2$ fragments can be produced by pepsin digestion of the antibody molecule, and Fab fragments can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries can be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse W. D. et al., Science 256:1275-1281 (1989), herein incorporated by reference in its entirety).

[0044] The bi- and tri-specific (or triple-specific) antibodies of the present invention are recombinant engineered antibodies such as those shown in Figure 7. These antibodies can bind two (bi-specific) or three (tri-specific) different epitopes. The antigen binding domains of two or three different antibodies are combined on a constant region frame to create bi- and tri-specific antibodies. As an example, the antigen binding domains of the following antibodies can be used: (i) bnAbs against glycan-dependent V1V2 and V3 related regions, e.g., N60-B1.1, PG9 and PGT128; (ii) bnAbs against CD4bs, e.g., b12 and VRC01; (iii) ADCC antibodies against the cluster A region of gp120, e.g., JR4 and JR48.1. Similarly, the constant region of any of these antibodies can be the frame that is engineered to contain the noted antigen binding domains. Techniques for production of such antibodies are well known in the art. These antibodies may have increased potency and broader specificity in neutralizing HIV-1 than a mixture of individual Abs. For example, such antibodies may exhibit simultaneous and synergetic binding to gp120.

Antibody Formulations

[0045] The skilled artisan will understand that the antibodies and fragments thereof defined in the present invention can be used in a variety of applications, including methods of treating HIV-1 infection, methods of preventing HIV-1 infections, methods of screening agonists and antagonists of HIV-1 infection, to name only a few. Thus, the invention includes pharmaceutical formulations comprising the antibodies and/or HIV-1 Env protein binding fragments thereof and a pharmaceutically acceptable carrier (also termed antibody formulations herein). The invention also includes a kit comprising one or more of the antibodies and/or HIV-1 Env protein binding fragments thereof and instructions for using the antibodies or fragments.

[0046] The pharmaceutical formulations comprising one or more of the antibodies or fragments of the invention and a pharmaceutically acceptable carrier may be administered to a subject, such as a human, for the treatment or prevention of HIV-1 infection. Suitable examples

of carriers are well known to those skilled in the art and include water, water-for-injection, saline, buffered saline, dextrose, glycerol, ethanol, propylene glycol, polysorbate 80 (Tween-80™), poly(ethylene)glycol 300 and 400 (PEG 300 and 400), PEGylated castor oil (e.g. Cremophor EL), poloxamer 407 and 188, hydrophilic and hydrophobic carriers, and combinations thereof. Hydrophobic carriers include, for example, fat emulsions, lipids, PEGylated phospholipids, polymer matrices, biocompatible polymers, lipospheres, vesicles, particles, and liposomes. The terms specifically exclude cell culture medium. The formulations may further comprise stabilizing agents, buffers, antioxidants and preservatives, tonicity agents, bulking agents, emulsifiers, suspending or viscosity agents, inert diluents, fillers, and combinations thereof.

[0047] The identity of the carrier(s) will also depend on the means used to administer pharmaceutical formulations comprising antibodies to a subject. For example, pharmaceutical formulations for intramuscular preparations can be prepared where the carrier is water-for-injection, 0.9% saline, or 5% glucose solution. Pharmaceutical formulations may also be prepared as liquid or powdered atomized dispersions for delivery by inhalation. Such dispersion typically contain carriers common for atomized or aerosolized dispersions, such as buffered saline and/or other compounds well known to those of skill in the art. The delivery of the pharmaceutical formulations via inhalation has the effect of rapidly dispersing the immunogenic formulation to a large area of mucosal tissues as well as quick absorption by the blood for circulation. One example of a method of preparing an atomized dispersion is described in U.S. Patent No. 6,187,344, entitled, "Powdered Pharmaceutical Formulations Having Improved Dispersibility," which is hereby incorporated by reference in its entirety.

[0048] Additionally, the pharmaceutical formulations may also be administered in a liquid form. The liquid can be for oral dosage, for ophthalmic or nasal dosage as drops, or for use as an enema or douche. When the pharmaceutical formulation is formulated as a liquid, the liquid can be either a solution or a suspension of the pharmaceutical formulation. There is a variety of suitable formulations for the solution or suspension of the pharmaceutical formulations that are well known to those of skill in the art, depending on the intended use thereof. Liquid formulations for oral administration prepared in water or other aqueous vehicles may contain various suspending agents such as methylcellulose, alginates, tragacanth, pectin, kelgin, carrageenan, acacia, polyvinylpyrrolidone, and polyvinyl alcohol. The liquid formulations may

also include solutions, emulsions, syrups and elixirs containing, together with the active compound(s), wetting agents, sweeteners, and coloring and flavoring agents.

Env Fusion Proteins

[0049] The invention is also directed to fusion proteins comprising a portion of the HIV-1 Env polypeptide and single-chain fragment V regions of enhancing antibodies and optionally a linker joining these two domains in a fusion protein. These Env fusion proteins include: gp120-b12-ScFV, gp120-VRC01-ScFV, gp120-JR4-ScFV and gp120-JR48.1-ScFV. Thus, the invention is directed to the gp120-b12-ScFV polypeptide comprising the amino acid sequence set forth in SEQ ID NO:8 and the polynucleotide sequence set forth in SEQ ID NO:7 which encodes the protein. The invention is also directed to the gp120-VRC01-ScFV polypeptide comprising the amino acid sequence set forth in SEQ ID NO:10 and the polynucleotide sequence set forth in SEQ ID NO:9 which encodes the protein. The invention is further directed to the gp120-JR4-ScFV polypeptide comprising the amino acid sequence set forth in SEQ ID NO:12 and the polynucleotide sequence set forth in SEQ ID NO:11 which encodes the protein. The invention is additionally directed to the gp120-JR48.1-ScFV polypeptide comprising the amino acid sequence set forth in SEQ ID NO:14 and the polynucleotide sequence set forth in SEQ ID NO:13 which encodes the protein.

[0050] The present invention is not limited to the four noted fusion proteins and variants thereof (discussed below). It encompasses all polypeptides comprising an HIV-1 Env peptide domain and a ScFV domain of an enhancing antibody, and an optional linker peptide domain for connecting these domains. The HIV-1 Env peptide domain will generally encompass amino acids of gp120 (amino acids 1-509 of SEQ ID NO:14) and gp140 (amino acids 1-693 of SEQ ID NO:14) of the HIV-1 Env polypeptide. The linker domain may be a GSA linker [27], such as GSSGGGGSGSGGGGSGGGAAA (SEQ ID NO:15) as a non-limiting example. Acceptable enhancing antibodies include, but are not limited to, CD4bs bnAbs (e.g., b12 [16], VRC01 [7]) as well as cluster A ADCC mAbs (e.g., JR4, JR48.1 [28]). The ScFV domain generally comprises a heavy chain variable domain and a light chain variable domain with a 15 amino acid GS linker (GGGGSGGGGSGGGGS; SEQ ID NO:16).

[0051] As with the antibodies above, the invention is also directed to variants of gp120-b12-ScFV, gp120-VRC01-ScFV, gp120-JR4-ScFV and gp120-JR48.1-ScFV. The skilled artisan will

readily appreciate that minor changes can be made to the fusion protein without altering the ability of the proteins to induce an immune response. Thus, the invention includes variants of the fusion proteins defined herein that include one or more of amino acid insertions, deletions and substitutions. In particular, the invention includes fusion protein variants having at least about 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity over the entire length with one of the four fusion proteins defined herein (i.e., gp120-b12-ScFV, gp120-VRC01-ScFV, gp120-JR4-ScFV and gp120-JR48.1-ScFV).

[0052] The invention also encompasses variants having about 80% or greater sequence identity within only one or two of the domains of the gp120-b12-ScFV, gp120-VRC01-ScFV, gp120-JR4-ScFV and gp120-JR48.1-ScFV polypeptides. For example, the amino acid changes may be limited to one or more of: (i) the HIV-1 Env peptide domain of the polypeptide, (ii) the linker domain of the polypeptide, and (iii) the ScFV domain of the polypeptide. The skilled artisan will appreciate that the linker domain is an especially suitable location for amino acid changes as changes therein would be expected to have very minor effects on ability of the polypeptide to induce particular immune responses in a subject. As with the changes over the entire length of the polypeptide discussed above, variants of the individual domains include those having at least about 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity over the entire length of a particular domain.

Fusion Protein Formulations

[0053] The Env fusion proteins and variants of the invention can be used to induce an immune response in a subject, such as a human. When the subject is a non-human animal, antibodies can be collected after immunization, processed appropriately, and then used in methods of treatment or prevention in humans. Alternatively, Env fusion proteins and variants of the invention can be administered directly to a human subject or non-human subject to induce endogenous production of therapeutic or protective HIV-1 binding antibodies. The Env fusion proteins and variants can therefore be used in the treatment of a subject suffering from or susceptible to HIV-1 infection. In most instances, the Env fusion proteins and variants will be prepared in an immunogenic formulation comprising one or more of the Env fusion proteins or variants and a pharmaceutically acceptable carrier and/or adjuvant. In a non-limiting example, an immunogenic formulation of the invention comprises: (a) one, two, three or all four of the

polypeptides gp120-b12-ScFV (SEQ ID NO:8), gp120-VRC01-ScFV (SEQ ID NO:10), gp120-JR4-ScFV (SEQ ID NO:12) and gp120-JR48.1-ScFV (SEQ ID NO:14) and a pharmaceutically acceptable carrier and/or adjuvant, or (b) the formulation of (a) wherein at least one of the polypeptides is a variant and wherein the variant has at least about 95% sequence identity with the full length reference sequence. In an exemplary, but not limiting aspect, the invention is directed to an immunogenic formulation comprising each of the polypeptides gp120-b12-ScFV (SEQ ID NO:8), gp120-VRC01-ScFV (SEQ ID NO:10), gp120-JR4-ScFV (SEQ ID NO:12) and gp120-JR48.1-ScFV (SEQ ID NO:14) and a pharmaceutically acceptable carrier and/or adjuvant. Pharmaceutically acceptable carriers and adjuvants are as described above.

Vectors

[0054] As discussed above, the Env fusion proteins and variants that are used to induce production of anti-HIV-1 antibodies can be administered directly to a subject, either "naked" or in the context of an immunogenic formulation. In addition, expression vectors encoding the Env fusion proteins and variants may be administered to the subject, whereupon the encoded fusion proteins and variants are produced, which in turn act as immunogens to induce production of anti-HIV-1 antibodies.

[0055] Thus, the invention also provides expression vectors encoding one or more of the Env fusion proteins of SEQ ID NOs:8, 10, 12 and 14 and/or one or more Env fusion protein variants. A particular vector can encode one or more than one of the fusion proteins and variants. When a single expression vector encodes more than one fusion protein or variant, the coding regions are arranged in 5' to 3' alignment on the vector with suitable spacing between the different coding regions.

[0056] The invention further provides pharmaceutical formulations comprising one or more of the vectors and a pharmaceutically acceptable carrier. In one aspect the invention provides pharmaceutical formulations comprising expression vectors encoding 1, 2, 3 or 4 of the fusion proteins and a pharmaceutically acceptable carrier. Other exemplary formulations include, but are not limited to, formulations comprising expression vectors encoding 1, 2, 3, 4 or more variants.

[0057] The skilled artisan will understand that there is a wide variety of expression vector combinations that may make up the pharmaceutical formulations. For example, a pharmaceutical

formulation may be prepared where all of the expression vectors therein have the same nucleotide sequence. As an illustration, the vector may encode only one of the fusion proteins of SEQ ID NOs:8, 10, 12 and 14, or the same vector may encode two, three or all four of the fusion proteins, arranged in 5' to 3' alignment on the vector with suitable spacing between the different coding regions. Alternatively, a pharmaceutical formulation may be prepared comprise expression vectors of two or more different sequences. Pharmaceutically acceptable carriers are as described above.

[0058] As will be described in detail below, the vectors and pharmaceutical formulations comprising the vectors can be used to induce HIV-1 antibody production in a subject, which in turn can be used in methods of treatment or prevention in the subject. The vectors and pharmaceutical formulations can therefore be used in the treatment of a subject suffering from or susceptible to HIV-1 infection.

Methods of Using Antibodies

[0059] The pharmaceutical formulations comprising the antibodies and/or HIV-1 Env protein binding fragments thereof can be used in a variety of applications. Such applications include, but are not limited to, passive immunization which results in the treatment or prevention of HIV-1 infection in a subject. Thus, the invention is directed to a method of treating an HIV-1 infection in a subject, comprising administering a therapeutically effective amount of a pharmaceutical formulation as defined herein to a subject having an HIV-1 infection, thereby treating an HIV-1 infection in a subject. In a related aspect, the invention includes methods of preventing an HIV-1 infection in a subject, comprising administering a therapeutically effective amount of a pharmaceutical formulation as defined herein to a subject at risk of developing an HIV-1 infection, thereby inhibiting an HIV-1 infection in a subject.

[0060] As used herein, the terms "treat", "treating" and "treatment" have their ordinary and customary meanings, and include one or more of, ameliorating a symptom of HIV-1 infection, blocking or ameliorating a recurrence of a symptom of HIV-1 infection, decreasing in severity and/or frequency a symptom of HIV-1 infection. Treatment means ameliorating, blocking, reducing, decreasing or inhibiting by about 1% to about 100% versus a subject to which the treatment has not been administered. Preferably, the ameliorating, blocking, reducing, decreasing or inhibiting is about 100%, about 99%, about 98%, about 97%, about 96%, about

95%, about 90%, about 80%, about 70%, about 60%, about 50%, about 40%, about 30%, about 20%, about 10%, about 5% or about 1%. The treatment may begin prior to, concurrent with, or after the onset of clinical symptoms of the infection. Thus, the subject may be infected with HIV-1 or merely be susceptible to the infection. The results of the treatment may be permanent or may continue for a period of days (such as 1, 2, 3, 4, 5, 6 or 7 days), weeks (such as 1, 2, 3 or 4 weeks) or months (such as 1, 2, 3, 4, 5, 6 or more months).

[0061] As used herein, the terms "prevent", "preventing" and "prevention" have their ordinary and customary meanings, and include one or more of, stopping, averting, avoiding, alleviating or blocking the occurrence of a symptom of HIV-1 infection, the recurrence of a symptom of HIV-1 infection, the development of HIV-1 infection or the progression of HIV-1 infection. Prevention means stopping by at least about 95% versus a subject to which the prevention has not been administered. Preferably, the stopping is about 100%, about 99%, about 98%, about 97%, about 96% or about 95%. The course of therapy may begin prior to, concurrent with, or after the onset of clinical symptoms of the infection. Thus, the subject may be infected with HIV-1 or merely be susceptible to the infection. The results of the prevention may be permanent or may continue for a period of days (such as 1, 2, 3, 4, 5, 6 or 7 days), weeks (such as 1, 2, 3 or 4 weeks) or months (such as 1, 2, 3, 4, 5, 6 or more months).

[0062] In each of the methods of treatment and prevention of the present invention the pharmaceutical formulations comprising the antibodies and/or HIV-1 Env protein binding fragments are administered in a pharmaceutically acceptable form and in substantially non-toxic quantities. These pharmaceutical formulations may be administered to a subject using different schedules, depending on the particular disease being treated or prevented, and severity thereof; the age and size of the subject; and the general health of the subject, to name only a few factors to be considered. In general, the pharmaceutical formulations may be administered once, or twice, three times, four times, five times, six times or more, over a course of treatment or prevention. The timing between each dose in a dosing schedule may range between days, weeks, months, or years, an includes administered once every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more weeks. The same quantity of protein in the formulation may be administered in each dose of the dosing schedule, or the amounts in each dose may vary. The identity of the particular antibodies and variants in the formulation may also vary or remain the same in each dose in a dosing schedule.

[0063] The amount of the antibody and/or variant administered to a subject in a dose when the methods of the present invention are practiced will again vary. However, the amount administered to a subject in a dose will be sufficient to effect treatment or prevention of HIV-1 infection in a subject. As an example, a therapeutically effective amount of antibody and/or variant in a dose of a pharmaceutical formulation of the present invention is typically between about 10 to about 200 mg per kg of body weight of the subject to which the dose of the pharmaceutical formulation is be administered.

[0064] Appropriate doses and dosing schedules can readily be determined by techniques well known to those of ordinary skill in the art without undue experimentation. Such a determination will be based, in part, on the tolerability and efficacy of a particular dose.

[0065] Administration of the pharmaceutical formulations may be via any of the means commonly known in the art of vaccine delivery. Such routes include intravenous, intraperitoneal, intramuscular, subcutaneous and intradermal routes of administration, as well as nasal application, by inhalation, ophthalmically, orally, rectally, vaginally, or by any other mode that results in the formulation contacting mucosal tissues.

Methods of Using Env Fusion Proteins

[0066] The invention provides methods of using the Env fusion proteins as immunogens to generate an immune response in a subject (active immunization). The methods comprise administering one or more of the Env fusion proteins, and/or one or more of the variants thereof, typically as an immunogenic formulation, to a subject in which it is desired that an immune response be generated. The Env fusion proteins and variants may be administered to the subject in the form of the vectors as defined above, or pharmaceutical compositions comprising the vectors. The Env fusion proteins and variants may also be administered to the subject in the form of the proteins themselves, whether alone or in an immunogenic formulation comprising the protein/variants and a pharmaceutically acceptable carrier and/or adjuvant.

[0067] Exemplary immunogenic formulations include, but are not limited to, formulations comprising SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NOs:8 and 10, SEQ ID NOs:10 and 12, SEQ ID NOs:12 and 14, SEQ ID NOs:8 and 14, SEQ ID NOs:8, 10 and 12, SEQ ID NOs:10, 12 and 14, SEQ ID NOs:8, 10 and 14, SEQ ID NOs:8, 12 and 14, or SEQ ID NOs:8, 10, 12 and 14.

[0068] In a non-limiting example, the invention is directed to a method of generating an immune response in a subject comprising administering an immunologically effective amount of an immunogenic formulation as defined herein to a subject, thereby generating an immune response in a subject. In a related aspect, the invention is directed to a method of generating a protective immune response in a subject comprising administering an immunologically effective amount of an immunogenic formulation as defined herein to a subject, thereby generating a protective immune response in a subject. Other exemplary formulations include, but are not limited to, formulations comprising 1, 2, 3, 4 or more variants.

[0069] In each of the methods of active immunization, the immunogenic formulations comprising the fusion proteins, variants and/or vectors are administered in a pharmaceutically acceptable form and in substantially non-toxic quantities. The immunogenic formulations may be administered to a subject using different schedules, depending on, for example, the contents of the immunogenic formulation; the age and size of the subject; and the general health of the subject, to name only a few factors to be considered. In general, the immunogenic formulations may be administered once, or boosted twice, three times, four times, five times, six times or more, over a course of immunization. The timing between each booster may range between days, weeks, months, or years, an includes administered once every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more weeks. The same quantity of protein in the formulation may be administered in each dose, or the amounts in each dose may vary. The identity of the particular fusion proteins, variants and vectors in the formulation may also vary or remain the same in each dose.

[0070] The amount of the fusion proteins, variants and vectors administered to a subject in a dose when immunization is practiced will vary. However, the amount administered to a subject in a dose will be sufficient to induce an immune response in the subject. As an example, a immunologically effective amount of fusion proteins, variants and/or vectors in a dose of an immunogenic formulation of the present invention is typically between about 10 to about 500 ug of protein.

[0071] Appropriate doses and booster schedules can readily be determined by techniques well known to those of ordinary skill in the art without undue experimentation. Such a determination will be based, in part, on the tolerability and efficacy of a particular dose.

[0072] Administration of the immunogenic formulations may be via any of the means commonly known in the art of vaccine delivery. Such routes include intravenous, intraperitoneal, intramuscular, subcutaneous and intradermal routes of administration, as well as nasal application, by inhalation, ophthalmically, orally, rectally, vaginally, or by any other mode that results in the formulation contacting mucosal tissues.

[0073] The term "subject" is intended to mean an animal, such birds or mammals, including humans and animals of veterinary or agricultural importance, such as dogs, cats, horses, sheep, goats, and cattle.

[0074] A kit comprising the necessary components for active immunization, including a immunogenic formulation comprising one or more fusion proteins or variants thereof that elicits an immune response and instructions for its use, is also within the purview of the present invention. In addition, a kit comprising the necessary components for passive immunization, including a pharmaceutical formulation comprising one or more antibodies or variants thereof with binding specificity for HIV-1 Env and instructions for its use, is within the purview of the present invention.

[0075] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

[0076] All documents, papers and published materials referenced herein, including books, journal articles, manuals, patent applications, published patent applications and patents, are expressly incorporated herein by reference in their entireties.

EXAMPLES

N60-B1.1 Antibody

[0077] A novel HIV-1 broadly neutralizing human antibody (bnAb) was cloned and termed N60-B1.1. The antibody was identified by neutralization screening of limiting dilution B cell cultures from a local volunteer NVS60 who has an ongoing broadly neutralizing antibody response and a low but detectable HIV-1 viral load of approximately 100 copies/ml [21, 22]. Limiting dilution B cell cultures were performed as previously described [22] and the neutralization assay was based on the standard TZM-bl assay. N60-B1.1 neutralized 40% of a

panel of 118 tier 2,3 viruses with an average IC50 of 0.44 ug/ml (Table 1). The neutralization pattern of N60-B1.1 is complementary to other bnAbs in that it potently neutralizes some viruses that are more resistant to other bnAbs (e.g., VRC01, VRCPG04) as shown in Table 2. This demonstrates the unique aspects of N60-B1.1 as a novel type of HIV-1 bnAb.

Table 1. Potency and breath of neutralization activity of N60-B1.1

	N60-B1.1	N60-B1.2	3BNC117	3BNC55	45-46	12A12	8ANC195	VRC01	VRCPG04	PG9	PG16	b12
# of tested viruses	122	110	119	119	119	119	119	85	55	73	73	120
Median IC50 (ug/ml)	0.37	1.51	0.09	0.55	0.07	0.22	0.87	0.21	0.18	0.10	0.03	3.20
% of neutralized viruses	40.2	21.8	89.1	65.5	89.1	94.1	66.4	88.2	72.7	83.6	84.9	38.3

Table 2 Unique neutralization pattern of N60-B1.1

Virus ID	Clade*	IC50 Titer (ug/ml) of antibodies											
		N60-B1.1	N60-B1.2	3BNC 117	3BNC 55	45-46	12A12	8ANC 195	VRC01	VRC-PG04	PG9	PG16	b12
SF162.LS	B-1A	0.019[d]	0.07[d]	NT	NT	NT	NT	NT	0.237[c]	0.088[d]	>50	>50	NT
BaL.26	B-1B	0.019[d]	0.04[d]	0.090[d]	>30	0.040[d]	0.017[d]	>50	0.100[d]	0.503[c]	0.034[d]	0.136[c]	0.200[c]
6535.3	B	0.019[d]	0.019[d]	0.550[c]	2.600[b]	0.140[c]	21.970[a]	0.200[c]	0.540[c]	0.840[c]	0.465[c]	>50	17.5[a]
AC10.0.29	B	0.14[c]	0.94[c]	13.840[a]	>50	0.420[c]	1.150[b]	0.880[c]	2.200[b]	>50	0.078[d]	0.02[d]	1.9[b]
THRO4156.18	B	0.30[c]	30.71[a]	1.760[b]	>50	1.590[b]	3.050[b]	>50	2.300[b]	>50	15.000[a]	0.975[c]	0.5[c]
CAAN5342.A2	B	0.019[d]	0.25[c]	0.420[c]	4.100[b]	0.110[c]	1.320[b]	>50	0.820[c]	1.660[b]	13.000[a]	7.430[b]	>50
1006_11_C3_1601	B (T/F)	0.07[d]	1.25[b]	0.030[d]	>50	0.050[d]	0.220[c]	0.430[c]	0.150[c]	NT	0.366[c]	>50	3.9[b]
ZM53M.PB12	C	0.019[d]	0.38[c]	0.210[c]	12.550[a]	0.190[c]	0.590[c]	9.630[b]	1.300[b]	4.280[b]	0.092[d]	0.009[d]	25.9[a]
Du172.17	C	7.08[b]	>50	1.190[b]	3.520[b]	>30	0.200[c]	10.800[a]	>50	0.218[c]	0.262[c]	0.030[d]	1[c]
Du422.1	C	0.18[c]	1.71[b]	>50	>50	>50	>50	>50	>50	>50	0.303[c]	0.02[d]	0.2[c]
T250-4	CRF02_AG	0.019[d]	0.019[d]	>15	0.240[c]	>30	>30	>50	>50	>50	0.001[d]	0.001[d]	>50
3817.v2.c59	CD	1.53[b]	NT	0.150[c]	>50	>50	23.740[a]	1.000[c]	>50	3.760[b]	0.007[d]	0.006[d]	>50
6540.v4.c1	AC	0.08[d]	2.16[b]	>50	>50	>50	0.110[c]	>50	>50	>50	0.035[d]	0.017[d]	46.1[a]
6545.v4.c1	AC	0.37[c]	NT	>50	>50	>50	0.250[c]	26.940[a]	>50	>50	0.095[d]	0.068[d]	>50
	Code (<):	50.00[a]	10.00[b]	1.00[c]	0.10[d]	* (T/F): Transmitted / Founder Virus					NT: Not tested		

[0078] The amino acid sequences of the variable regions of N60-B1.1 are shown in Figure 1, along with the light chain variable region (VK) of the clone N60-B1.2 (the un-mutated germline VK gene) and the heavy chain variable region (VH) of the un-mutated germline VH gene IGHV4-39*01. The VK and VH sequences were determined by IMGT/V-QUEST (see the website having the url beginning with "www" and ending with "imgt.org/IMGT_vquest/vquest") sequence analysis. Further, the DNA and amino acid sequences of the VK chain of N60-B1.1 are provided in SEQ ID NOs:1 and 2, respectively, while the DNA and amino acid sequences of the VH chain of N60-B1.1 are provided in SEQ ID NOs:3 and 4, respectively.

[0079] N60-B1.1 is encoded by VH4-39*07 that is somatically mutated 13.3% at the nucleotide level and 24.5% at the amino acid level, which is within the normal somatic mutation rate of an antibody response *in vivo*. It uses a single point mutated light chain of germline (CDRL3) VK3-15*01. This is distinct from other bnAbs, which have exceptionally high rates of somatic mutation in this chain. Therefore, the induction of an Ab response of N60-B1.1-like antibodies should not be limited by the bottle-neck of somatic mutation that is required for other bnAbs against HIV-1.

[0080] N60-B1.1 binds to monomeric gp120 with low affinity and the binding was detectable most reproducibly by ELISA [22]. The antibody binds to intact virions as evidenced by fluorescence correlation spectroscopy and binds cell surface expressed gp160 as detected by flow cytometry. The antibody also binds virions attached to target cells with biphasic kinetics as showed by time course immunofluorescence confocal microscopy.

[0081] Mutagenesis studies indicate that N60-B1.1 binds a novel epitope in gp120 that is different from other reported bnAbs. As shown in Figure 2, mutations in the V1/V2 loop (dV1V2), the co-receptor binding site (CoRbs, I420R), and the V3 loop (dV3) affect N60-B1.1 binding to monomeric gp120. It does not bind to linear peptides of Env. Its binding to gp120 can be modulated by CD4 interaction with gp120 (FLSC) but the epitope is not affected by mutation (D368R) that abrogate CD4 binding site epitopes. In early studies, it was established that N60-B1.1 does not bind to linear Env peptides. Further, it was found that N60-B1.1 binds to monomeric BaL gp120 with low avidity and its binding is detectable most reproducibly by capture ELISA [22].

[0082] To map the epitope of N60-B1.1, its binding to a large panel of gp120 mutants was tested by capture ELISA [22]. As shown in Table 3, mutants affecting the co-receptor binding

site (CoRbs, I420R, I423A), deletion of the V1/V2 loop (dV1V2) and deletion of the V3 loop (dV3) and a minimal V3 loop mutant (mV3) [23] all abrogate N60-B1.1 binding to monomeric gp120.

Table 3.Mapping N60-B1.1 in gp120 mutants

% Binding		N60-B1.1	17b	b12	2G12	N12-O3.1
Wild-type	BaL-gp120	100	100	100	100	100
N-terminal Extension	-E31A	103	100	86	80	100
	-E32A	108	113	104	93	100
	-K33A	76	48[d]	89	83	100
	-W35A	132[c]	140[c]	107	99	100
	-Y39A	121	113	90	89	100
	-Y40A	139[c]	143[c]	106	93	100
Beta-Sandwich 1	-V44A	114	105	94	93	100
	-W45A	84	37[d]	96	92	100
	-K46A	50[d]	26[d]	93	92	100
	-E47A	78	26[d]	100	96	100
	-E47AE91A	73[d]	11[a]	86	86	100
Layer 1	-F53A	90	48[d]	91	99	100
	-D57A	98	85	95	91	100
	-D57AD62A	76	48[d]	103	105	100
	-D57AD78A	103	74[d]	96	95	100
	-R58AK59A	92	76	101	88	100
	-Y61A	92	87	85	81	100
	-D62A	80	49[d]	98	86	100
	-V68A	68[d]	53[d]	83	82	100
	-W69A	84	30[d]	97	87	100
	-V75A	104	64[d]	93	89	100
	-P76A	102	76	96	90	100
	-D78A	108	74[d]	92	87	100
Layer 2	-H105A	88	85	100	89	100
	-E106AD107A	36[d]	6[a]	80	79	100
Outer Domain	-D368R	99	117	0[b]	82	100
	-I420R	22[a]	0[b]	101	93	100
	-I423A	0[b]	0[b]	96	94	100
	-T455A	73[d]	93	90	87	100

Layer 3	-R476A	57[d]	20[a]	101	98	100
	-R476AR480A	5[b]	0[b]	68[d]	83	100
	-K487AK490A	31[d]	5[a]	85	83	100
Beta-Sandwich 3	-I491A	85	47[d]	98	92	100
	-E492A	113	96	105	93	100
	-P493A	92	70[d]	95	93	100
C-terminal Extension	-L494A	99	96	95	79	100
	-G495A	106	106	90	93	100
	-V496A	107	114	100	96	100
	-P498A	101	100	97	93	100
	-T499A	102	81	107	100	100
	-K500A	94	100	93	92	100
Variable Loop Deletions	-dV1	0[b]	134[c]	90	93	100
	-dV2	0[b]	174[c]	87	82	100
	-dV3	0[b]	0[b]	109	100	0[b]
	-mV3	0[b]	85	100	100	0[b]
N/C-terminal Deletions	core-D7	0[b]	0[b]	106	100	0[b]
	core-V1V2-D7	0[b]	0[b]	103	100	0[b]
	core-V3-D7	0[b]	285[c]	102	86	100
Glycan mutant	S158A	3[b]	62[d]	80	80	100
	T162A	100	101	110	87	100
	S200A	22[a]	55[d]	86	92	100
	T303A	2[b]	100	92	88	100
	S334A	91	95	nd[c]	16[a]	100
sCD4 complex	FLSC	10[a]	266[c]	24[a]	104	100

Scale <75[d] <25[a] <5[b] >125[c] 0: OD = background

[0083] N60-B1.1 is a glycan-dependent bnAb as the removal of selected glycosylation sites at V1V2 region (S158A and S200A) and V3 region (T303A) substantially decrease its binding abilities. Removal of N160 and N332 glycosylation sites do not affect its binding, which indicates that N60-B1.1 is different from other reported glycan-dependent PG9-like and PGT series bnAbs. Interestingly, N60-B1.1 binding to gp120 is decreased on gp120-CD4 complexes as evidenced by the substantially decreased binding to gp120-sCD4 fusion protein FLSC. In addition, the N60-B1.1 epitope is not affected by mutation (D368R) that abrogates CD4bs epitopes. Collectively, these results indicate that N60-B1.1 binds a glycan dependent co-receptor associated epitope involving the V1V2 and V3 region, which is different from that of other

reported bnAbs, such as PG9, PG16, PGT128 and VRC01. N60-B1.1 is not a traditional CD4i or CD4bs mAb because it does not bind well to FLSC or BaL-gp120-I420R mutants, but binds well to a BaL-gp120-D368R mutant. It is not a PG9-like bnAb because N60-B1.1 binds reasonably well to monomeric gp120 and shows a different neutralization profile from that of PG9 (for example, it potently neutralizes the PG9 resistant SF162 virus).

[0084] Importantly, N60-B1.1 is a protective bnAb without auto-reactivity as shown in Figure 3A. Most reported HIV-1 bnAbs showed auto/poly-reactivity [7, 8, 10]. Further, there is evidence that auto-reactivity can delete "2F4 and 4E10 like" anti-gp41 B cell response *in vivo* [12, 24, 25]. For this reason, the auto-reactivity of N60-B1.1 was tested. As shown in Figure 3A, N60-B1.1 is not auto-reactive, in contrast to the well-studied bnAbs of 4E10 and 2F5. N60-B1.1 is also not as poly-reactive as PG9. It should be noted that while the thresholds of detrimental auto/poly-reactivity for B cell responses *in vivo* is not yet well-defined, it is well known that high affinitive auto-reactive and ploy-reactive B cells are negatively selected during B cell development and the germinal center reaction [12, 24, 25]. Therefore, it is unlikely that N60-B1.1-like bnAb responses will be impacted by clonal deletion or anergy during B cell development.

[0085] To determine whether N60-B1.1 can provide protection *in vivo*, passive immunization studies were performed in rhesus macaque/SHIV162p3 models. As shown in Figure 3B, in screening study, a single injection of N60-B1.1 could transiently reduce viral loads by 2 logs in SHIV162p3 infected rhesus macaques. This result indicates that N60-B1.1 is a potential protective bnAb. To test if N60-B1.1 can afford sterilizing immunity, passive immunization was performed in naive rhesus macaques. After establishing mAb decay *in vivo* in a PK study, eight macaques per experimental group were intravenously-infused with N60-B1.1 at 25mg/kg body weight eight hours before an intra-rectal challenge with a SHIV162P3 stock at a 1:100 dilution. This challenge dose was previously determined to infect approximately 100% of the rhesus macaques used for in vivo titration. Monoclonal antibody b12 was used as a positive control for protection. As shown in Figure 3C, the infusion of N60-B1.1 afforded statistically significant sterilizing immunity against the intra-rectal challenge of SHIV162P3. This is thought to be the first study showing that a bnAb can passively protect against a rectal challenge of this hard-to-neutralize virus. It should be noted that the challenge dose is slightly greater than the *in vivo* infection curve from the titration study. So it is not surprising that one animal out of the 8

animals of the control group remained uninfected. These studies demonstrated that N60-B1.1 is a novel protective bnAb and support the use N60-B1.1 as template for HIV-1 vaccine design.

[0086] Collectively, these data show that N60-B1.1 recognizes a novel epitope, the co-receptor associated region (CAR) of Env that involves V1/V2 and V3 regions, and is distinct from the other known bnAbs, such as PG9, PG16, PGT128 and VRC01.

Env Fusion Proteins

[0087] Characterization of N60-B1.1 binding to antigen revealed that it could define a common transitional conformation structure of HIV-1 Env. Competition ELISA was performed with a 50% binding dose of biotin-labeled N60-B1.1 together with a serial diluted amount of competing antibody in above capture ELISA. The result showed that the N60-B1.1 binding to gp120 was competed by V1V2 mAbs 2158 and 697-20D, V3 mAbs (N10-O2.1, N12-O3.1) and traditional CD4i mAb (17b, N60-i1.1) that binds CoRBS (Figure 4A). This is consistent to the above mutagenesis studies that showed N60-B1.1 recognizes a novel co-receptor associated region (CAR) that involves V1/V2 and V3 regions.

[0088] Interestingly, binding of N60-B1.1 to gp120 was inhibited by non-broadly neutralizing CD4bs mAbs (b13, F105, N12-B2), while broadly neutralizing CD4bs mAbs, like b12 and VRC01, do not compete N60-B1.1 binding to gp120 (Figure 4B). Instead, b12 and VRC01 substantially enhance N60-B1.1 binding to gp120, as shown in Figure 4B. This unique feature of N60-B1.1 that could differentiate non-broadly neutralizing CD4bs mAbs (b13, F105, N12-B2, etc.) from broadly neutralizing CD4bs mAbs (b12, VRC01), therefore, defines a common transitional conformation structure of gp120, to which both bnAbs of CD4bs and N60-B1.1 can bind simultaneously. Remarkably, this observation also indicated that the common transitional conformation structure of gp120 can be stabilized by bnAb against gp120, like b12, VRC01, as well as non-neutralizing ADCC functional Abs against conserved regions of HIV-1 Env, like JR4, C11, JR48.1 (Figure 4C), because of the enhanced binding of N60-B1.1 to complex of gp120 with these mAbs.

[0089] Non-broadly neutralizing CD4bs mAbs compete the binding of N60-B1.1 to gp120. In contrast, broadly neutralizing CD4bs mAbs enhance the binding of N60-B1.1 to gp120. This observation also indicates that bnAbs against HIV-1 gp120 may share a common neutralizing mechanism in that they neutralize HIV-1 by locking the Env trimers on virion in a transitional

conformation structure, which N60-B1.1 and VRC01 can bind to, that is unable to proceed to the fusion step of HIV-1 infection.

[0090] Notably, these competitive mAbs of V1V3, V3, CD4i and CD4bs do not block neutralizing activity of N60-B1.1. Therefore, characterization of N60-B1.1 defined a common transitional conformation structure of Env that is a novel target for HIV-1 bnAbs and HIV-1 vaccine.

[0091] This observation that selected mAbs enhance the binding of N60-B1.1 to gp120 led to the design of vaccine immunogens that preferentially expose the epitope of the bnAb N60-B1.1 and that can potentially induce bnAb responses similar to that which resulted in N60-B1.1.

[0092] The following immunogen design strategy was followed. Recombinant fusion protein constructs of Env linked to single-chain fragment V region (ScFV) of enhancing mAbs were designed that can expose the binding site of N60-B1.1 These Env-ScFV proteins (for example: gp120-b12-ScFV (SEQ ID NO:8), gp120-VRC01-ScFV (SEQ ID NO:10), gp120-JR4-ScFv (SEQ ID NO:12) and gp120-JR48.1-ScFV (SEQ ID NO:14) substantially expose the epitopes of N60-B1.1 as proved by ELISA. As shown in Figure 5, fusion protein of gp120-b12-ScFv and gp120-VRC01-ScFv enhanced the exposure of epitope of bnAb N60-B1.1 compared with that of wild type gp120 protein. Notably, they also enhanced the exposure of epitopes of other bnAbs PG9 and PGT128. These observations highlight the potential value of this type of fusion protein as a vaccine candidate for inducing bnAb response against HIV-1. They also further support the above observation that bnAbs against HIV-1 gp120 may share a common neutralizing mechanism. They neutralize HIV-1 by locking the Env trimers on virion in a common inactive transitional conformation structure that different types of bnAbs against gp120, N60-B1.1, VRC01, PG9 and PGT128, etc., can simultaneously bind to.

[0093] gp120-ScFv fusion proteins were designed for the four mAbs that enhance the exposure of the N60-B1.1 epitope on gp120. As predicted (Figure 5), these gp120-ScFV proteins (gp120-b12-ScFV, gp120-VRC01-ScFV, gp120-JR4-ScFv and gp120-JR48.1-ScFV) stably expose the epitope recognized by N60-B1.1 as compared with the wild type gp120 protein. Further, they also enhanced the exposure of epitopes recognized by other bnAbs, like PG9 and PGT128 (Fig. 6 and Table 4).

Table 4. Profile of epitopes exposure of gp120-ScFv proteins (different from FLSC)

Fold of Ab needed for ELISA binding OD equal to half max on gp120														
	V1V2/V3 related bnAb			CD4bs bnAb			CD4i Ab	Cluster A ADCC Ab					Glycan	V3 Ab
Antigen	N60-B1.1	PG9	PGT128	b12	VRC01	PG04	N12-i2	A32	N5-i5	JR4	C11	N12-i3	2G12	N12-O3.1
Bal-gp120	1	1	1	1	1	1	1	1	1	1	1	1	1	1
FLSC R/T	16.45[b]	8.02[b]	5.07[b]	46.27[b]	17.04[b]	>100[b]	0.01[a]	0.04[a]	0.02[a]	0.11[a]	0.37[a]	0.35[a]	0.91	1
gp120-b12-ScFV	0.01[a]	0.06[a]	0.09[a]	>100[b]	51.44[b]	>100[b]	36.92[b]	0.06[a]	0.02[a]	0.09[a]	0.36[a]	0.23[a]	0.56	1
gp120-VRCO1-ScFv	0.03[a]	0.09[a]	0.23[a]	>100[b]	36.02[b]	>100[b]	0.01[a]	0.20[a]	0.11[a]	0.32[a]	0.32[a]	0.36[a]	0.66	1
gp120-JR4-ScFv	0.04[a]	0.02[a]	0.13[a]	0.47[a]	0.44[a]	0.26[a]	0.01[a]	>100[b]	>100[b]	>100[b]	>100[b]	>100[b]	0.61	1
gp120-JR48.1-ScFv	0.14[a]	0.07[a]	0.14[a]	0.56	0.53	0.36[a]	0.07[a]	0.10[a]	0.08[a]	0.21[a]	17.17[b]	86.81[b]	0.58	1

Scale: Enhancing[a] Inhibiting[b]

[0094] This contrasts with the gp120-sCD4 (FLSC) protein [26, 27] to which these bnAbs bind poorly. In addition, the fusion proteins also enhance the exposure of epitopes recognized by potent ADCC mAbs that were described previously [28]. Similar to FLSC, the new type of fusion protein of gp120 with ScFV of enhancing mAbs, can substantially expose the epitopes recognized by traditional co-receptor binding site (CoRbs) CD4i Abs (N12-i2), with the exception of gp120-b12-ScFV, as summarized in Table 4. The gp120-b12-ScFv stands unique in this regard in that it blocks the exposure of epitopes recognized by traditional CD4i mAbs (N12-i2). Interestingly, these four gp120-ScFV fusion proteins are different in the exposure of epitopes recognized by cluster A ADCC mAbs. The fusion proteins gp120-b12-ScFV and gp120-VRC01-ScFV can substantially expose the epitopes recognized by all three subgroups of cluster A ADCC mAbs [28]: A32 like Abs (N5-i5), C11 like Abs (N12-i3) and Abs competing with both A32 and C11 (JR4) (Table 4). By contrast, the fusion protein gp120-JR4-ScFv is not recognized by any cluster A ADCC mAbs, whereas the gp120-JR48.1-ScFv fusion protein exposes the epitopes recognized by A32-like antibodies (N5-i5) and antibodies competing with both A32 and C11 (JR4), but inhibits epitopes of C11-like Abs (N12-i3). As expected, the fusion proteins of gp120-b12-ScFV and gp120-VRC01-ScFV do not bind CD4bs bnAbs (Table 4) as compared with gp120. On the other hand, the gp120-JR4-ScFV and gp120-JR48.1-ScFV fusion proteins stably express the epitopes recognized by CD4bs bnAbs (b12, VRC01 and PG04).

[0095] In addition, these fusion proteins enhance the exposure of epitopes for potent ADCC mAbs that were described recently [28]. Similar to the gp120-sCD4 full-length single chain (FLSC) fusion protein that was shown to be a promising HV-1 vaccine candidate[26, 27], this new type of fusion protein of gp120 with ScFV of bnAb enhancing mAbs, for example, gp120-b12-ScFV and gp120-VRC01-ScFV, can substantially expose the epitopes of all three subgroups of cluster A ADCC mAbs [28], A32 like Abs (N5-i5), C11 like Abs (N12-i3) and Abs competing with both the A32 and C11 (JR4) (Figure 6). The gp120-VRC01-ScFv also enhances the exposure of epitopes of traditional co-receptor binding CD4i mAbs (N12-i2), similar to the FLSC [26, 27]. The gp120-b12-ScFv stands unique in this feature that it blocks the exposure of epitopes of traditional co-receptor binding CD4i mAbs (N12-i2).

[0096] These Env-ScFV proteins (for example: gp120-b12-ScFV and gp120-VRC01-ScFV) substantially expose the epitopes of bnAbs like N60-B1.1, PG9 and PGT128, as well as epitopes

of potent ADCC mAbs like C11-like Ab N5-i5, A32-like AbN12-i3 and N26-i1-like Ab 3.5B. These attractive features of these fusion proteins, therefore, mark them as promising Env vaccine candidates. Immunization with these fusion proteins (individual, combination and sequential) will potentially induce bnAb responses and cluster A ADCC Ab responses against HIV-1.

Bi-specific and Triple-specific Abs against HIV-1

[0097] The findings presented herein that CD4bs bnAbs (b12, VRC01, etc.) as well as cluster A ADCC mAbs (JR4, JR48.1, etc) can enhance the binding of glycan-dependent V1V2 and V3 related bnAbs (N60-B1.1, PG9, PGT128, etc.) to Env also indicate that these three types of non-overlap protective mAbs can be engineered to make bi-specific and triple-specific recombinant human antibodies (Figure 7). The activity of bi-specific and tri-specific anti-HIV-1 antibodies can be optimized by optimal linker (length) between ScFV and the VH/VL of IgG1 that can result in simultaneous binding of two/three binding domains of the antibodies to the same Env protein. For example, a b12-N60-B1.1 bi-specific Ab (or VRC01-N60-B1.1, JR4-N60-B1.1, b12-PG9, VRC01-PG9, JR4-PG9, etc.) will be much more potent and broad in neutralizing HIV-1 than mixture of individual Abs of b12 and N60-B1.1 (or mixture of VRC01/N60-B1.1, JR4/N60-B1.1, b12/PG9, VRC01/PG9, JR4/PG9, etc. respectively). A JR4-b12-N60-B1.1 triple-specific Ab (or JR4-VRC01-N60-B1.1, JR4-b12-PG9, JR4-VRC01-PG9, etc.) will be much more potent and broad in neutralizing HIV-1 than mixture of individual Abs of JR4, b12 and N60-B1.1 (or mixture of JR4/VRC01/N60-B1.1, JR4/b12/PG9, JR4/ VRC01/PG9, etc. respectively).

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[0098] All patents and publications mentioned in this specification are indicative of the level of skill of those skilled in the art to which the invention pertains. Each cited patent and publication is incorporated herein by reference in its entirety. All of the following references have been cited in this application:

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WHAT IS CLAIMED IS:

1. An immunogenic formulation comprising:

(a) at least one polypeptide selected from the group consisting of gp120-b12-ScFV (SEQ ID NO:8), gp120-VRC01-ScFV (SEQ ID NO:10), gp120-JR4-ScFV (SEQ ID NO:12) and gp120-JR48.1-ScFV (SEQ ID NO:14) and a pharmaceutically acceptable carrier and/or adjuvant, or

(b) the formulation of (a) wherein at least one of the polypeptides is a variant and wherein the variant has at least about 95% sequence identity with the full length reference sequence.

2. An immunogenic formulation comprising:

(a) at least two polypeptides selected from the group consisting of gp120-b12-ScFV (SEQ ID NO:8), gp120-VRC01-ScFV (SEQ ID NO:10), gp120-JR4-ScFV (SEQ ID NO:12) and gp120-JR48.1-ScFV (SEQ ID NO:14) and a pharmaceutically acceptable carrier and/or adjuvant, or

(b) the formulation of (a) wherein at least one of the polypeptides is a variant and wherein the variant has at least about 95% sequence identity with the full length reference sequence.

3. An immunogenic formulation comprising:

(a) at least three polypeptides selected from the group consisting of gp120-b12-ScFV (SEQ ID NO:8), gp120-VRC01-ScFV (SEQ ID NO:10), gp120-JR4-ScFV (SEQ ID NO:12) and gp120-JR48.1-ScFV (SEQ ID NO:14) and a pharmaceutically acceptable carrier and/or adjuvant, or

(b) the formulation of (a) wherein at least one of the polypeptides is a variant and wherein the variant has at least about 95% sequence identity with the full length reference sequence.

4. An immunogenic formulation comprising:

(a) polypeptides gp120-b12-ScFV (SEQ ID NO:8), gp120-VRC01-ScFV (SEQ ID NO:10), gp120-JR4-ScFV (SEQ ID NO:12) and gp120-JR48.1-ScFV (SEQ ID NO:14) and a pharmaceutically acceptable carrier and/or adjuvant, or

(b) the formulation of (a) wherein at least one of the polypeptides is a variant and wherein the variant has at least about 95% sequence identity with the full length reference sequence.

5. An immunogenic formulation comprising polypeptides gp120-b12-ScFV (SEQ ID NO:8), gp120-VRC01-ScFV (SEQ ID NO:10), gp120-JR4-ScFV (SEQ ID NO:12) and gp120-JR48.1-ScFV (SEQ ID NO:14) and a pharmaceutically acceptable carrier and/or adjuvant.

6. A method of generating an immune response in a subject comprising administering an immunologically effective amount of an immunogenic formulation of any one of claims 1-5 to a subject, thereby generating an immune response in a subject.

7. A method of generating a protective immune response in a subject comprising administering an immunologically effective amount of an immunogenic formulation of any one of claims 1-5 to a subject, thereby generating a protective immune response in a subject.

8. A polypeptide selected from the group consisting of gp120-b12-ScFV (SEQ ID NO:8), gp120-VRC01-ScFV (SEQ ID NO:10), gp120-JR4-ScFV (SEQ ID NO:12) and gp120-JR48.1-ScFV (SEQ ID NO:14).

9. An isolated antibody or an HIV-1 Env protein binding fragment thereof, wherein the antibody comprises:

(a) a VK domain comprising the amino acid sequence of SEQ ID NO:2,

(b) a VH domain comprising the amino acid sequence of SEQ ID NO:4,

(c) a VK domain comprising an amino acid sequence with 95% sequence identity to SEQ ID NO:2, or

(d) a VH domain comprising an amino acid sequence with 95% sequence identity to SEQ ID NO:4.

10. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 9, wherein the antibody comprises a VK domain having the amino acid sequence of SEQ ID NO:2 and a VH domain having the amino acid sequence of SEQ ID NO:4.

11. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 9, wherein the antibody is a bi-specific antibody or a tri-specific antibody.

12. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 9, wherein the antibody comprises a VK domain comprising the amino acid sequence of SEQ ID

NO:2 and a VH domain comprising an amino acid sequence with 95% sequence identity to SEQ ID NO:4.

13. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 9, wherein the antibody comprises a VK domain comprising an amino acid sequence with 95% sequence identity to SEQ ID NO:2 and a VH domain comprising the amino acid sequence of SEQ ID NO:4.

14. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 9, wherein the antibody comprises a VK domain comprising an amino acid sequence with 95% sequence identity to SEQ ID NO:2 and a VH domain comprising an amino acid sequence with 95% sequence identity to SEQ ID NO:4.

15. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 10, wherein the VK domain comprises amino acids 1-95 of SEQ ID NO:2 or the VH domain comprises amino acids 1-102 of SEQ ID NO:4.

16. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 10, wherein the VK domain comprises amino acids 1-95 of SEQ ID NO:2 and the VH domain comprises amino acids 1-102 of SEQ ID NO:4

17. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 9 or 10, wherein the antibody has binding specificity for HIV-1 Env protein.

18. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 9 or 10, wherein the antibody is a fully human antibody or a chimeric antibody.

19. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 9 or 10, wherein the antibody is a monoclonal antibody.

20. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 9 or 10, wherein the antibody is a recombinant antibody.

21. The isolated antibody or an HIV-1 Env protein binding fragment thereof of claim 9 or 10, wherein the fragment is a Fab fragment, $F(ab')_2$ fragment, and single chain Fv (scFv).

22. A pharmaceutical formulation comprising the isolated antibody or an HIV-1 Env protein binding fragment thereof of any one of claims 9-11 and a pharmaceutically acceptable carrier.

23. A method of preventing an HIV-1 infection in a subject, comprising administering a therapeutically effective amount of a pharmaceutical formulation of claim 22 to a subject at risk of developing an HIV-1 infection, thereby inhibiting an HIV-1 infection in a subject.

24. A method of treating an HIV-1 infection in a subject, comprising administering a therapeutically effective amount of a pharmaceutical formulation of claim 22 to a subject having an HIV-1 infection, thereby treating an HIV-1 infection in a subject.

Figure 1. Sequences of Heavy and Light Chains of N60-B1.1

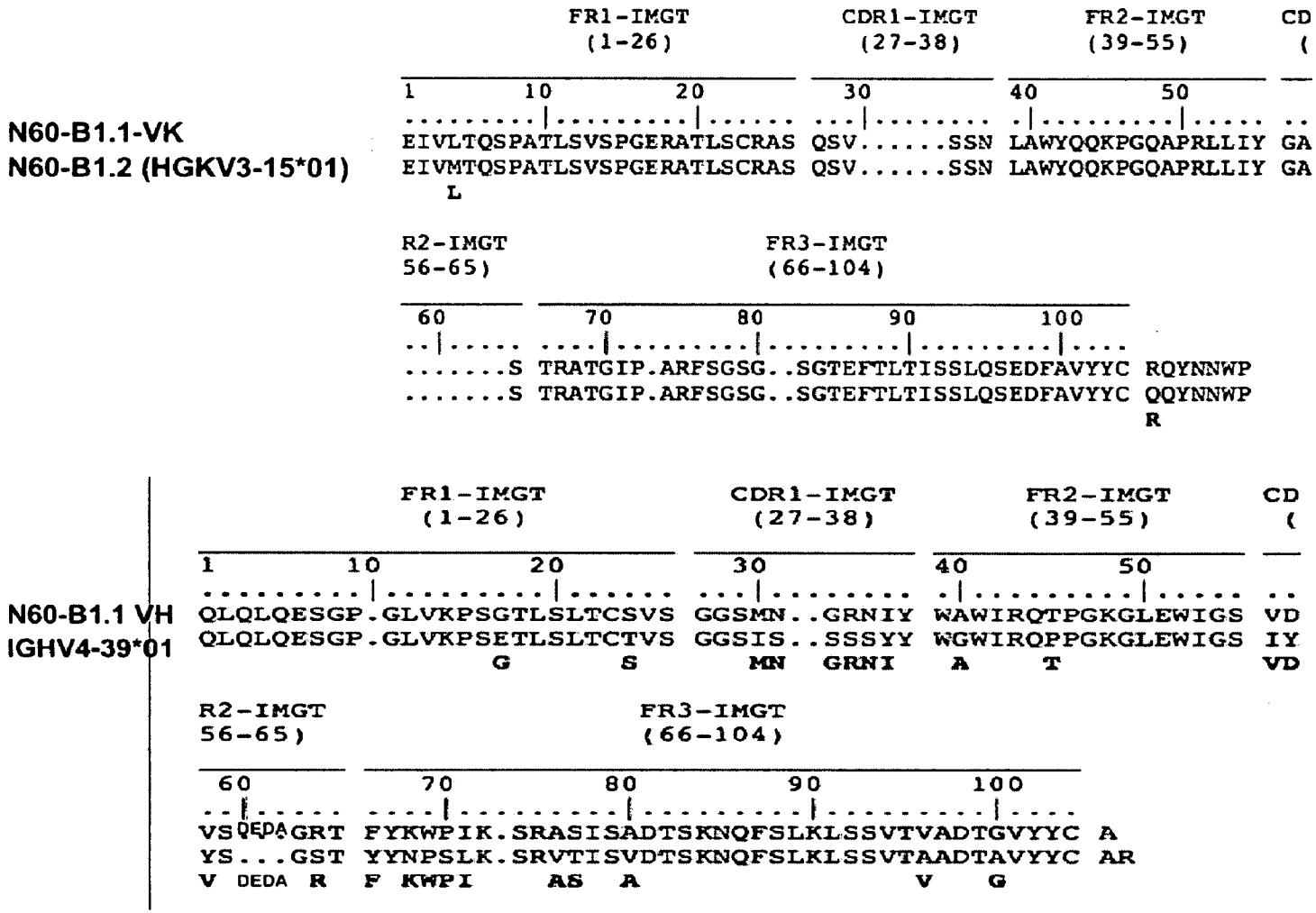
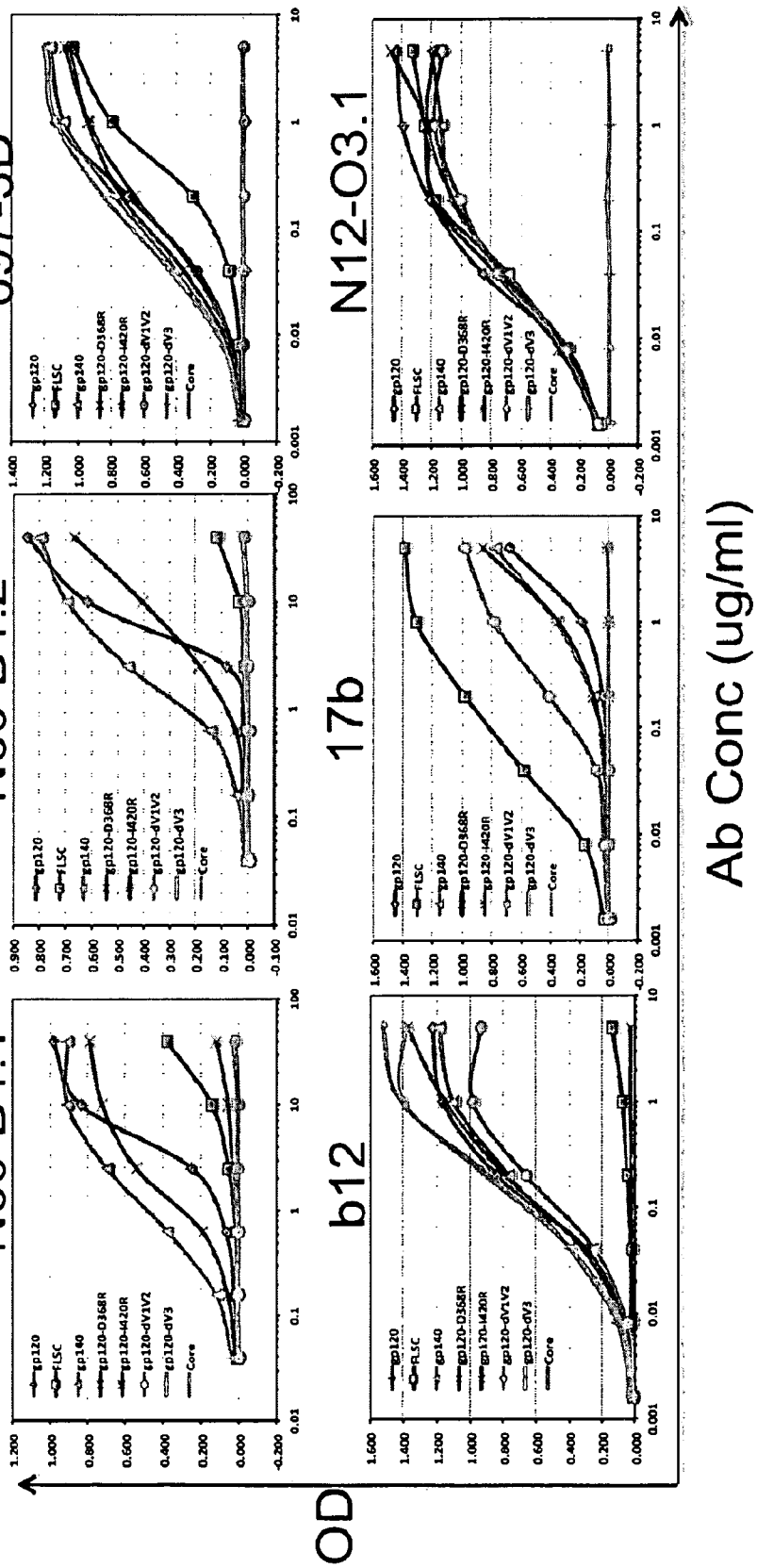


Figure 2. N60-B1.1 against an epitope of co-receptor-associated-region involving V1/V2 and V3



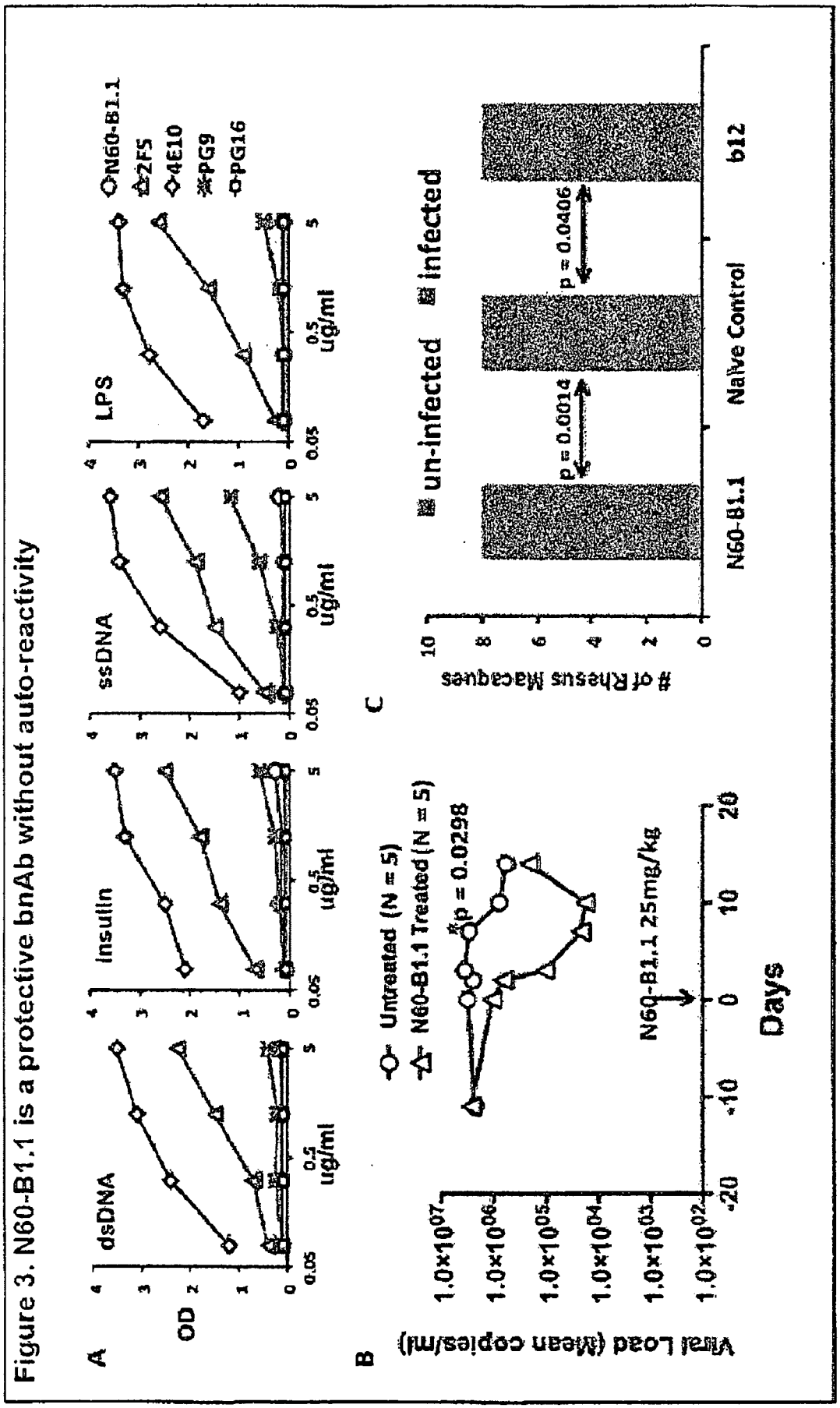


Figure 3. N60-B1.1 is a protective bnAb without auto-reactivity
A
dsDNA
insulin
ssDNA
LPS
OD
ug/ml
N60-B1.1
2F5
4E10
PG9
PG16
B
Untreated (N = 5)
N60-B1.1 Treated (N = 5)
p = 0.0298
N60-B1.1 25mg/kg
Viral Load (Mean copies/ml)
Days
C
un-infected
infected
# of Rhesus Macaques
p = 0.0014
p = 0.0406
N60-B1.1
Naïve Control
b12

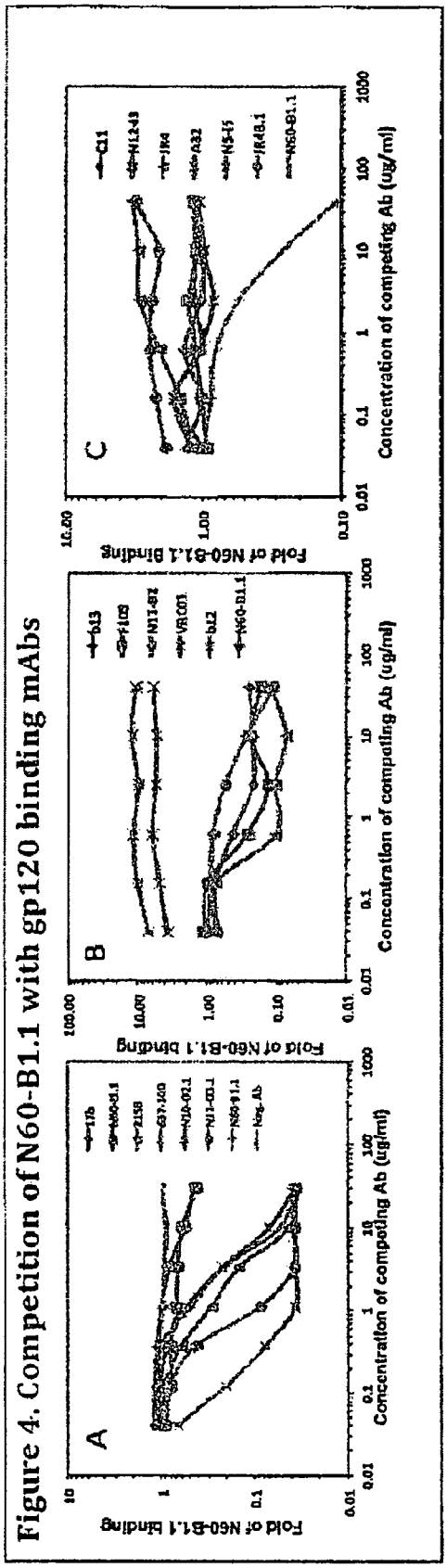


Figure 4. Competition of N60-B1.1 with gp120 binding mAbs

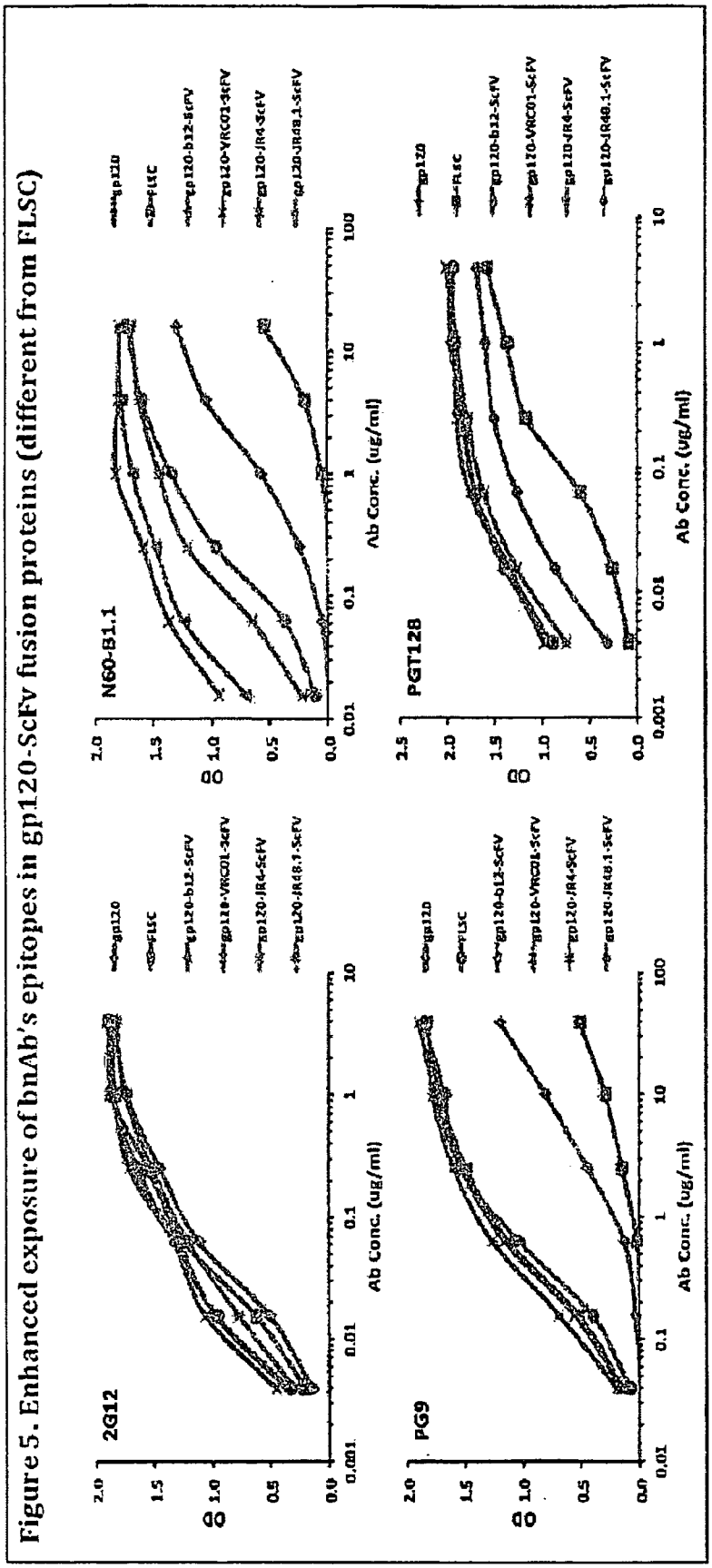


Figure 5. Enhanced exposure of bnAb's epitopes in gp120-ScFv fusion proteins (different from FLSC)

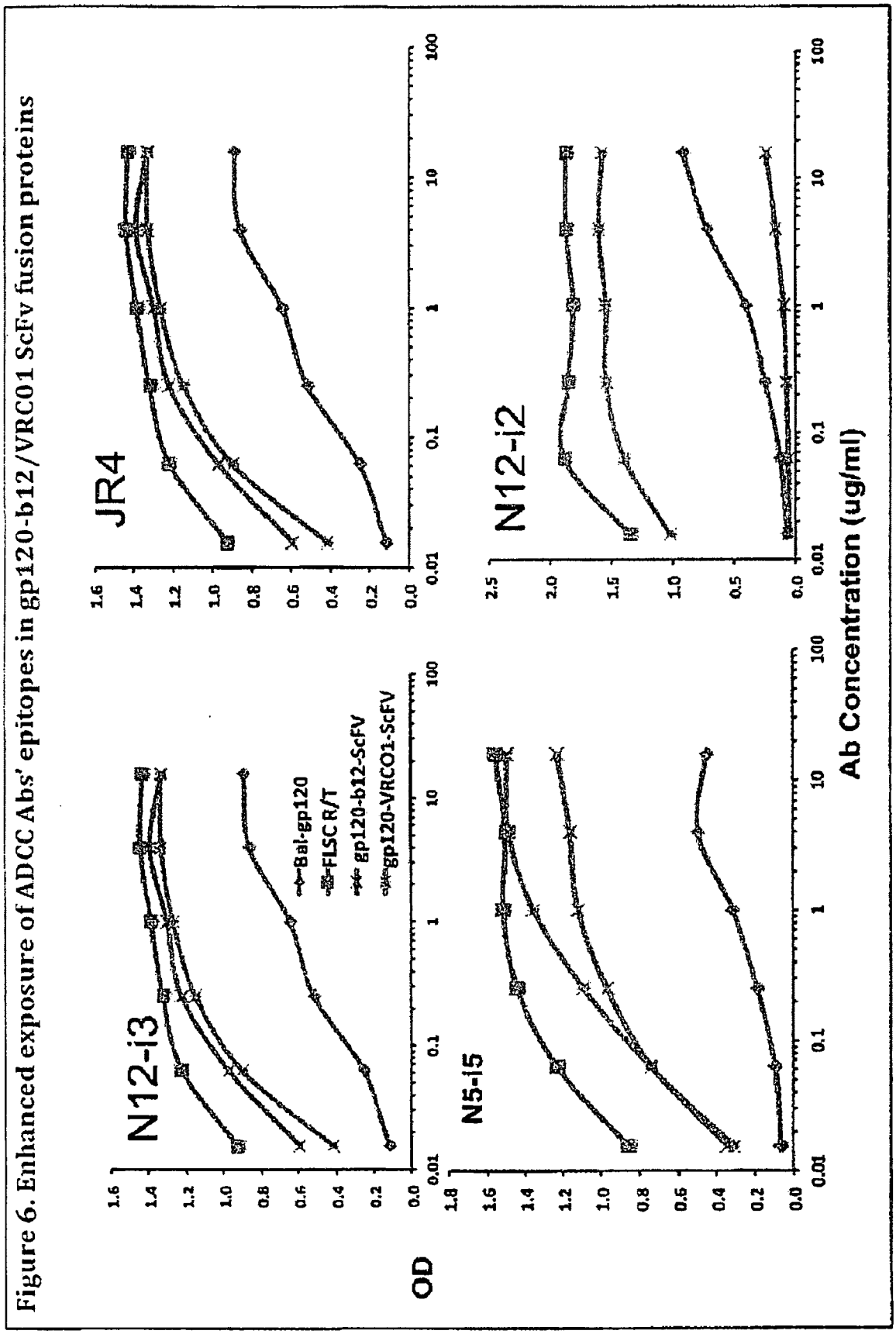


Figure 6. Enhanced exposure of ADCC Abs' epitopes in gp120-b12/VRC01 ScFv fusion proteins

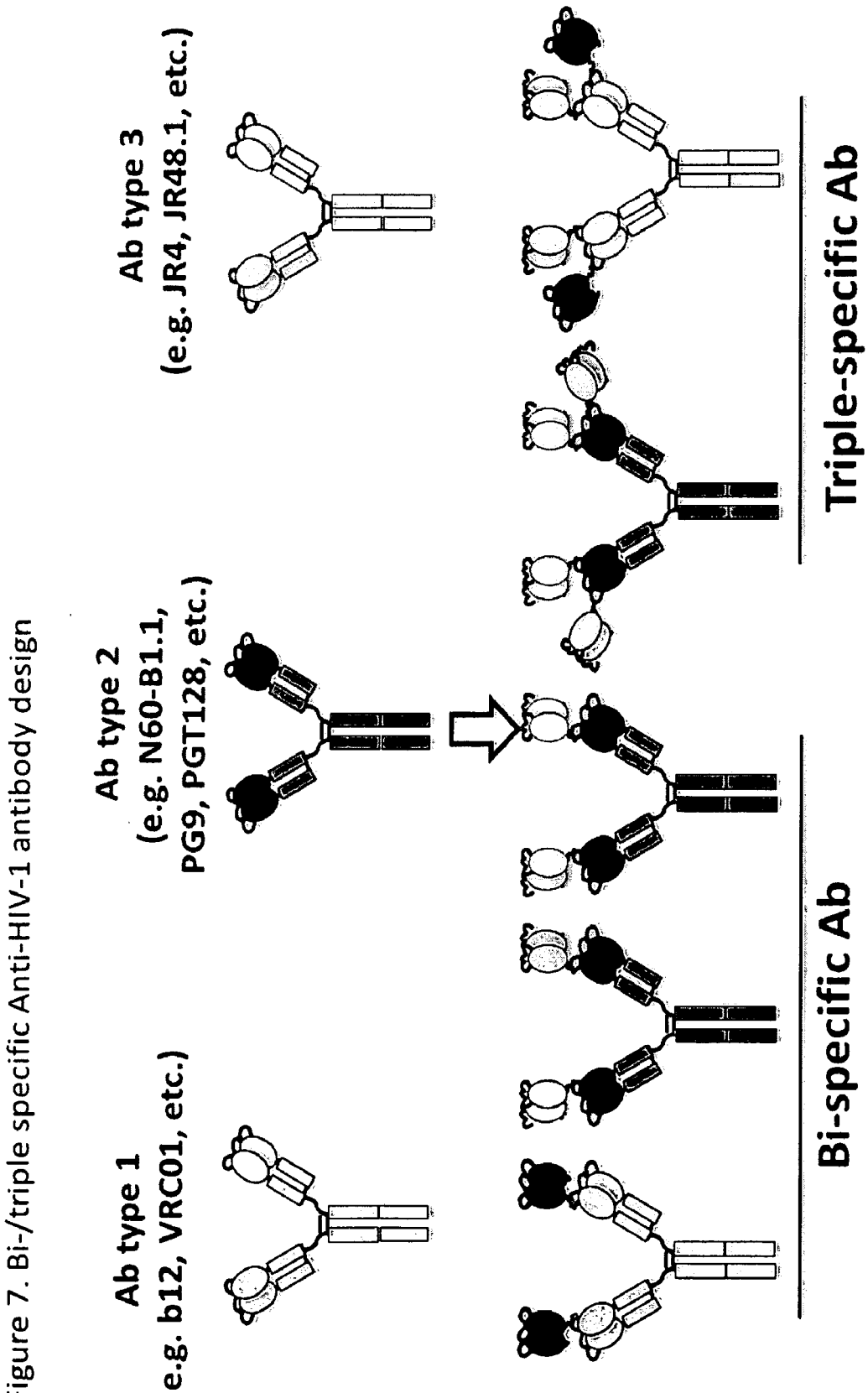


Figure 7. Bi-/triple specific Anti-HIV-1 antibody design

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/073003

A. CLASSIFICATION OF SUBJECT MATTER

C07K 19/00(2006.01)i, C07K 14/155(2006.01)i, C07K 16/10(2006.01)i

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 19/00; A61K 39/395; C07K 16/10; C07K 16/00; C07K 16/46; A61K 39/42; C07K 14/155

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:polypeptide, antibody, HIV-1 Env protein binding fragment, pharmaceutical formulation, neutralizing, fragment, fusion protein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6924360 B2 (GREEN, LARRY L. et al.) 02 August 2005 See claims 4, 49-51; and columns 3, 9, 13 and 16.	9,11,18-22
A		1-5,8,10,12-17
X	US 8026344 B2 (ZHOU, QING et al.) 27 September 2011 See claims 1-18 and sequence list, SEQ ID NO:64.	9,11,18-22
A	NCBI, Genbank accession no. ABA26068.1 (31 December 2005) See the whole document.	1-5,8-22
A	WO 2011-038290 A2 (THE U. S. A., AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES) 31 March 2011 See the whole document.	1-5,8-22
A	WO 2012-065055 A2 (THE ROCKEFELLER UNIVERSITY) 18 May 2012 See the whole document.	1-5,8-22

☐ 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	Date of mailing of the international search report
25 March 2014 (25.03.2014)	**26 March 2014 (26.03.2014)**

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/073003

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 6-7,23-24
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 6-7 and 23-24 pertain to methods for treatment of the human body by therapy and thus relate to a subject matter which this International Seraching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/073003

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 2012-065055 A2	18/05/2012	EP 2638072 A2	18/09/2013
		US 2012-0121597 A1	17/05/2012
		US 8637024 B2	28/01/2014
		WO 2012-065055 A3	12/07/2012