STABILIZED HUMAN IMMUNODEFICIENCY VIRUS (HIV) ENVELOPE (ENV) TRIMER VACCINES AND METHODS OF USING SAME

Statement as to Federally Funded Research

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Background of the Invention

Vaccines that elicit cellular immune responses against viruses seek to reflect global viral diversity in order to effectively treat or prevent viral infection. For HIV vaccines, the initiation of robust and diverse human immunodeficiency virus (HIV)-specific T cell responses is desirable for an effective HIV vaccine. The highly variable Envelope protein (Env) is the primary target for neutralizing antibodies against HIV, and vaccine antigens may be tailored accordingly to elicit these antibody responses. To this end, immunogens mimicking the trimeric structure of Env on the native HIV virion are actively being pursued as antibody-based HIV vaccines. However, it has proven difficult to produce biochemically stable trimeric Env immunogens that elicit diverse neutralizing antibody responses.

Thus, there is an unmet need in the field for the development of vaccines that include novel, optimized trimeric Env immunogens, which can elicit broadly neutralizing antibody responses in order to allow for more successful HIV vaccination outcomes.

Summary of the Invention

In a first aspect, the invention features a stabilized trimer having three gp140 polypeptides in which at least one (e.g., two or each) of the gp140 polypeptides includes an amino acid sequence having at least 90% identity (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 2 (mEnv+).

In a related second aspect, the invention features a stabilized trimer having three gp140 polypeptides in which at least one (e.g., two or each) of the gp140 polypeptides includes an amino acid sequence having substantially the sequence of (e.g., 99% or more identity), or the sequence of, SEQ ID NO: 1 (mEnv) or SEQ ID NO: 3 (cEnv).

In some embodiments, the stabilized trimers are heterotrimers. The stabilized polypeptide heterotrimers may include two mosaic Env1 gp140 polypeptides (e.g., mEnv and/or mEnv+) each including an amino acid sequence having at least 90% identity (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 1 or 2, and one clade C Env gp140 polypeptide (e.g., "cEnv" having SEQ ID NO: 3) including an amino acid sequence having at least 90% identity (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 3 (cEnv) (e.g., two mEnv and one cEnv; two mEnv+ and one cEnv; or one mEnv, one mEnv+, and one cEnv). In other embodiments, the stabilized heterotrimers may include one mosaic Env1 gp140 polypeptide (e.g., mEnv and/or mEnv+) including an amino acid sequence having at least 90% identity (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence

of, SEQ ID NO: 1 or 2, and two clade C Env gp140 polypeptides (e.g., cEnv) each including an amino acid sequence having at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 3 (e.g., one mEnv and two cEnv; one mEnv+ and two cEnv). In some embodiments, the stabilized heterotrimer includes a combination of two different mosaic Env1 sequences (e.g., one mEnv and two mEnv+; two mEnv and one mEnv+; or one mEnv, one mEnv+, and cEnv). In some embodiments, the stabilized heterotrimer includes cEnv and two of the same Env1 polypeptides (e.g., two mEnv and one cEnv; two mEnv+ and one cEnv). In other embodiments, the stabilized heterotrimer includes one cEnv and two different mosaic Env1 polypeptides (e.g., one cEnv, one mEnv, and one mEnv+). In yet other embodiments, the stabilized heterotrimer includes two cEnv polypeptides and one mosaic Env1 polypeptide (e.g., two cEnv and one mEnv; or two cEnv and one mEnv+).

Alternatively, stabilized gp140 Env trimers can be prepared in which one or two of the gp140 Env polypeptides in the trimer has a sequence of SEQ ID NO: 4 (mosaic gp140 Env2, "mEnv2") or SEQ ID NO: 5 (mosaic gp140 Env3, "mEnv3"). In another embodiment said stabilized trimers have three gp140 polypeptides in which at least one (e.g., two or each) of the gp140 polypeptides includes an amino acid sequence having at least 90% identity (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 4 or 5. Preferably, mEnv2 or mEnv3 is modified in a similar manner to that of mEnv, mEnv+, or cEnv, which each possess a trimerization domain, as discussed herein below. Therefore, in some embodiments of the invention, stabilized gp140 Env trimers can be prepared which have the following constituent polypeptides: one mEnv and two mEnv2; two mEnv and one mEnv2; one mEnv+ and two mEnv2; two mEnv+ and one mEnv2; one cEnv and two mEnv2; two cEnv and one mEnv2; one mEnv, one mEnv+, and one mEnv2; one mEnv, one cEnv, and mEnv2; one mEnv+, one cEnv, and one mEnv2; one mEnv and two mEnv3; two mEnv and one mEnv3; one mEnv+ and two mEnv3; two mEnv+ and one mEnv3; one cEnv and two mEnv3; two cEnv and one mEnv3; one mEnv, one mEnv+, and one mEnv3; one mEnv, one cEnv, and mEnv3; one mEnv+, one cEnv, and one mEnv3; one mEnv, one mEnv2, and one mEnv3; one mEnv+, one mEnv2, and one mEnv3; or one cEnv, one mEnv2, and one mEnv3.

In a third aspect, the invention features a composition including a stabilized trimer of the first or second aspect. In an embodiment, the composition of the third aspect includes one or more different stabilized trimer(s). In other embodiments, the different stabilized trimer(s) has three gp140 polypeptides in which at least one (e.g., two or each) of the gp140 polypeptides comprises an amino acid sequence having at least 90% identity (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NOs: 1, 2, 3, 4 or 5. In other embodiments, the different stabilized trimer(s) may be a homotrimer or a heterotrimer. In some embodiments, the compositions of the third aspect further include a pharmaceutically acceptable carrier, excipient, or diluent, and/or an adjuvant.

In a fourth aspect, the invention features a vaccine including any one of the compositions of the third aspect. In some embodiments, the vaccine is used for treating or reducing the risk of a human immunodeficiency virus (HIV) infection in a subject in need thereof. In some embodiments, the vaccine elicits production of neutralizing anti-HIV antisera (e.g., neutralizing anti-HIV-1 antisera) after administration to the subject. The anti-HIV antisera can neutralize HIV (e.g., HIV-1), for example, selected from any one or more of clade A, clade B, and clade C.

In a fifth aspect, the invention features a nucleic acid molecule having a nucleotide sequence that encodes at least one (e.g., two, or three or more) gp140 polypeptide, wherein the at least one gp140 polypeptide includes: (a) an amino acid sequence having at least 95% identity (e.g., at least 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 1; (b) an amino acid sequence having at least 95% identity (e.g., at least 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 2; or (c) an amino acid sequence having the sequence of SEQ ID NO: 3; (d) an amino acid sequence having the sequence of SEQ ID NO: 4; (e) an amino acid sequence having the sequence of SEQ ID NO: 5 or combinations thereof. In some embodiments, the nucleic acid molecule further includes a nucleotide sequence that encodes one or more different (e.g., a second, third, or fourth) gp140 polypeptides (e.g., gp140 polypeptides having at least 95% identity (e.g., at least 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 1, 2, 3, 4 and/or 5). In some embodiments, the nucleic acid molecule includes one or more internal ribosome entry site (IRES) sequences to allow for the expression of multiple peptide or polypeptide chains from the single nucleic acid molecule transcript.

In a sixth aspect, the invention features a vector including one or more nucleic acid molecules of the fifth aspect. In some embodiments, the vector is an adenovirus vector or a poxvirus vector. The adenovirus vector may be derived, for example, from a recombinant adenovirus serotype 11 (Ad11), adenovirus serotype 15 (Ad15), adenovirus serotype 24 (Ad24), adenovirus serotype 26 (Ad26), adenovirus serotype 34 (Ad34), adenovirus serotype 35 (Ad35), adenovirus serotype 48 (Ad48), adenovirus serotype 49 (Ad49), adenovirus serotype 50 (Ad50), Pan9 (AdC68), or a chimeric variant thereof (e.g., adenovirus serotype 5 HVR48 (Ad5HVR48)). The poxvirus vector may be derived, for example, from modified vaccinia virus Ankara (MVA).

In a seventh aspect, the invention provides a method of treating or reducing the risk of an HIV infection in a subject in need thereof by administering a therapeutically effective amount of a composition of the invention (e.g., any one of the stabilized trimers of the first or second aspect, the compositions of the third aspect, the vaccines of the fourth aspect, the nucleic acid molecules of the fifth aspect, and/or the vectors of the sixth aspect) to the subject, such as a mammal, for example, a human. Treating, according to this seventh aspect of the invention, can be therapeutic or prophylactic.

In an eighth aspect, the invention provides a method of reducing an HIV-mediated activity in a subject infected with HIV by administering a therapeutically effective amount of a composition of the invention (e.g., any one of the stabilized trimers of the first or second aspect, the compositions of the third aspect, the vaccines of the fourth aspect, the nucleic acid molecules of the fifth aspect, and/or the vectors of the sixth aspect) to the subject. In some embodiments, the HIV-mediated activity is viral spread, infection, or cell fusion. Cell fusion may be, for example, target cell entry or syncytial formation. In some embodiments, the HIV titer in the subject infected with HIV is decreased (e.g., by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more compared to HIV titer of the subject prior to treatment or a control subject infected with HIV but not treated with the composition(s) of the invention) after administration of the vaccine to the subject, such as a mammal, for example, a human.

In some embodiments, the composition (e.g., a vaccine) is administered intramuscularly, intravenously, intradermally, percutaneously, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally,

intrarectally, topically, intratumorally, peritoneally, subcutaneously, subconjunctivally, intravesicularlly, mucosally, intrapericardially, intraumbilically, intraocularly, orally, topically, locally, by inhalation, by injection, by infusion, by continuous infusion, by localized perfusion bathing target cells directly, by catheter, by lavage, by gavage, in cremes, or in lipid compositions. In some embodiments, the subject is administered at least one dose (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more doses) of the composition or is administered at least one dose (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more doses) daily, weekly, monthly, or yearly. The administration period may be defined (e.g., 1-4 weeks, 1-12 months, 1-20 years) or may be for the life of the subject. In other embodiments, the subject is administered at least two doses (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more doses) of the composition. In yet another embodiment, the composition is administered to said subject as a prime or a boost composition or in a prime-boost regimen. In a preferred embodiment, one or more composition(s) (e.g., a vaccine) of the invention is administered as a boost.

In another preferred embodiment, the invention features a method of treating or reducing the risk of an HIV infection in a subject by administering, as the prime composition in a prime-boost vaccination regimen, a vaccine that includes a first polypeptide having at least 85% amino acid sequence identity (e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 6, or at least a first vector (e.g., an adenoviral or poxvirus vector) that includes a first nucleic acid molecule that encodes this first polypeptide. Optionally, a second polypeptide having at least 85% identity (e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 7 may also be administered in combination with the first polypeptide, or, if a first vector encoding the first polypeptide is administered, a second vector (e.g., an adenoviral or poxvirus vector) including a second nucleic acid molecule that encodes the second polypeptide may be administered in combination with the first vector. The boost composition in this prime-boost regimen may include one or more of the composition(s) of the invention (e.g., any one of the stabilized trimers of the first or second aspect, the compositions of the third aspect, the vaccines of the fourth aspect, the nucleic acid molecules of the fifth aspect, and/or the vectors of the sixth aspect). In still other embodiments, the prime composition in this prime-boost regimen may include polypeptide(s) having the sequence(s) of any one of SEQ ID NOs: 8-32, or one or more vectors including nucleic acid molecules that encode any one of SEQ ID NOs: 8-32, followed by a boost including one or more of the composition(s) of the invention (e.g., any one of the stabilized trimers of the first or second aspect, the compositions of the third aspect, the vaccines of the fourth aspect, the nucleic acid molecules of the fifth aspect, and/or the vectors of the sixth aspect).

In still other embodiments, one or more composition(s) of the invention (e.g., a vaccine) is administered as the prime composition in a prime-boost regimen and the boost composition is a different vaccine composition, e.g., a vaccine that includes one or more polypeptides having at least 85% amino acid sequence identity (e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, any one or more of SEQ ID NOs: 6-32 (preferably the polypeptide of SEQ ID NO: 6 and/or 7), or one or more vectors (e.g., adenoviral or poxvirus vectors) each of which includes a nucleic acid molecule that encodes one or more polypeptides having at least 85% identity (e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or

the sequence of, one or more of SEQ ID NOs: 8-32 (preferably the vector encodes the polypeptide of SEQ ID NO: 6 and/or 7).

In some embodiments, the subject may, for example, be administered polypeptide compositions of the invention (e.g., stabilized gp140 Env trimers of the invention) in a non-vectored composition. The polypeptide composition administered may include between approximately 1 μg and 1 mg of stabilized Env trimers, and more preferably between 50 μg and 300 μg of stabilized Env trimers of the invention.

In other embodiments wherein the delivery vector is a virus, the subject can be administered at least about $1x10^3$ viral particles (vp)/dose or between $1x10^1$ and $1x10^{14}$ vp/dose, preferably between $1x10^3$ and $1x10^{12}$ vp/dose, and more preferably between $1x10^5$ and $1x10^{11}$ vp/dose. The composition may be administered, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 35, 40, 45, 50, 55, or 60 minutes, 2, 4, 6, 10, 15, or 24 hours, 2, 3, 5, or 7 days, 2, 4, 6 or 8 weeks, or even 3, 4, or 6 months pre-exposure or pre-diagnosis, or may be administered to the subject 15-30 minutes or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 20, 24, 48, or 72 hours, 2, 3, 5, or 7 days, 2, 4, 6 or 8 weeks, 3, 4, 6, or 9 months, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 years or longer post-diagnosis or post-exposure or to HIV. The subject is administered one or more doses of the composition once daily, weekly, monthly, or yearly. When treating an HIV infection, the composition(s) of the invention (e.g., any one of the stabilized trimers of the first, second, or third aspect, the compositions of the fourth or fifth aspect, the vaccines of the sixth aspect, the nucleic acid molecules of the seventh aspect, and/or the vectors of the eighth aspect) may be administered to the subject either before the occurrence of symptoms of an HIV infection or disease/syndrome (e.g., acquired immune deficiency syndrome (AIDS)) or a definitive diagnosis, or after diagnosis or symptoms become evident. The composition(s) may be administered, for example, immediately after diagnosis or the clinical recognition of symptoms or 2, 4, 6, 10, 15, or 24 hours, 2, 3, 5, or 7 days, 2, 4, 6 or 8 weeks, or even 3, 4, or 6 months after diagnosis or detection of symptoms.

In a ninth aspect, the invention provides methods of manufacturing a vaccine for treating or reducing the risk of an HIV infection in a subject in need thereof. The method includes the steps of: (a) contacting a nucleic acid of the second aspect of the invention (e.g., a nucleic acid that further includes a vector of the eighth aspect) with a cell; and (b) expressing the nucleic acid in the cell to form a stabilized trimer. In some embodiments, the method is performed *in vitro* or *ex vivo*. In some embodiments, the cell is a bacterial, plant, or mammalian cell (e.g., a human or non-human mammalian cell). In a preferred embodiment, the mammalian cell is a 293T cell.

In a final aspect, the invention features a kit including: (a) a composition of the invention (e.g., any one of the stabilized trimers of the first, second, or third aspect, the compositions of the fourth or fifth aspect, the vaccines of the sixth aspect, the nucleic acid molecules of the seventh aspect, and/or the vectors of the eighth aspect, e.g., a vaccine including mEnv and/or mEnv+ trimers and cEnv trimers); (b) a pharmaceutically acceptable carrier, excipient, or diluent; and (c) instructions for use thereof. The kit may optionally include an adjuvant.

In preferred embodiments of all aspects of the invention, the subject is a mammal, preferably a primate, such as a human.

Definitions

As used herein, the term "about" means +/- 10% of the recited value.

By "adenovirus" is meant a medium-sized (90-100 nm), non-enveloped icosahedral virus that includes a capsid and a double-stranded linear DNA genome. The adenovirus can be a naturally occurring, but isolated, adenovirus (e.g., sAd4287, sAd4310A, or sAd4312) or a recombinant adenovirus (e.g., replication-defective or replication competent sAd4287, sAd4310A, or sAd4312, or a chimeric variant thereof).

As used herein, "administering" is meant a method of giving a dosage of a pharmaceutical composition (e.g., a composition of the invention, such as any one of the vaccines of the first or fourth aspects, the compositions of the third aspect, the nucleic acid molecules of the fifth aspect, and/or the vectors of the sixth aspect) to a subject. The compositions utilized in the methods described herein can be administered, for example, intramuscularly, intravenously, intradermally, percutaneously, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, topically, intratumorally, peritoneally, subcutaneously, subconjunctivally, intravesicularlly, mucosally, intrapericardially, intraumbilically, intraocularly, orally, topically, locally, by inhalation, by injection, by infusion, by continuous infusion, by localized perfusion bathing target cells directly, by catheter, by lavage, by gavage, in cremes, or in lipid compositions. The preferred method of administration can vary depending on various factors (e.g., the components of the composition being administered and the severity of the condition being treated).

As used herein, the term "clade" refers to related human immunodeficiency viruses (HIVs) classified according to their degree of genetic similarity. There are currently three groups of HIV-1 isolates: M, N and O. Group M (major strains) consists of at least ten clades, A through J. Group O (outer strains) may consist of a similar number of clades. Group N is a new HIV-1 isolate that has not been categorized in either group M or O. In certain exemplary embodiments, a composition of the invention (e.g., any one of the vaccines of the first or fourth aspects, the compositions of the third aspect, the nucleic acid molecules of the fifth aspect, and/or the vectors of the sixth aspect) as described herein will recognize and raise an immune response (e.g., neutralizing anti-HIV antisera) against two, three, four, five, six, seven, eight, nine, ten or more clades and/or two or more groups of HIV.

Throughout this specification and claims, the word "comprise," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

As used herein, the term "envelope glycoprotein" refers, but is not limited to, the glycoprotein that is expressed on the surface of the envelope of HIV virions and the surface of the plasma membrane of HIV infected cells. The *env* gene encodes gp160, which is proteolytically cleaved into the gp120 and gp41 Envelope (Env) proteins. Gp120 binds to the CD4 receptor on a target cell that has such a receptor, such as, e.g., a T-helper cell. Gp41 is non-covalently bound to gp120, and provides the second step by which HIV enters the cell. It is originally buried within the viral envelope, but when gp120 binds to a CD4 receptor, gp120 changes its conformation causing gp41 to become exposed, where it can assist in fusion with the host cell.

By "gene product" is meant to include mRNAs transcribed from a gene as well as polypeptides translated from those mRNAs.

By "heterologous nucleic acid molecule" or "heterologous gene" is meant any exogenous nucleic acid molecule (e.g., a nucleic acid molecule encoding an optimized gp140 Env polypeptide of the invention) that can be inserted into the a vector of the invention (e.g., an adenovirus or poxvirus vector) for transfer into a cell, tissue, or organism, for subsequent expression of a gene product of interest or fragment thereof encoded by the heterologous nucleic acid molecule or gene. In a preferred embodiment, the heterologous nucleic acid molecule, which can be administered to a cell or subject as part of the present invention, can include, but is not limited to, a nucleic acid molecule encoding at least one optimized mosaic Env polypeptide (e.g., a mosaic Env1 polypeptide, such as mEnv and mEnv+) and/or a clade C Env polypeptide (e.g., a clade C Env1 polypeptide, such as cEnv).

By "human immunodeficiency virus" or "HIV" is meant a virus of the genus *Lentivirinae*, part of the family of *Retroviridae*, and includes, but is not limited to, HIV type 1 (HIV-1) and HIV type 2 (HIV-2), two species of HIV that infect humans.

By "immune response" is meant any response to an antigen or antigenic determinant by the immune system of a subject (e.g., a human). Exemplary immune responses include humoral immune responses (e.g., production of antigen-specific antibodies, e.g., neutralizing antibodies (NAbs)) and cell-mediated immune responses (e.g., lymphocyte proliferation).

As used herein, the term "reducing" with respect to HIV refers to a reduction or decrease of an HIV-mediated activity (e.g., infection, fusion (e.g., target cell entry and/or syncytia formation), viral spread, etc.) and/or a decrease in viral titer. HIV-mediated activity and/or HIV titer may be decreased by 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or more compared to that of a control subject (e.g., an untreated subject or a subject treated with a placebo).

By "neutralizing antibody" or "NAb" is meant an antibody which either is purified from, or is present in, serum and which recognizes a specific antigen (e.g., HIV Env glycoprotein, such as a gp140 polypeptide or a gp120 polypeptide) and inhibits the effect(s) of the antigen in the host (e.g., a human). As used herein, the antibody can be a single antibody or a plurality of antibodies.

"Nucleic acid" or "polynucleotide," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase, or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after synthesis, such as by conjugation with a label. Other types of modifications include, for example, "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example,

proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid or semi-solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and a basic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), "(O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

By "optimized" is meant an immunogenic polypeptide that is not a naturally-occurring peptide, polypeptide, or protein, such as a non-naturally occurring viral polypeptide (e.g., a gp140 polypeptide of the invention). Optimized viral polypeptide sequences are initially generated by modifying the amino acid sequence of one or more naturally-occurring viral gene products (e.g., peptides, polypeptides, and proteins, e.g., a viral Env polypeptide, e.g., a viral Env1, Env2, and/or Env3 polypeptide) to increase the breadth, intensity, depth, or longevity of the antiviral immune response (e.g., cellular or humoral immune responses) generated upon immunization (e.g., when incorporated into a composition of the invention, e.g., vaccine of the invention) of a subject (e.g., a human). Thus, the optimized viral polypeptide may correspond to a "parent" viral gene sequence; alternatively, the optimized viral polypeptide may not correspond to a specific "parent" viral gene sequence but may correspond to analogous sequences from various strains or quasi-species of a virus. Modifications to the viral gene sequence that can be included in an optimized viral polypeptide include amino acid additions, substitutions, and deletions. In one embodiment of the invention, the optimized polypeptide is a mosaic envelope protein, such as mosaic Env1 gp140 (see, e.g., U.S. Patent Publication No. 2012/0076812, herein incorporated by reference), or an optimized version thereof, which has been further altered to include a leader/signal sequence for maximal protein expression, cleavage site mutation(s), a factor Xa site, and/or a foldon trimerization domain (see, e.g., SEQ ID NO: 2). Methods of generating an optimized viral polypeptide are described in, e.g., Fisher et al. "Polyvalent Vaccine for Optimal Coverage of Potential T-Cell Epitopes in Global HIV-1 Variants," *Nat. Med.* 13(1):100-106 (2007) and International Patent Application Publication WO 2007/024941, herein incorporated by reference. Once the optimized viral polypeptide sequence is generated, the corresponding polypeptide can be produced or administered by standard techniques (e.g., recombinant viral vectors, such as the adenoviral vectors disclosed in International Patent Application

Publications WO 2006/040330 and WO 2007/104792, herein incorporated by reference) and optionally assembled in conjunction with one or more other viral polypeptides of the invention to form a stabilized polypeptide trimer.

By "pharmaceutically acceptable diluent, excipient, carrier, or adjuvant" is meant a diluent, excipient, carrier, or adjuvant which is physiologically acceptable to the subject while retaining the therapeutic properties of the pharmaceutical composition with which it is administered. One exemplary pharmaceutically acceptable carrier is physiological saline. Other physiologically acceptable diluents, excipients, carriers, or adjuvants and their formulations are known to one skilled in the art (see, e.g., U.S. Pub. No. 2012/0076812).

By "recombinant," with respect to a composition (e.g., a vector of the invention, such as an adenovirus or poxvirus vector), is meant a composition that has been manipulated *in vitro* (e.g., using standard cloning techniques) to introduce changes (e.g., changes to the composition, e.g., adenovirus or poxvirus genome of an adenovirus or poxvirus vector, respectively) that enable binding to or containment of a therapeutic agent and/or that promote the introduction of a therapeutic agent into a subject (e.g., a human) or a host cell. The recombinant composition of the invention may therefore be an adenoviral or poxviral transport vector (e.g., a replication-defective adenoviral or poxviral vector) for delivery of one or more of the stabilized Env polypeptide trimers of the invention.

By "sequence identity" or "sequence similarity" is meant that the identity or similarity between two or more amino acid sequences, or two or more nucleotide sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of "percentage (%) identity," wherein the higher the percentage, the more identity shared between the sequences. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similarity shared between the sequences. Homologs or orthologs of nucleic acid or amino acid sequences possess a relatively high degree of sequence identity/similarity when aligned using standard methods. Sequence identity may be measured using sequence analysis software on the default setting (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software may match similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

As used herein, the term "stabilized polypeptide trimer" refers, but is not limited to, an oligomer that includes a protein and/or polypeptide sequence that increases the stability (e.g., via the presence of one or more oligomerization domains) of the trimeric structure (e.g., reduces dissociation of a trimer into monomeric units). In particular, the stabilized polypeptide trimer is composed of three mosaic Env proteins (e.g., Env1, Env2, and/or Env3), three clade C Env proteins, or a combination of one or more mosaic Env proteins and one or more clade C Env proteins, in which at least one Env protein includes an oligomerization domain. An "oligomerization domain" refers, but is not limited to, a polypeptide sequence that can be used to increase the stability of an oligomeric envelope protein such as, e.g., to increase the stability of a HIV gp140 trimer. Oligomerization domains can be used to increase the stability of homooligomeric polypeptides as well as heterooligomeric polypeptides. Oligomerization domains are well known in the art, and include "trimerization domains." A trimerization domain refers to an oligomerization domain that stabilizes trimeric polypeptides (e.g., trimers consisting of one or more of the gp140

polypeptides of the invention). Examples of trimerization domains include, but are not limited to, the T4-fibritin "foldon" trimerization domain; the coiled-coil trimerization domain derived from GCN4 (Yang et al. (2002) J. Virol. 76:4634); and the catalytic subunit of *E. coli* aspartate transcarbamoylase as a trimer tag (Chen et al. (2004) J. Virol. 78:4508).

A "subject" is a vertebrate, such as a mammal (e.g., a human). Mammals also include, but are not limited to, farm animals (such as cows), sport animals (e.g., horses), pets (such as cats and dogs), mice, and rats. A subject to be treated according to the methods described herein (e.g., a subject having an HIV infection or a subject at risk of an HIV infection) may be one who has been diagnosed by a medical practitioner as having such a condition. Diagnosis may be performed by any suitable means. A subject in whom the risk of an HIV infection is to be reduced or prevented may or may not have received such a diagnosis. One skilled in the art will understand that a subject to be treated according to the present invention may have been subjected to standard tests or may have been identified, without examination, as one at high risk due to the presence of one or more risk factors (e.g., a needle stick or known exposure to HIV or an HIV infected individual).

By "having substantially the sequence of" with respect to constructs of the invention is meant having at least 99% sequence identity to a recited reference sequence (e.g., having no more than 7 amino acid residue differences, e.g., 1, 2, 3, 4, 5, or 6 amino acid residue differences (e.g., additions, deletions, or conservative amino acid substitutions), relative to a recited reference sequence).

By "therapeutically effective amount" is meant an amount of a therapeutic agent that alone, or together with one or more additional (optional) therapeutic agents, produces beneficial or desired results upon administration to a mammal. The therapeutically effective amount depends upon the context in which the therapeutic agent is applied. For example, in the context of administering a vaccine composition including a therapeutic agent such as a stabilized gp140 trimer of the invention, the therapeutically effective amount of the vaccine composition is an amount sufficient to achieve a reduction in the level of HIV (e.g., as measured by a stabilization or decrease in HIV titer compared to a non-treated control), and/or an increase in the level of neutralizing anti-HIV antisera (e.g., as measured by an increase in serum neutralizing antibody levels relative to a non-treated control in a luciferase-based virus neutralization assay) as compared to a response obtained without administration of a composition of the invention (e.g., a vaccine composition), and/or to prevent the propagation of an infectious virus (e.g., HIV) in a subject (e.g., a human) having an increased risk of viral infection. Ideally, a therapeutically effective amount provides a therapeutic effect without causing a substantial cytotoxic effect in the subject. In general, a therapeutically effective amount of a composition administered to a subject (e.g., a human) will vary depending upon a number of factors associated with that subject, for example the overall health of the subject, the condition to be treated, or the severity of the condition. A therapeutically effective amount of a composition can be determined by varying the dosage of the product and measuring the resulting therapeutic response.

As used herein, and as well understood in the art, "treatment" is an approach for obtaining beneficial or desired results, such as clinical results. Beneficial or desired results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions; diminishment of extent of disease, disorder, or condition; stabilization (i.e., not worsening) of a state of disease, disorder, or condition; prevention of spread of disease, disorder, or condition; delay or slowing the progress of the

disease, disorder, or condition; amelioration or palliation of the disease, disorder, or condition; and remission (whether partial or total), whether detectable or undetectable. "Palliating" a disease, disorder, or condition means that the extent and/or undesirable clinical manifestations of the disease, disorder, or condition are lessened and/or time course of the progression is slowed or lengthened, as compared to the extent or time course in the absence of treatment.

The term "vaccine," as used herein, is defined as material used to provoke an immune response (e.g., the production of neutralizing anti-HIV antisera). Administration of the vaccine to a subject may confer at least partial immunity against HIV infection.

As used herein, the term "vector" is meant to include, but is not limited to, a virus (e.g., adenovirus or poxvirus), naked DNA, oligonucleotide, cationic lipid (e.g., liposome), cationic polymer (e.g., polysome), virosome, nanoparticle, or dentrimer. By "adenovirus vector" is meant a composition that includes one or more genes (non-structural or structural), or fragments thereof, from an adenoviral species (e.g., adenovirus serotype 11 (Ad11), adenovirus serotype 15 (Ad15), adenovirus serotype 24 (Ad24), adenovirus serotype 26 (Ad26), adenovirus serotype 34 (Ad34), adenovirus serotype 35 (Ad35), adenovirus serotype 48 (Ad48), adenovirus serotype 49 (Ad49), adenovirus serotype 50 (Ad50), Pan9 (AdC68), or a chimeric variant thereof (e.g., adenovirus serotype 5 HVR48 (Ad5HVR48))) that may be used to transmit one or more heterologous genes (e.g., one or more of the optimized gp140 polypeptides of the invention) from a viral or non-viral source to a subject or a host. The nucleic acid material of the viral vector may be encapsulated, e.g., in a lipid membrane or by structural proteins (e.g., capsid proteins), that may include one or more viral polypeptides (e.g., an envelope glycoprotein). The viral vector can be used to infect cells of a subject, which, in turn, promotes the translation of the heterologous gene(s) of the viral vector into a protein product (e.g., one or more of the gp140 Env polypeptides described herein, such that a stabilized trimer of the invention is formed).

The term "virus," as used herein, is defined as an infectious agent that is unable to grow or reproduce outside a host cell and that infects mammals (e.g., humans) or birds.

Other features and advantages of the invention will be apparent from the following Detailed Description, the drawings, and the claims.

Brief Description of the Drawings

Figure 1A shows the amino acid sequence of a mosaic human immunodeficiency virus (HIV) gp140 Envelope (Env) polypeptide ("mEnv"; SEQ ID NO: 1) of the invention. The boxed region identifies the signal/leader sequence; the underlined region identifies gp120; the plain text region identifies the gp41 ectodomain; and the double-underscored region identifies the T4-fibritin "foldon" trimerization/oligomerization domain.

Figure 1B shows the amino acid sequence of a mosaic HIV gp140 Env polypeptide ("mEnv+"; SEQ ID NO: 2) of the invention. This polypeptide sequence has been further optimized and includes a different signal/leader sequence to maximize protein expression (boxed region); the addition of cleavage site-inactivating mutations (E/E substitution mutations) (circled residues); and the addition of a Factor Xa site (zig-zag underlined region). Other regions are noted as in Figure 1A.

Figure 1C shows the amino acid sequence of an optimized clade C Env polypeptide (cEnv; SEQ ID NO: 3) of the invention. All regions are noted as in Figure 1B.

Figure 2 is a Western blot showing the expression levels of mEnv and mEnv+ in lanes 3 and 4, respectively, compared to cEnv and an expression vector control (pVRC8400) in lanes 1 and 2, respectively.

Figure 3 is a gel filtration chromatograph depicting the uniform elution of mEnv+ trimers six days post-PEI transfection of 293T cells in roller bottles (750-ml of supernatant).

Figure 4 is an image of a 4-16% gradient SDS-PAGE showing the peak fractions of mEnv+ following gel filtration elution. The final protein yield per purification is approximately 8.44 mg following gel filtration. The final concentration is approximately 5.62 mg/ml.

Figure 5A is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with clade C gp140 Env (cEnv) homotrimer tested against a multi-clade panel of tier 1 neutralization-sensitive isolates including clade B (SF162.LS and Bal.26) and clade C (MW965.26 and TV1.21) HIV-1 Envelope pseudoviruses, as well as Murine lukemia virus (MuLV) (negative control).

Figure 5B is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with mosaic gp140 Env version-1 (mEnv) homotrimer tested against a multi-clade panel of tier 1 neutralization-sensitive isolates including clade B (SF162.LS and Bal.26) and clade C (MW965.26 and TV1.21) HIV-1 Envelope pseudoviruses, as well as Murine lukemia virus (MuLV) (negative control).

Figure 5C is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with both cEnv and mEnv trimers tested against a multi-clade panel of tier 1 neutralization-sensitive isolates including clade B (SF162.LS and Bal.26) and clade C (MW965.26 and TV1.21) HIV-1 Envelope pseudoviruses, as well as Murine lukemia virus (MuLV) (negative control).

Figure 6A is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with cEnv homotrimer, mEnv homotrimer, or both cEnv and mEnv trimers tested against a Tier 1B intermediate neutralization-sensitive clade A HIV-1 Envelope pseudovirus, MS208.A1.

Figure 6B is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with cEnv homotrimer, mEnv homotrimer, or both cEnv and mEnv trimers tested against a Tier 1B intermediate neutralization-sensitive clade A HIV-1 Envelope pseudovirus, Q23.17.

Figure 7A is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with cEnv homotrimer, mEnv homotrimer, or both cEnv and mEnv trimers tested against a Tier 1A highly neutralization-sensitive clade B HIV-1 Envelope pseudovirus, SF162.LS.

Figure 7B is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with cEnv homotrimer, mEnv homotrimer, or both cEnv and mEnv trimers tested against a Tier 1B intermediate neutralization-sensitive clade B HIV-1 Envelope pseudovirus, BaL.26.

Figure 7C is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with cEnv

homotrimer, mEnv homotrimer, or both cEnv and mEnv trimers tested against a Tier 1B intermediate neutralization-sensitive clade B HIV-1 Envelope pseudovirus, SS1196.1.

Figure 7D is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with cEnv homotrimer, mEnv homotrimer, or both cEnv and mEnv trimers tested against a Tier 1B intermediate neutralization-sensitive clade B HIV-1 Envelope pseudovirus, 6535.3.

Figure 8A is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with cEnv homotrimer, mEnv homotrimer, or both cEnv and mEnv trimers tested against a Tier 1A highly neutralization-sensitive clade C HIV-1 Envelope pseudovirus, MW965.26.

Figure 8B is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with cEnv homotrimer, mEnv homotrimer, or both cEnv and mEnv trimers tested against a Tier 1B intermediate neutralization-sensitive clade C HIV-1 Envelope pseudovirus, TV1.21.

Figure 8C is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) with cEnv homotrimer, mEnv homotrimer, or both cEnv and mEnv trimers tested against a Tier 1B intermediate neutralization-sensitive clade C HIV-1 Envelope pseudovirus, ZM109F.PB4.

Figure 8D is a graph showing a quantitative analysis of ID_{50} titer measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post) cEnv homotrimer, mEnv homotrimer, or both cEnv and mEnv trimers tested against a Tier 1B intermediate neutralization-sensitive clade C HIV-1 Envelope pseudovirus, ZM197M.PB7.

Figure 9A is a Western blot showing expression of HIV-1 mEnv, mEnv2, and mEnv3 48-hours after transient transfection of 293T cells. cEnv (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111; Nkolola et al. (2010) J. Virol. 84:3270) and Clade A (92UG037.8) gp140 (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111) were used as positive controls.

Figure 9B is a graph showing the size-exclusion chromatography profile of purified mEnv.

Figure 9C is a graph showing the size exclusion chromatography profile of mEnv after freeze-thaw and incubation at 4°C for 7 days. The signals for freeze-thaw and 4°C incubation for 7 days overlap closely.

Figure 10A is a graph showing the surface plasmon resonance (SPR) binding profile of mEnv to CD4. Soluble, two-domain CD4 was irreversibly coupled to a CM5 chip and mEnv flowed over the chip at concentrations of 62.5-1000 nM. Sensorgrams are presented in black and kinetic fits are presented in gray; the two signals overlap closely. RU = response units.

Figure 10B is a graph showing the SPR binding profile of mEnv to 17b IgG. 17b IgG was captured and mEnv trimer flowed over bound IgG at a concentration of 1000 nM in the presence (gray trace) or absence (black trace) of CD4 bound to the immunogen.

Figures 10C and 10D are graphs showing the SPR binding profiles of mEnv to VRC01 and 3BNC117. VRC01 IgG (C) or 3BNC117 IgG (D) were captured and mEnv trimer flowed over the bound IgGs at concentrations of 62.5-1000 nM. Sensorgrams are presented in black and kinetic fits are presented in gray; in both graphs, the two signals overlap closely. RU = response units.

Figures 11A – 11F are graphs showing the SPR binding profiles of mEnv to glycan-dependent bnAbs PGT121, PGT126 and quaternary dependent bnAbs PG9 and PG16. For all experiments, protein A was irreversibly coupled to a CM5 chip and IgGs were captured. Mosaic M gp140 was flowed over bound (A) PGT121 IgG and (B) PGT126 IgG at concentrations of 62.5-1000 nM. Mosaic gp140 was flowed over bound (C) PG9 IgG and (D) PG16 IgG. Mosaic gp120 was also flowed over bound (E) PG9 IgG and (F) PG16 IgG. Sensorgrams are presented in black and kinetic fits are presented in gray; in all graphs, the two signals overlap closely. RU = response units.

Figure 12A is a sequence alignment of the 2F5 and 4E10 epitope sequences to the mEnv trimer sequence.

Figure 12B is a Western blot of 2F5 and 4E10 bound to (1) mEnv (2) 92UG037.8 gp140 (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111; Nkolola et al. (2010) J. Virol. 84:3270) (positive control) and (3) mosaic M gp120 (negative control).

Figure 12C is a set of graphs showing 2F5 and 4E10 binding ELISA of mEnv. Both mEnv (mosaic gp140) and mosaic gp120 show no detectable signal. Clade A (92UG037.8) intermediate-gp41 (Frey et al. (2008) Proc. Natl. Acad. Sci. U.S.A. 105:3739) (positive control) is presented as squares, mEnv is presented as triangles. Dotted line indicates assay background threshold.

Figure 12D is a set of graphs showing SPR binding of protein-A captured 2F5 and 4E10 to mEnv, with GCN4 gp41 positive control in black and mEnv in gray.

Figure 13 is a graph showing mEnv pseudovirion infection of TZM.bl cells. Pseudovirions generated with full length mosaic M gp160 Env were used to infect target TZM.bl cells expressing CD4 and co-receptors CCR5/CXCR4 in the TZM.bl assay. Broken horizontal line indicates background of TZM.bl cells alone without virus (negative control). RLU, relative luminescence units.

Figures 14A and 14B are graphs showing ELISA end-point binding antibody responses. Sera obtained 4 weeks after each immunization in guinea pigs immunized with clade C gp140 (cEnv), mEnv or bivalent cEnv + mEnv (C+M) gp140s in (A) Matrix M or (B) CpG/Emulsigen adjuvants were tested in endpoint ELISAs against mEnv and cEnv trimer antigens. Data are presented as geometric mean titers at each time point +/- standard deviations. The horizontal broken line indicates assay background threshold. *P<0.05; unpaired t-test

Figures 15A and 15B are graphs showing neutralizing antibody titers against panels of tier 1 HIV-1 isolates in the TZM.bl assay, using Matrix M adjuvant. Sera obtained post-third-trimer vaccination in cEnv (C), mEnv (M), or bivalent cEnv + mEnv (C+M) vaccinated guinea pigs were tested against a multi-clade panel of tier 1 (MW965.26, SF162.LS, Bal.26, DJ263.8, TV1.21, MS208, Q23.17, SS1196.1, 6535.3, ZM109.F, ZM197M) HIV-1 Env pseudoviruses in TZM.bl neutralization assays. Horizontal lines indicate median titers. Broken horizontal line indicates assay background threshold. Sera from pre-vaccination time points showed minimal background. *P<0.05; Mann-Whitney test.

Figures 15C and 15D are graphs showing neutralizing antibody titers against panels of tier 1 HIV-1 isolates in the TZM.bl assay, using CpG/Emulsigen adjuvant. Sera obtained post-third-trimer vaccination in cEnv (C), mEnv (M), or bivalent cEnv + mEnv (C+M) vaccinated guinea pigs were tested against a multi-clade panel of tier 1 (MW965.26, SF162.LS, Bal.26, DJ263.8, TV1.21, MS208, Q23.17, SS1196.1, 6535.3, ZM109.F, ZM197M) HIV-1 Env pseudoviruses in TZM.bl neutralization assays.

Horizontal lines indicate median titers. Broken horizontal line indicates assay background threshold. Sera from pre-vaccination time points showed minimal background. *P<0.05; Mann-Whitney test.

Figures 16A and 16B are graphs showing neutralizing antibody titers of guinea pig sera against HIV-1 tier 2 clade B and C isolates in the A3R5 neutralization assay. Sera obtained pre-vaccination (Pre) and post-third-trimer vaccination (Post) in Matrix M adjuvanted cEnv, mEnv or bivalent cEnv + mEnv (C+M) vaccinated guinea pigs were tested against (A) tier 2 clade C isolates Ce_1086_B2, Ce2010 and Du422.1 and (B) tier 2 clade B isolates SC22.3C2, SUMA and REJO in A3R5 neutralization assays. Broken horizontal line indicates assay background threshold. *P<0.05; Mann-Whitney test.

Figure 17A is a graph showing robust purity of the optimized version of mosaic M gp140 referred to as "mEnv+" in a 2-liter fresh preparation.

Figure 17B is a graph showing robust stability of mEnv+ after freeze/thaw or incubation at 4°C for 1 week. The signals for freeze-thaw and 4°C incubation for 7 days overlap closely.

Figure 17C is an SDS-PAGE gel showing robust stability of mEnv+ after freeze/thaw or incubation at 4 degrees C for 1 week.

Figures 18A – 18B are graphs showing immunogenicity of mEnv using Adju-Phos as the adjuvant compared to mEnv+ using various adjuvants. mEnv and mEnv+ show comparable immunogenicity as measured by binding antibodies by ELISA.

Figure 19 is a set of graphs showing quantitative analyses of ID_{50} titers, measuring TZM.bl neutralizing antibody responses in guinea pigs pre-vaccination (Pre) and post-vaccination (Post). mEnv and mEnv+ showed comparable immunogenicity in these assays regardless of adjuvant.

Detailed Description of the Invention

Most antibodies induced by human immunodeficiency virus (HIV) (e.g., HIV type 1 (HIV-1)) are ineffective at preventing initiation or spread of infection, as they are either non-neutralizing or narrowly isolate-specific. One of the biggest challenges in HIV vaccine development is to design a HIV envelope immunogen that can induce protective, neutralizing antibodies effective against the diverse HIV strains that characterize the global pandemic. Indeed, the generation of "broadly neutralizing" antibodies that recognize relatively conserved regions on the envelope glycoprotein are rare. The present invention is based in part on the discovery of stabilized trimeric HIV envelope (Env) proteins and combinations thereof that elicit a surprisingly broad neutralizing antibody response *in vivo*.

Stabilized gp140 Env Trimers of the Invention

The invention features novel stabilized HIV gp140 Env polypeptide trimers. Stabilized trimers of the invention feature optimized gp140 Env polypeptides. These polypeptides may have, or may be modified to include, one or more of the following domains and/or mutations. The gp140 Env polypeptide constituents may include a T4-fibritin "foldon" trimerization domain sequence to support stable trimer formation (see, e.g., Figures 1A, 1B, and 1C, depicting the amino acid sequences of mEnv (SEQ ID NO: 1), mEnv+ (SEQ ID NO: 2), and cEnv (SEQ ID NO: 3), respectively, which each include a C-terminal trimerization domain). The optimized gp140 Env polypeptides may also include cleavage site mutations to enhance stability, for example, by eliminating cleavage by a peptidase (see, e.g., Figures 1B and 1C, which depict the mutated residues as circled residues in the mEnv+ and cEnv amino acid sequence,

respectively, between the gp120 and gp41 moieties). The optimized gp140 Env polypeptides may additionally have a signal/leader sequence to maximize protein expression (see, e.g., the signal/leader sequence of mEnv+ or cEnv, demarcated in Figures 1B and 1C, respectively). Further, the optimized gp140 Env polypeptides may include a Factor Xa cleavage site (SRIEGR), which may, for example, be incorporated upstream of (N-terminal to) the trimerization domain (see, e.g., Figures 1B and 1C, which depict the location of the Factor Xa cleavage site in the amino acid sequence of mEnv+ and cEnv, respectively). As discussed herein below, the stabilized trimers of the invention are preferably homotrimers (e.g., trimers composed of three identical polypeptides). Heterotrimers (e.g., trimers composed of three polypeptides that are not all identical) of the invention are also envisioned.

The stabilized trimers of the invention are preferably stabilized homotrimers that include, for example, three gp140 polypeptides, wherein each of the gp140 polypeptides includes an amino acid sequence having at least 90% identity (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 2 (mEnv+). The invention also features stabilized homotrimers including three gp140 polypeptides, wherein each of said gp140 polypeptides includes an amino acid sequence having substantially the sequence of (e.g., 99% or more identity), or the sequence of, SEQ ID NO: 1 (mEnv) or SEQ ID NO: 3 (cEnv) or SEQ ID NO: 4 or SEQ ID NO: 5. Exemplary homotrimers of the invention include Trimers 1, 2, and 3 in Table 1 below.

Alternatively, the stabilized trimer of the invention may be a stabilized heterotrimer. For example, the stabilized trimer may be a stabilized heterotrimer that includes a combination of two different mosaic Env1 sequences (e.g., one mEnv and two mEnv+; two mEnv and one mEnv+; or one mEnv, one mEnv+, and cEnv). In some instances, the stabilized heterotrimer includes cEnv and two of the same Env1 polypeptides (e.g., two mEnv and one cEnv; two mEnv+ and one cEnv). In other instances, the stabilized heterotrimer includes one cEnv and two different mosaic Env1 polypeptides (e.g., one cEnv, one mEnv, and one mEnv+).

Alternatively, the stabilized heterotrimer may include one, two or three constituent Env polypeptides including an amino acid sequence of SEQ ID NO: 4 (mosaic gp140 Env2, "mEnv2") or SEQ ID NO: 5 (mosaic gp140 Env3, "mEnv3"). Preferably, mEnv2 or mEnv3 is modified in a similar manner to that of mEnv, mEnv+, or cEnv, which each possess a trimerization domain, as discussed above and as depicted in Figures 1A-1C. Therefore, other stabilized heterotrimers of the invention include trimers having the following constituent polypeptides: one mEnv and two mEnv2; two mEnv and one mEnv2; one mEnv+ and two mEnv2; two mEnv+ and one mEnv2; one cEnv and two mEnv2; two cEnv and one mEnv2; one mEnv, one mEnv+, and one mEnv2; one mEnv, one cEnv, and mEnv2; one mEnv+, one cEnv, and one mEnv2; one mEnv and two mEnv3; two mEnv and one mEnv3; one mEnv+ and two mEnv3; two mEnv+ and one mEnv3; one cEnv and two mEnv3; two cEnv and one mEnv3; one mEnv, one mEnv+, and one mEnv3; one mEnv, one cEnv, and mEnv3; one mEnv+, one cEnv, and one mEnv3; one mEnv, one mEnv2, and one mEnv3; one mEnv+, one mEnv2, and one mEnv3; or one cEnv, one mEnv2, and one mEnv3. Exemplary heterotrimers of the invention include Trimers 4-31 in Table 1 below.

Table 1.

Exemplary Trimer	Constituent Polypeptides		
	Polypeptide 1	Polypeptide 2	Polypeptide 3
Trimer 1	SEQ ID NO: 1	SEQ ID NO: 1	SEQ ID NO: 1
Trimer 2	SEQ ID NO: 2	SEQ ID NO: 2	SEQ ID NO: 2
Trimer 3	SEQ ID NO: 3	SEQ ID NO: 3	SEQ ID NO: 3
Trimer 4	SEQ ID NO: 1	SEQ ID NO: 2	SEQ ID NO: 2
Trimer 5	SEQ ID NO: 1	SEQ ID NO: 1	SEQ ID NO: 2
Trimer 6	SEQ ID NO: 1	SEQ ID NO: 3	SEQ ID NO: 3
Trimer 7	SEQ ID NO: 1	SEQ ID NO: 1	SEQ ID NO: 3
Trimer 8	SEQ ID NO: 2	SEQ ID NO: 3	SEQ ID NO: 3
Trimer 9	SEQ ID NO: 2	SEQ ID NO: 2	SEQ ID NO: 3
Trimer 10	SEQ ID NO: 1	SEQ ID NO: 2	SEQ ID NO: 3
Trimer 11	SEQ ID NO: 1	SEQ ID NO: 4	SEQ ID NO: 4
Trimer 12	SEQ ID NO: 1	SEQ ID NO: 1	SEQ ID NO: 4
Trimer 13	SEQ ID NO: 2	SEQ ID NO: 4	SEQ ID NO: 4
Trimer 14	SEQ ID NO: 2	SEQ ID NO: 2	SEQ ID NO: 4
Trimer 15	SEQ ID NO: 3	SEQ ID NO: 4	SEQ ID NO: 4
Trimer 16	SEQ ID NO: 3	SEQ ID NO: 3	SEQ ID NO: 4
Trimer 17	SEQ ID NO: 1	SEQ ID NO: 2	SEQ ID NO: 4
Trimer 18	SEQ ID NO: 1	SEQ ID NO: 3	SEQ ID NO: 4
Trimer 19	SEQ ID NO: 2	SEQ ID NO: 3	SEQ ID NO: 4
Trimer 20	SEQ ID NO: 1	SEQ ID NO: 5	SEQ ID NO: 5
Trimer 21	SEQ ID NO: 1	SEQ ID NO: 1	SEQ ID NO: 5
Trimer 22	SEQ ID NO: 2	SEQ ID NO: 5	SEQ ID NO: 5
Trimer 23	SEQ ID NO: 2	SEQ ID NO: 2	SEQ ID NO: 5
Trimer 24	SEQ ID NO: 3	SEQ ID NO: 5	SEQ ID NO: 5
Trimer 25	SEQ ID NO: 3	SEQ ID NO: 3	SEQ ID NO: 5
Trimer 26	SEQ ID NO: 1	SEQ ID NO: 2	SEQ ID NO: 5
Trimer 27	SEQ ID NO: 1	SEQ ID NO: 3	SEQ ID NO: 5
Trimer 28	SEQ ID NO: 2	SEQ ID NO: 3	SEQ ID NO: 5
Trimer 29	SEQ ID NO: 1	SEQ ID NO: 4	SEQ ID NO: 5
Trimer 30	SEQ ID NO: 2	SEQ ID NO: 4	SEQ ID NO: 5
Trimer 31	SEQ ID NO: 3	SEQ ID NO: 4	SEQ ID NO: 5

Stabilized gp140 Env Trimer Compositions of the Invention

Any one of the stabilized gp140 Env trimers of the invention, such as those described above, can be included in compositions (e.g., pharmaceutical compositions). Accordingly, the invention features a composition including at least one of the stabilized gp140 Env trimers described above (e.g., at least 2, 3, 4, 5, or more different types of stabilized gp140 Env trimers may be included in a single composition or vaccine). For example, a composition including a homotrimer of mEnv or mEnv+ may additionally include an additional stabilized trimer form, for example, an additional stabilized trimer form that includes three gp140 polypeptides, wherein each of the gp140 polypeptides comprises an amino acid sequence having at least 90% identity (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 3 (cEnv).

The compositions may include a stabilized homotrimer including three mosaic Env1 polypeptides, for example, three polypeptides of mEnv or three polypeptides of mEnv+ or three optimized clade C Env polypeptides, such as the cEnv polypeptide of SEQ ID NO: 3.

Alternatively, the compositions may also include a stabilized heterotrimer. For example, the composition (e.g., a vaccine) may include at least one stabilized heterotrimer that includes a combination of two different mosaic Env1 sequences (e.g., one mEnv and two mEnv+; and two mEnv and one mEnv+). In some embodiments, the composition (e.g., a vaccine) includes at least one stabilized heterotrimer that includes cEnv and Env1 polypeptide (e.g., two mEnv and one cEnv; two mEnv+ and one cEnv; two cEnv and one mEnv; and two cEnv and one mEnv+). In other embodiments, the compositions include at least one stabilized heterotrimer that includes one cEnv and two different mosaic Env1 polypeptides (e.g., one cEnv, one mEnv, and one mEnv+).

Optionally, the compositions may include at least one stabilized heterotrimer having one, two or three constituent Env polypeptides including an amino acid sequence of SEQ ID NO: 4 (mosaic gp140 Env2, "mEnv2") or SEQ ID NO: 5 (mosaic gp140 Env3, "mEnv3"). As noted above, preferably, mEnv2 or mEnv3 may be, and is preferably, modified in a similar manner to that of mEnv, mEnv+, or cEnv, which each possess a trimerization domain, as discussed above and depicted in Figures 1A-1C. Therefore, other vaccines of the invention may include stabilized heterotrimers having the following constituent polypeptides: one mEnv and two mEnv2; two mEnv and one mEnv2; one mEnv+ and two mEnv2; two mEnv+ and one mEnv2; one cEnv and two mEnv2; two cEnv and one mEnv2; one mEnv, one mEnv+, and one mEnv2; one mEnv, one cEnv, and mEnv2; one mEnv+, one cEnv, and one mEnv2; one mEnv and two mEnv3; two mEnv and one mEnv3; one mEnv+ and two mEnv3; two mEnv+ and one mEnv3; one cEnv and two mEnv3; two cEnv and one mEnv3; one mEnv, one mEnv+, and one mEnv3; one mEnv, one cEnv, and mEnv3; one mEnv+, one cEnv, and one mEnv3; one mEnv, one mEnv2, and one mEnv3; one mEnv+, one mEnv2, and one mEnv3; or one cEnv, one mEnv2, and one mEnv3.

Any one of the compositions of the invention may further include a pharmaceutically acceptable carrier, excipient, or diluent, and/or an adjuvant.

Stabilized gp140 Env Trimer Vaccines of the Invention

The invention features vaccines including at least one of the compositions of the invention described herein and above. The vaccine may be used for treating or reducing the risk of a human immunodeficiency virus (HIV) infection in a subject in need thereof. For example, the vaccine may elicit production of neutralizing anti-HIV antisera (e.g., neutralizing anti-HIV-1 antisera) after administration to the subject. The anti-HIV antisera may also be able to neutralize HIV (e.g., HIV-1), for example, selected from any one or more of clade A, clade B, and clade C.

Nucleic Acid Molecules of the Invention

In some embodiments, the vaccines of the invention include one or more nucleic acid molecules of the invention, such as a nucleic acid molecule having a nucleotide sequence that encodes a gp140 polypeptide, in which the gp140 polypeptide includes (a) an amino acid sequence having at least 95% identity (e.g., 96%, 97%, 98%, 99%, or 100% identity) to SEQ ID NO: 1, (b) an amino acid sequence having at least 95% identity (e.g., 96%, 97%, 98%, 99%, or 100% identity) to SEQ ID NO: 2, and/or (c) an amino acid sequence having the sequence of SEQ ID NO: 3, (d) an amino acid sequence having the sequence of SEQ ID NO: 4. (e) an amino acid sequence having the sequence of SEQ ID NO: 5 and/or combinations thereof. As discussed below, vectors (e.g., viral vectors, such as an adenovirus or poxvirus

vector) of the invention can include one or more of these nucleic acid molecules. Accordingly, vaccines of the invention may include one or more of these vectors. The stabilized gp140 Env trimer polypeptides of the invention, as well as vaccines, nucleic acids, and vectors that incorporate one or more optimized gp140 Env polypeptides, can be recombinantly expressed in a cell or organism, or can be directly administered to a subject (e.g., a human) infected with, or at risk of becoming infected with, HIV (e.g., HIV-1).

Vectors of the Invention

As noted above, the invention features vectors including one or more of the nucleic acid molecules of the invention. The vector can be, for example, a carrier (e.g., a liposome), a plasmid, a cosmid, a yeast artificial chromosome, or a virus (e.g., an adenovirus vector or a poxvirus vector) that includes one or more of the nucleic acid molecules of the invention.

An adenovirus vector of the invention can be derived from a recombinant adenovirus serotype 11 (Ad11), adenovirus serotype 15 (Ad15), adenovirus serotype 24 (Ad24), adenovirus serotype 26 (Ad26), adenovirus serotype 34 (Ad34), adenovirus serotype 35 (Ad35), adenovirus serotype 48 (Ad48), adenovirus serotype 49 (Ad49), adenovirus serotype 50 (Ad50), Pan9 (AdC68), or a chimeric variant thereof (e.g., adenovirus serotype 5 HVR48 (Ad5HVR48)). A poxvirus vector of the invention may be derived, for example, from modified vaccinia virus Ankara (MVA). These vectors can include additional nucleic acid sequences from several sources.

Vectors of the invention can be constructed using any recombinant molecular biology technique known in the art. The vector, upon transfection or transduction of a target cell or organism, can be extrachromosomal or integrated into the host cell chromosome. The nucleic acid component of a vector can be in single or multiple copy number per target cell, and can be linear, circular, or concatamerized. The vectors can also include internal ribosome entry site (IRES) sequences to allow for the expression of multiple peptide or polypeptide chains from a single nucleic acid transcript (e.g., a polycistronic vector, e.g., a bi- or tri-cistronic vector).

Vectors of the invention can also include gene expression elements that facilitate the expression of the encoded polypeptide(s) of the invention (e.g., SEQ ID NOs: 1 (mEnv), 2 (mEnv+), 3 (cEnv), 4 and/or 5 or polypeptides having amino acids sequences with at least 90%, 91%, 92$, 93&, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 1 or 2). Gene expression elements include, but are not limited to, (a) regulatory sequences, such as viral transcription promoters and their enhancer elements, such as the SV40 early promoter, Rous sarcoma virus LTR, and Moloney murine leukemia virus LTR; (b) splice regions and polyadenylation sites such as those derived from the SV40 late region; and (c) polyadenylation sites such as in SV40. Also included are plasmid origins of replication, antibiotic resistance or selection genes, multiple cloning sites (e.g., restriction enzyme cleavage loci), and other viral gene sequences (e.g., sequences encoding viral structural, functional, or regulatory elements, such as the HIV long terminal repeat (LTR)).

Exemplary vectors are described below.

Adenovirus Vectors

Recombinant adenoviruses offer several significant advantages for use as vectors for the expression of, for example, one or more of the optimized gp140 Env polypeptides of the invention. The viruses can be prepared to high titer, can infect non-replicating cells, and can confer high-efficiency transduction of target cells *ex vivo* following contact with a target cell population. Furthermore, adenoviruses do not integrate their DNA into the host genome. Thus, their use as expression vectors has a reduced risk of inducing spontaneous proliferative disorders. In animal models, adenoviral vectors have generally been found to mediate high-level expression for approximately one week. The duration of transgene expression (expression of a nucleic acid molecule of the invention) can be prolonged by using cell or tissue-specific promoters. Other improvements in the molecular engineering of the adenovirus vector itself have produced more sustained transgene expression and less inflammation. This is seen with so-called "second generation" vectors harboring specific mutations in additional early adenoviral genes and "gutless" vectors in which
virtually all the viral genes are deleted utilizing a Cre-Lox strategy (Engelhardt et al., *Proc. Natl. Acad. Sci. USA* 91:6196 (1994) and Kochanek et al., *Proc. Natl. Acad. Sci. USA* 93:5731 (1996), each herein incorporated by reference).

The rare serotype and chimeric adenoviral vectors disclosed in International Patent Application Publications WO 2006/040330 and WO 2007/104792, each incorporated by reference herein, are particularly useful as vectors of the invention. For example, recombinant adenovirus serotype 11 (Ad11), adenovirus serotype 15 (Ad15), adenovirus serotype 24 (Ad24), adenovirus serotype 26 (Ad26), adenovirus serotype 34 (Ad34), adenovirus serotype 35 (Ad35), adenovirus serotype 48 (Ad48), adenovirus serotype 49 (Ad49), adenovirus serotype 50 (Ad50), Pan9 (AdC68), or a chimeric variant thereof (e.g., adenovirus serotype 5 HVR48 (Ad5HVR48) can encode and/or deliver one or more of the optimized gp140 Env polypeptides of the invention to facilitate formation and presentation of gp140 Env trimer formation. In some embodiments, one or more recombinant adenovirus vectors can be administered to the subject in order to express gp140 Env polypeptides for formation of stabilized trimers of the invention.

Adeno-Associated Virus (AAV) Vectors

Adeno-associated viruses (AAV), derived from non-pathogenic parvoviruses, can also be used to facilitate delivery and/or expression of one or more of the optimized gp140 Env polypeptides of the invention as these vectors evoke almost no anti-vector cellular immune response, and produce transgene expression lasting months in most experimental systems.

Stabilized trimers of the invention may be produced upon expression of the gp140 Env polypeptides described herein using an AAV vector.

Retrovirus Vectors

Retroviruses are useful for the expression of optimized gp140 Env polypeptides of the invention. Unlike adenoviruses, the retroviral genome is based in RNA. When a retrovirus infects a cell, it will introduce its RNA together with several enzymes into the cell. The viral RNA molecules from the retrovirus will produce a double-stranded DNA copy, called a provirus, through a process called reverse

transcription. Following transport into the cell nucleus, the proviral DNA is integrated in a host cell chromosome, permanently altering the genome of the transduced cell and any progeny cells that may derive from this cell. The ability to permanently introduce a gene into a cell or organism is the defining characteristic of retroviruses used for gene therapy. Retroviruses include lentiviruses, a family of viruses including human immunodeficiency virus (HIV) that includes several accessory proteins to facilitate viral infection and proviral integration. Current, "third-generation," lentiviral vectors feature total replication incompetence, broad tropism, and increased gene transfer capacity for mammalian cells (see, e.g., Mangeat and Trono, *Human Gene Therapy* 16(8):913 (2005) and Wiznerowicz and Trono, *Trends Biotechnol.* 23(1):42 (2005), each herein incorporated by reference).

Stabilized trimers of the invention may be produced upon expression of the gp140 Env polypeptides described herein using a retrovirus vector.

Other Viral Vectors

Besides adenoviral and retroviral vectors, other viral vectors and techniques are known in the art that can be used to facilitate delivery and/or expression of one or more of the optimized gp140 Env polypeptides of the invention in a cell (e.g., a blood cell, such as a lymphocyte) or subject (e.g., a human) in order to promote formation of the trimers of the invention. These viruses include poxviruses (e.g., vaccinia virus and modified vaccinia virus Ankara (MVA); see, e.g., U.S. Patent Nos. 4,603,112 and 5,762,938, each incorporated by reference herein), herpesviruses, togaviruses (e.g., Venezuelan Equine Encephalitis virus; see, e.g., U.S. Patent No. 5,643,576, incorporated by reference herein), picornaviruses (e.g., poliovirus; see, e.g., U.S. Patent No. 5,639,649, incorporated by reference herein), baculoviruses, and others described by Wattanapitayakul and Bauer (*Biomed. Pharmacother.* 54:487 (2000), incorporated by reference herein).

Naked DNA and Oligonucleotides

Naked DNA or oligonucleotides encoding one or more of the optimized gp140 Env polypeptides of the invention can also be used to express these polypeptides in a cell or a subject (e.g., a human) in order to promote formation of the trimers of the invention. See, e.g., Cohen, *Science* 259:1691-1692 (1993); Fynan et al., *Proc. Natl. Acad. Sci. USA*, 90:11478 (1993); and Wolff et al., *BioTechniques* 11:474485 (1991), each herein incorporated by reference. This is the simplest method of non-viral transfection. Efficient methods for delivery of naked DNA exist, such as electroporation and the use of a "gene gun," which shoots DNA-coated gold particles into a cell using high pressure gas and carrier particles (e.g., gold).

Lipoplexes and Polyplexes

To improve the delivery of a nucleic acid encoding one or more of the optimized gp140 Env polypeptides of the invention into a cell or subject in order to promote formation of the trimers of the invention, lipoplexes (e.g., liposomes) and polyplexes can be used to protect the nucleic acid from undesirable degradation during the transfection process. The nucleic acid molecules can be covered with lipids in an organized structure like a micelle or a liposome. When the organized structure is complexed with the nucleic acid molecule it is called a lipoplex. There are three types of lipids: anionic (negatively-

charged), neutral, or cationic (positively-charged). Lipoplexes that utilize cationic lipids have proven utility for gene transfer. Cationic lipids, due to their positive charge, naturally complex with the negatively-charged nucleic acid. Also as a result of their charge they interact with the cell membrane, endocytosis of the lipoplex occurs, and the nucleic acid is released into the cytoplasm. The cationic lipids also protect against degradation of the nucleic acid by the cell.

Complexes of polymers with nucleic acids are called polyplexes. Most polyplexes consist of cationic polymers and their production is regulated by ionic interactions. One large difference between the methods of action of polyplexes and lipoplexes is that polyplexes cannot release their nucleic acid load into the cytoplasm, so, to this end, co-transfection with endosome-lytic agents (to lyse the endosome that is made during endocytosis) such as inactivated adenovirus must occur. However, this is not always the case; polymers such as polyethylenimine have their own method of endosome disruption as does chitosan and trimethylchitosan.

Exemplary cationic lipids and polymers that can be used in combination with one or more of the nucleic acid molecules encoding one or more of the optimized gp140 Env polypeptides of the invention to form lipoplexes or polyplexes include, but are not limited to, polyethylenimine, lipofectin, lipofectamine, polylysine, chitosan, trimethylchitosan, and alginate.

Hybrid methods

Several hybrid methods of gene transfer combine two or more techniques. Virosomes, for example, combine lipoplexes (e.g., liposomes) with an inactivated virus. This approach has been shown to result in more efficient gene transfer in respiratory epithelial cells compared to either viral or liposomal methods alone. Other methods involve mixing other viral vectors with cationic lipids or hybridizing viruses. Each of these methods can be used to facilitate transfer of one or more of the nucleic acid molecules of the invention encoding one or more of the optimized gp140 Env polypeptides of the invention into a cell or subject in order to promote formation of the trimers of the invention.

Dendrimers

Dendrimers may be also be used to transfer one or more of the nucleic acid molecules of the invention encoding one or more of the optimized gp140 Env polypeptides of the invention into a cell or subject in order to promote formation of the trimers of the invention. A dendrimer is a highly branched macromolecule with a spherical shape. The surface of the particle may be functionalized in many ways, and many of the properties of the resulting construct are determined by its surface. In particular it is possible to construct a cationic dendrimer (i.e., one with a positive surface charge). When in the presence of genetic material (e.g., a nucleic acid molecule), charge complimentarity leads to a temporary association of the nucleic acid with the cationic dendrimer. On reaching its destination the dendrimer-nucleic acid complex is then taken into the cell via endocytosis.

Methods of Treatment using the Compositions of the Invention

***In vivo* Administration**

The invention features methods for the *in vivo* administration of a therapeutically effective amount of one or more of the compositions (i.e., vaccines, vectors, stabilized trimer(s), nucleic acids,

polypeptides, stabilized trimer, or other composition thereof described herein) of the invention to a subject (e.g., a human, e.g., a human infected with HIV or a human at risk of an HIV infection) in need thereof. Upon administering one or more of the compositions of the invention to the subject, the stabilized trimers of the invention can elicit protective or therapeutic immune responses (e.g., cellular or humoral immune responses, e.g., neutralizing anti-HIV antisera production, e.g., anti-HIV antisera that neutralizes HIV selected from clade A, clade B, and/or clade C HIV) directed against the viral immunogens.

The method may be used to treat or reduce the risk of an HIV infection in a subject in need thereof. The subject may be infected with HIV or may be at risk of exposure to HIV. The compositions of the invention can be administered to a subject infected with HIV to treat AIDS. Examples of symptoms of diseases caused by a viral infection, such as AIDS, that can be treated using the compositions of the invention include, for example, fever, muscle aches, coughing, sneezing, runny nose, sore throat, headache, chills, diarrhea, vomiting, rash, weakness, dizziness, bleeding under the skin, in internal organs, or from body orifices like the mouth, eyes, or ears, shock, nervous system malfunction, delirium, seizures, renal (kidney) failure, personality changes, neck stiffness, dehydration, seizures, lethargy, paralysis of the limbs, confusion, back pain, loss of sensation, impaired bladder and bowel function, and sleepiness that can progress into coma or death. These symptoms, and their resolution during treatment, may be measured by, for example, a physician during a physical examination or by other tests and methods known in the art.

In cases in which the subject is infected with HIV, the method may be used to reduce an HIV-mediated activity (e.g., infection, fusion (e.g., target cell entry and/or syncytia formation), viral spread, etc.) and/or to decrease HIV titer in the subject. HIV-mediated activity and/or HIV titer may be decreased, for example, by 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or more compared to that of a control subject (e.g., an untreated subject or a subject treated with a placebo).

One or more of the compositions of the invention may also be administered in the form of a vaccine for prophylactic treatment of a subject (e.g., a human) at risk of an HIV infection.

The compositions utilized in the methods described herein can be formulated, for example, for administration intramuscularly, intravenously, intradermally, percutaneously, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, topically, intratumorally, peritoneally, subcutaneously, subconjunctivally, intravesicularlly, mucosally, intrapericardially, intraumbilically, intraocularly, orally, topically, locally, by inhalation, by injection, by infusion, by continuous infusion, by localized perfusion bathing target cells directly, by catheter, by lavage, by gavage, in cremes, or in lipid compositions.

The preferred method of administration can vary depending on various factors (e.g., the components of the composition being administered and the severity of the condition being treated). Formulations suitable for oral or nasal administration may consist of liquid solutions, such as an effective amount of the composition dissolved in a diluent (e.g., water, saline, or PEG-400), capsules, sachets, tablets, or gels, each containing a predetermined amount of the chimeric Ad5 vector composition of the invention. The pharmaceutical composition may also be an aerosol formulation for inhalation, for

example, to the bronchial passageways. Aerosol formulations may be mixed with pressurized, pharmaceutically acceptable propellants (e.g., dichlorodifluoromethane, propane, or nitrogen). In particular, administration by inhalation can be accomplished by using, for example, an aerosol containing sorbitan trioleate or oleic acid, for example, together with trichlorofluoromethane, dichlorofluoromethane, dichlorotetrafluoroethane, or any other biologically compatible propellant gas.

Immunogenicity of the composition of the invention may be significantly improved if it is co-administered with an immunostimulatory agent or adjuvant. Suitable adjuvants well-known to those skilled in the art include, for example, aluminum phosphate, aluminum hydroxide, QS21, Quil A (and derivatives and components thereof), calcium phosphate, calcium hydroxide, zinc hydroxide, glycolipid analogs, octodecyl esters of an amino acid, muramyl dipeptides, polyphosphazene, lipoproteins, ISCOM matrix, DC-Chol, DDA, cytokines, Adju-Phos, Matrix M, CpG/Emulsigen, and other adjuvants and derivatives thereof.

Compositions according to the invention described herein may be formulated to release the composition immediately upon administration (e.g., targeted delivery) or at any predetermined time period after administration using controlled or extended release formulations. Administration of the composition in controlled or extended release formulations is useful where the composition, either alone or in combination, has (i) a narrow therapeutic index (e.g., the difference between the plasma concentration leading to harmful side effects or toxic reactions and the plasma concentration leading to a therapeutic effect is small; generally, the therapeutic index, TI, is defined as the ratio of median lethal dose (LD_{50}) to median effective dose (ED_{50})); (ii) a narrow absorption window at the site of release (e.g., the gastro-intestinal tract); or (iii) a short biological half-life, so that frequent dosing during a day is required in order to sustain a therapeutic level.

Many strategies can be pursued to obtain controlled or extended release in which the rate of release outweighs the rate of metabolism of the pharmaceutical composition. For example, controlled release can be obtained by the appropriate selection of formulation parameters and ingredients, including, for example, appropriate controlled release compositions and coatings. Suitable formulations are known to those of skill in the art. Examples include single or multiple unit tablet or capsule compositions, oil solutions, suspensions, emulsions, microcapsules, microspheres, nanoparticles, patches, and liposomes.

The compositions of the invention may be administered to provide pre-infection prophylaxis or after a subject has been diagnosed with an HIV infection or a disease with an etiology traceable to an HIV infection (e.g., AIDS). The composition may be administered, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 35, 40, 45, 50, 55, or 60 minutes, 2, 4, 6, 10, 15, or 24 hours, 2, 3, 5, or 7 days, 2, 4, 6 or 8 weeks, or even 3, 4, or 6 months pre-infection or pre-diagnosis, or may be administered to the subject 15-30 minutes or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 20, 24, 48, or 72 hours, 2, 3, 5, or 7 days, 2, 4, 6 or 8 weeks, 3, 4, 6, or 9 months, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 years or longer post-diagnosis or post-infection to HIV. The subject can be administered a single dose of the composition(s) (or, e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more doses) or the subject can be administered at least one dose (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more doses) daily, weekly, monthly, or yearly. The administration period may be defined (e.g., 1-4 weeks, 1-12 months, 1-20 years) or may be for the life of the subject. The composition(s) may also be administered to said subject as a prime or a boost composition or in a prime-boost regimen. In a

preferred embodiment, the composition (e.g., vaccine) of the invention is administered as a boost following administration of an additional composition (e.g., vaccine) as a prime, where the prime includes at least a first vector including a first nucleic acid molecule that encodes a polypeptide having at least 85% amino acid sequence identity (e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 6, and optionally a second vector including a second nucleic acid molecule that encodes a polypeptide having at least 85% identity (e.g., 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 7. The boost in this regimen includes one or more of the composition(s) of the invention (e.g., any one of the stabilized trimers, the compositions, the vaccines, the nucleic acid molecules, and/or the vectors of the invention). In still other embodiments, the prime includes at least a first vector including a nucleic acid molecule that encodes a polypeptide having the sequence of any one of SEQ ID NOs: 8-32. Alternatively, the composition (e.g., vaccine) of the invention is administered as a prime. In some embodiments where the composition of the invention is administered as a prime, a different vaccine (e.g., a vaccine including at least a first vector including a first nucleic acid molecule that encodes a polypeptide having at least 85% amino acid sequence identity (e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 6, and optionally a second vector including a second nucleic acid molecule that encodes a polypeptide having at least 85% identity (e.g., 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 7; or a vaccine including at least a first vector including a nucleic acid molecule that encodes a polypeptide having the sequence of any one of SEQ ID NOs: 8-32) is administered as a boost.

When treating disease (e.g., AIDS), the compositions of the invention may be administered to the subject either before the occurrence of symptoms or a definitive diagnosis or after diagnosis or symptoms become evident. For example, the composition may be administered, for example, immediately after diagnosis or the clinical recognition of symptoms or 2, 4, 6, 10, 15, or 24 hours, 2, 3, 5, or 7 days, 2, 4, 6 or 8 weeks, or even 3, 4, or 6 months after diagnosis or detection of symptoms.

For in vivo treatment of human and non-human patients infected with HIV, the patient may be administered or provided a pharmaceutical formulation including a composition of the present invention. When used for in vivo therapy, compositions of the invention may be administered to the patient in therapeutically effective amounts (i.e., amounts that eliminate or reduce the patient's viral burden). The compositions may be administered to a human patient in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intraarticular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The compositions may be administered parenterally, when possible, at the target cell site, or intravenously. Intravenous or subcutaneous administration of the composition is preferred in certain embodiments. Therapeutic compositions of the invention may be administered to a patient or subject systemically, parenterally, or locally.

The compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation may be administered in powder form or combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably between 5

and 9 or between 6 and 8, and most preferably between 7 and 8, such as 7 to 7.5. The resulting compositions in solid form may be packaged in multiple single dose units, each containing a fixed amount of any one or more of the optimized gp140 Env nucleic acids required to support formation of one or more of the stabilized trimers of the invention and/or one or more of the stabilized trimers of the invention of the invention and, if desired, one or more immunomodulatory agents, such as in a sealed package of tablets or capsules, or in a suitable dry powder inhaler (DPI) capable of administering one or more doses.

Dosages

The dose of a composition of the invention (e.g., a vaccine including one or more of the stabilized gp140 Env trimers of the invention) or the number of treatments using a composition of the invention may be increased or decreased based on the severity of, occurrence of, or progression of, the HIV infection and/or disease related to the HIV infection (e.g., AIDS) in the subject (e.g., based on the severity of one or more symptoms of HIV infection/AIDS described above).

The stabilized gp140 Env trimer compositions of the invention can be administered in a therapeutically effective amount that provides an immunogenic and/or protective effect against HIV or target protein of HIV (e.g., gp140). The subject may, for example, be administered polypeptide compositions of the invention (e.g., stabilized gp140 Env trimers of the invention) in a non-vectored composition. The polypeptide composition administered may include between approximately 1 µg and 1 mg of stabilized Env trimers, and more preferably between 50 µg and 300 µg of stabilized Env trimers of the invention.

Alternatively, the subject may be administered, in the form of a viral vector, at least about $1x10^3$ viral particles (vp)/dose or between $1x10^1$ and $1x10^{14}$ vp/dose, preferably between $1x10^3$ and $1x10^{12}$ vp/dose, and more preferably between $1x10^5$ and $1x10^{11}$ vp/dose.

Viral particles include nucleic acid molecules encoding one or more of the optimized gp140 Env polypeptides of the invention and are surrounded by a protective coat (a protein-based capsid with hexon and fiber proteins). Viral particle number can be measured based on, for example, lysis of vector particles, followed by measurement of the absorbance at 260 nm (see, e.g,. Steel, Curr. Opin. Biotech., 1999).

The dosage administered depends on the subject to be treated (e.g., the age, body weight, capacity of the immune system, and general health of the subject being treated), the form of administration (e.g., as a solid or liquid), the manner of administration (e.g., by injection, inhalation, dry powder propellant), and the cells targeted (e.g., epithelial cells, such as blood vessel epithelial cells, nasal epithelial cells, or pulmonary epithelial cells). The composition is preferably administered in an amount that provides a sufficient level of the stabilized gp140 Env trimer gene product (e.g., a level of stabilized gp140 Env trimer that elicits an immune response without undue adverse physiological effects in the subject caused by the immunogenic trimer).

In addition, single or multiple administrations of the compositions of the present invention may be given (pre- or post-infection and/or pre- or post-diagnosis) to a subject (e.g., one administration or administration two or more times). For example, subjects who are particularly susceptible to, for example, HIV infection may require multiple treatments to establish and/or maintain protection against the virus. Levels of induced immunity provided by the pharmaceutical compositions described herein can be

monitored by, for example, measuring amounts of neutralizing anti-HIV secretory and serum antibodies. The dosages may then be adjusted or repeated as necessary to trigger the desired level of immune response. For example, the immune response triggered by a single administration (prime) of a composition of the invention may not be sufficiently potent and/or persistent to provide effective protection. Accordingly, in some embodiments, repeated administration (boost), such that a prime-boost regimen is established, may significantly enhance humoral and cellular responses to the antigen of the composition.

Alternatively, as applies to recombinant therapy, the efficacy of treatment can be determined by monitoring the level of the one or more optimized gp140 Env trimers expressed by or present in a subject (e.g., a human) following administration of the compositions of the invention. For example, the blood or lymph of a subject can be tested for the immunogenic trimer(s) using, for example, standard assays known in the art (see, e.g., Human Interferon-Alpha Multi-Species ELISA kit (Product No. 41105) and the Human Interferon-Alpha Serum Sample kit (Product No. 41110) from Pestka Biomedical Laboratories (PBL), Piscataway, New Jersey).

A single dose of one or more of the compositions of the invention may achieve protection, pre-infection or pre-diagnosis. In addition, a single dose administered post-infection or post-diagnosis can function as a treatment according to the present invention.

A single dose of one or more of the compositions of the invention can also be used to achieve therapy in subjects being treated for a disease. Multiple doses (e.g., 2, 3, 4, 5, or more doses) can also be administered, in necessary, to these subjects.

Carriers, Excipients, Diluents

Therapeutic formulations of the compositions of the invention (e.g., vaccines, vectors, stabilized trimer(s), nucleic acid molecules, etc.) may be prepared using standard methods known in the art by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences (20^{th} edition), ed. A. Gennaro, 2000, Lippincott, Williams & Wilkins, Philadelphia, PA). Acceptable carriers, include saline, or buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagines, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, PLURONICS™, or PEG.

Optionally, but preferably, the formulation contains a pharmaceutically acceptable salt, preferably sodium chloride, and preferably at about physiological concentrations. Optionally, the formulations of the invention can contain a pharmaceutically acceptable preservative. In some embodiments the preservative concentration ranges from 0.1 to 2.0%, typically v/v. Suitable preservatives include those known in the pharmaceutical arts. Benzyl alcohol, phenol, m-cresol, methylparaben, and propylparaben are preferred preservatives. Optionally, the formulations of the invention can include a pharmaceutically acceptable surfactant at a concentration of 0.005 to 0.02%.

Adjuvants

Any one of the compositions of the invention (e.g., vaccines, vectors, stabilized trimer(s), nucleic acid molecules, etc.) can be formulated to include, be administered concurrently with, and/or be administered in series with one or more pharmaceutically acceptable adjuvants to increase the immunogenicity of the composition (e.g., upon administration to a subject in need thereof, e.g., a subject infected with HIV or at risk of an HIV infection). Adjuvants approved for human use include aluminum salts (alum). These adjuvants have been useful for some vaccines including hepatitis B, diphtheria, polio, rabies, and influenza. Other useful adjuvants include Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA), muramyl dipeptide (MDP), synthetic analogues of MDP, N-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanine-2-[1,2-dipalmitoyl-s-gly- cero-3-(hydroxyphosphoryloxy)]ethylamide (MTP-PE), Adju-Phos, Matrix M, CpG/Emulsigen, and compositions containing a metabolizable oil and an emulsifying agent, wherein the oil and emulsifying agent are present in the form of an oil-in-water emulsion having oil droplets substantially all of which are less than one micron in diameter.

Ex Vivo Transfection and Transduction

The present invention also provides for the *ex vivo* transfection or transduction of cells, followed by administration of these cells back into a subject (e.g., human) to allow for the expression of one or more of the optimized gp140 Env polypeptides of the invention that have immunogenic properties. In one embodiment, the cells are autologous to the treated subject. Cells can be transfected or transduced *ex vivo* with, for example, one or more vectors of the invention to allow for the temporal or permanent expression of one or more of the optimized gp140 Env polypeptides in the treated subject. Upon administering these modified cells to the subject, the one or more vectors of the invention will be expressed, eliciting protective or therapeutic immune responses (e.g., cellular or humoral immune responses, e.g., production of neutralizing anti-HIV antisera) directed against the gp140 immunogenic trimer or trimers that form.

Cells that can be isolated and transfected or transduced *ex vivo* according to the methods of invention include, but are not limited to, blood cells, skin cells, fibroblasts, endothelial cells, skeletal muscle cells, hepatocytes, prostate epithelial cells, and vascular endothelial cells. Stem cells are also appropriate cells for transduction or transfection with a vector of the invention. Totipotent, pluripotent, multipotent, or unipotent stem cells, including bone marrow progenitor cells and hematopoietic stem cells (HSC), can be isolated and transfected or transduced with, for example, a vector of the invention, and administered to a subject according to the methods of the invention.

The method of transfection or transduction has a strong influence on the strength and longevity of protein expression (e.g., stabilized gp140 trimer expression) in the transfected or transduced cell, and subsequently, in the subject (e.g., human) receiving the cell. The present invention provides vectors that are temporal (e.g., adenoviral vectors) or long-lived (e.g., retroviral vectors) in nature. Regulatory sequences (e.g., promoters and enhancers) are known in the art that can be used to regulate protein expression. The type of cell being transfected or transduced also has a strong bearing on the strength

and longevity of protein expression. For example, cell types with high rates of turnover can be expected to have shorter periods of protein expression.

Combination Therapies

Other therapeutic regimens can be combined with the administration of compositions of the present invention. The combined administration includes co-administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preferably such combined therapy results in a synergistic therapeutic effect. In certain embodiments, it is desirable to combine administration of a composition of the invention with an agent (e.g., an antibody or antibody fragment) directed against an antigen associated with the infectious agent. Passive immunization has proven to be an effective and safe strategy for the prevention and treatment of viral diseases (see Keller et al., Clin. Microbiol. Rev. 13:602- 14 (2000); Casadevall, Nat. Biotechnol. 20: 114 (2002); *Shibata et al.*, Nat. Med. 5:204-10 (1999); *and Igarashi et al.*, Nat. Med. 5:211-16 (1999), each of which are incorporated herein by reference). Passive immunization using human monoclonal antibodies, provide an immediate treatment strategy for emergency prophylaxis and treatment of HIV, and can be used in combination alongside the compositions of the present invention. HIV antibodies and fragments thereof specifically bind or preferentially bind to infected cells, as compared to normal control uninfected cells and tissue. Thus, these HIV antibodies can be used to selectively target infected cells or tissues in a patient, biological sample, or cell population, e.g., as part of a combination therapy with compositions of the present invention.

For example, US Provisional Application No. 61/886,932, incorporated herein by reference in its entirety, discloses HIV clade C Env trimers, the polypeptides of which may be used in combination with polypeptides disclosed herein to form trimers of the present invention. US Provisional Application No. 61/884,414, also incorporated herein by reference, discloses methods of treating subjects infected with HIV and blocking HIV infections in subjects at risk of HIV infection using H332 glycan-dependent antibodies. PCT application WO 2012/030904, also incorporated herein by reference in its entirety, discloses broadly neutralizing antibodies against HIV, any of which can be used in methods of treating HIV in a subject in need thereof according to the present invention. Exemplary antibodies include VRC01, PGT121, PGT126, PG9, PG16, and 3BNC117.

Kits

The invention provides kits that include a pharmaceutical composition containing a vaccine, vector, stabilized trimer, or optimized viral polypeptide of the invention, and a pharmaceutically-acceptable carrier, in a therapeutically effective amount for preventing or treating a viral infection. The kits include instructions to allow a clinician (e.g., a physician or nurse) to administer the composition contained therein.

Preferably, the kits include multiple packages of the single-dose pharmaceutical composition(s) containing an effective amount of a vaccine, vector, stabilized trimer, or optimized viral polypeptide of the invention. Optionally, instruments or devices necessary for administering the pharmaceutical

composition(s) may be included in the kits. For instance, a kit of this invention may provide one or more pre-filled syringes containing an effective amount of a vaccine, vector, stabilized trimer, or optimized viral polypeptide of the invention. Furthermore, the kits may also include additional components such as instructions or administration schedules for a patient infected with or at risk of being infected with a virus to use the pharmaceutical composition(s) containing a vaccine, vector, stabilized trimer, or optimized viral polypeptide of the invention.

It will be apparent to those skilled in the art that various modifications and variations can be made in the compositions, methods, and kits of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

Examples

The present invention is illustrated by the following examples, which are in no way intended to be limiting of the invention.

Example 1. Materials and Methods

Western Blot Immunodetection

Volumes containing 10-µg equivalents of DNA expression vectors pVRC8400 empty, pVRC8400 mosaic gp140 version-1 (expression vector for a polypeptide including the amino acid sequence of SEQ ID NO: 1), or pVRC8400 mosaic gp140 version-2 (expression vector for a polypeptide including the amino acid sequence of SEQ ID NO: 2) were each made up to 100 µl with Dulbeco's Modified Eagle Medium (DMEM; Invitrogen). 40 µl of Lipofectamine (Invitrogen) transfection reagent was then added 60 µl DMEM and 100 µl of this mix added to each DNA vector followed by gentle agitation and incubation at room temperature for 30 minutes. 293T cells grown to approximately 70-80% confluency in T-25 flasks were washed once with 2.5 ml DMEM, 2.3 ml of DMEM added followed by 200 µl DNA/Lipofectamine mix. Cells were then incubated at 37°C, 10% CO_2 for 48 hours. 48 hours post-transfection, 0.5ml of supernatant from each T-25 flask was harvested, briefly spun and 20 µl placed in a fresh eppendorf tube. 5 µl of 5x reducing sample buffer (Pierce) was added to each tube, each sample heated for 5 minutes at 100°C and then place on ice to cool. 20 µl of each sample was loaded on a 4-15% pre-cast SDS-PAGE (Biorad), and the gel run at 150V for approximately 70 minutes. Transfer of protein from gel to membrane was performed using the iblot dry blotting system (Invitrogen) as per vendor protocol using PVDF gel transfer stacks. Membrane blocking was performed overnight at 4°C in 20 ml of PBS-T Block (i.e., Dulbeco's phosphate buffered saline (Invitrogen), containing 0.2% V/V Tween 20 (Sigma) and 5% W/V non-fat milk powder) on an orbital shaker. 10 µl of monoclonal HRP conjugated anti-His tag antibody (Qiagen) was then added to 20 ml PBS-T Block (1:2000 dilution) followed by incubation on an orbital shaker at room temperature for 1 hour. Membranes were washed 5 times in PBS-T block, membranes touch dried on absorbent paper to remove excess block, and for detection, Amersham ECL Plus Western Blotting Detection System (GE Healthcare) was utilized.

Roller Bottle Transfection and Protein Purification

DMEM growth media supplemented with 10% Fetal Bovine Serum (FBS) was used to grow 293T to confluence in Cell Bind® roller bottles (Corning), growth media removed, followed by addition of 250 ml of pre-warmed Freestyle 293 expression medium (Invitrogen) and incubation for 2 hours at 37°C, 5% CO_2. 250 µg of DNA expression vector pVRC8400 mosaic gp140 version-2 was mixed with 320 µl of polyethylenimine (PEI) (1 mg/ml) added to 20 ml of room temperature freestyle 293 medium, incubated at room temperature for 20 minutes and then added in each roller bottle followed by incubation for 6 days in 37°C, 5% CO_2. The cell supernatant was harvested at 6 days after medium change. The Histidine-tagged optimized mosaic gp140 Env version-2 protein, including SEQ ID NO: 2, was purified by Ni-NTA (Qiagen) followed by size-exclusion chromatography. Briefly, after a clarifying spin and the addition of imidazole to the final concentration of 10 mM, the cell supernatant was loaded onto a nickel column at a flow rate of 0.8 mL/min and was washed with 20 mM imidazole in PBS followed by further washing with 40 mM imidazole in PBS. The protein then was eluted with 300 mM imidazole in PBS. The fractions containing the purified protein were pooled, concentrated, and further purified by gel-filtration chromatography on Superose 6 (GE Healthcare) in a column running buffer containing 25 mM Tris (pH 7.5) and 150 mM NaCl. The purified proteins were concentrated, frozen in liquid nitrogen, and stored at –80 °C.

Animals and Immunizations

Outbred female Hartley guinea pigs (Elm Hill Labs) were housed at the Animal Research Facility of Beth Israel Deaconess Medical Center under protocols approved by the Institutional Animal Care and Use Committee. Guinea pigs were immunized by bilateral intramuscular injections in the upper quadriceps with clade C gp140 Env polypeptide (i.e., homotrimer of three molecules including the amino acid sequence of cEnv (SEQ ID NO: 3)), mosaic gp140 Env (i.e., homotrimer of three molecules including the amino acid sequence of mEnv (SEQ ID NO: 1)), or a clade C gp140 Env/mosaic gp140 Env mixture (100 µg/animal) at 4-week intervals (weeks 0, 4, and 8) using 500 µl of a dual adjuvant combination comprising 15% (v/v) oil-in-water Emulsigen (MVP Laboratories)/PBS and 50 µg of immunostimulatory di-nucleotide CpG DNA (5'-TCGTCGTTGTCGTTTTGTCGTT-3') (Midland Reagent Company). The clade C gp140 Env/mosaic gp140 Env mixture contained 50 µg of each protein. Serum samples were obtained from the vena cava of anesthetized animals 4 weeks after each immunization.

Neutralizing Antibody Assay in TZM.bl Cells

Neutralizing antibody responses against HIV-1 Env pseudoviruses were measured using luciferase-based virus neutralization assays in TZM.bl cells. These assays measure the reduction in luciferase reporter gene expression in TZM-bl cells following a single round of virus infection. The ID_{50} was calculated as the serum dilution that resulted in a 50% reduction in relative luminescence units compared with the virus control wells after the subtraction of cell control relative luminescence units. Briefly, threefold serial dilutions of serum samples were performed in duplicate (96-well flat-bottomed plate) in 10% DMEM growth medium (100 µl per well). Virus was added to each well in a volume of 50µl, and the plates were incubated for 1 hour at 37 °C. Then TZM.bl cells were added ($1x10^4$ per well in 100 µl volume) in 10% DMEM growth medium containing diethylaminoethyldextran (Sigma) at a final

concentration of 11 µg/ml. Murine leukemia virus (MuLV) negative controls were included in all assays. HIV-1 Envelope pseudoviruses included clade A (MS208.A1 and Q23.17) isolates, clade B (SF162.LS, BaL.26, SS1196.1 and 6535.3), and clade C (MW965.26, TV1.21, ZM109F.PB4 and ZM197M.PB7) isolates.

Example 2. Generation of optimized mosaic gp140 Env1 trimers of the invention

mEnv+ (polypeptide including the amino acid sequence of SEQ ID NO: 2) has been modified from mEnv (polypeptide including the amino acid sequence of SEQ ID NO: 1) in the following manner. First, the leader peptide secretion sequence has been made identical to that used in the stabilized clade C gp140 Env (cEnv) trimer polypeptide constituent (SEQ ID NO: 3). Second, cleavage site mutations have been incorporated between gp120 and gp41 moieties to further enhance stability. Third, a factor Xa protease cleavage site (SRIEGR) has been incorporated upstream of the foldon trimerization domain. The amino acid sequences of the three Env polypeptides (SEQ ID NOs: 1-3) and the specific modifications present in each are depicted in Figures 1A-1C.

Surprisingly, these modifications resulted in a remarkably stabilized gp140 Env1 trimer (e.g., an mEnv+ trimer of the invention). In order to assess stability, we first compared the expression levels of mEnv+ relative to mEnv by Western blot analysis. To this end, T-25 flasks containing 80% confluent 293T cells were transfected with eukaryotic expression vector pVRC8400 expressing mEnv or mEnv+ using lipofectamine 2000 (Invitrogen) and 10µl of each supernatant analyzed by Western blot immunodetection using anti-Histidine tag HRP (Qiagen). Figure 2 depicts a Western blot showing the expression levels of mEnv and mEnv+ in lanes 3 and 4, respectively. Notably, the expression levels of mEnv+ were remarkably higher compared to that of mEnv or cEnv, which was used as a positive control (see lane 1). In this experiment, empty pVRC8400 was used as a negative control (see lane 2).

As noted above, the mEnv+ was expressed in 293T cells and purified following cell lysis and clarification by virtue of a His-tag using a Ni-NTA (Qiagen) column. The collected fractions following imidazole elution were pooled, concentrated, and further purified by gel-filtration chromatography on Superose 6 (GE Healthcare) in a column running buffer containing 25 mM Tris (pH 7.5) and 150 mM NaCl. A chromatography trace of depicting mEnv+ elution from the Superose 6 column is depicted in Figure 3. The peak fractions (i.e., the fractions obtained under the peak curve in Figure 3) were then individually analyzed on a 4-15% pre-case SDS-PAGE gel (Figure 4). The SDS-PAGE gel demonstrates that the gel-filtration purification succesfully resulted in the isolation of a homogenous population of mEnv+ polypeptides. As described further herein, the immunogenicity of these stabilized gp140 Env trimers (both homotrimers of mEnv and mEnv+, as well as a combination of mEnv and cEnv homotrimers) was assessed in guinea pigs using a panel of tier 1 isolates from clades A, B, and C.

Example 3. Analysis of neutralizing antibody responses

Preclinical evaluation of candidate Env immunogens is critical for concept testing and for prioritization of vaccine candidates. Luciferase-based virus neutralization assays in TZM.b1 cells (Li et al. (2005) J. Virol. 79:10108; Montefiori (2005) Curr. Prot. Immunol. Chapter 12: Unit 1211) have been developed as high throughput assay that can be standardized (Montefiori (2009) Methods Mol. Biol. 485:395; Polonis et al. (2008) Virology 375:315). A luciferase reporter gene assay was performed in

TZM-b1 cells (a genetically engineered cell line that expresses CD4, CXCR4 and CCR5 and contains Tat-inducible Luc and β-Gal reporter reporter genes) based on single round infection with molecularly cloned Env-pseudotyped viruses. This assay resulted in a high success rate in single round infections, increased assay capacity (e.g., a two day assay), increased precision (e.g., accurately measured 50% neutralization), and an improved level of standardization (e.g., a stable cell line). The luciferase reporter gene assay was optimized and validated.

To assess the neutralization profile afforded by the stabilized gp140 Env trimers of the invention, TZM.b1 assays were performed in which guinea pig sera obtained pre-vaccination (Pre) and four weeks after the third vaccination (Post) with cEnv homotrimers, mEnv homotrimers, or both cEnv and mEnv homotrimers were tested against a multi-clade panel of tier 1 neutralization-sensitive isolates including clade B (SF162.LS and Bal.26), and clade C (MW965.26 and TV1.21) HIV-1 Envelope pseudoviruses and Murine lukemia virus (MuLV) (negative control) (Figures 5A-5C).

TZM.b1 assays were also performed in which guinea pig sera obtained pre-vaccination (Pre) and four weeks after the third vaccination (Post) using cEnv homotrimers, mEnv homotrimers, or both cEnv and mEnv homotrimers were tested against HIV-1 Envelope pseudoviruses of intermediate neutralization-sensitive tier-1 (Tier 1B) clade A isolates (MS208.A1 and Q23.17) (Figures 6A-6B), highly neutralization sensitive (Tier 1A) and Tier 1B clade B isolates (SF162.LS, BaL.26, SS1196.1, and 6535.3) (Figures 7A-7D), and Tier 1A and Tier 1B clade C isolates (MW965.26, TV1.21, ZM109F.PB4, and ZM197M.PB7) (Figures 8A-8D).

Unexpectedly, quatitation of ID_{50} titer data collectively demonstrate that the combination of cEnv and mEnv homotrimers induced neutralizing antibody responses that were superior to either cEnv or mEnv alone. Specifically, the combination of cEnv and mEnv was particularly surprising in terms of expanding the breadth of neutralizing antibody responses induced. Such an expansion of neutralizing antibody breadth has not previously been described and is a major unmet need in the field.

Example 4. Treating or reducing the risk of an HIV infection in a subject using the compositions of the invention

The composition of the invention (e.g., a vaccine of the invention) may be administered to a subject (e.g., a human infected with HIV or at risk of an HIV infection) in a prime-boost vaccination regimen to treat or reduce the risk of an HIV infection in a subject in need thereof. For example, one or more of the compositions of the invention, such as vaccine including mEnv, mEnv+, or cEnv trimers, or a combination of mEnv with cEnv or mEnv+ with cEnv trimers may be administered as a boost. Prior to administration of the boost, the subject is administered as a prime vaccination at least a first vector including a first nucleic acid molecule that encodes a polypeptide having at least 85% amino acid sequence identity (e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 6, and optionally a second vector including a second nucleic acid molecule that encodes a polypeptide having at least 85% identity (e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) to, or the sequence of, SEQ ID NO: 7.

The composition is preferably administered in an amount that provides a sufficient level of the stabilized gp140 Env trimer gene product (e.g., a level of stabilized gp140 Env trimer that elicits an

immune response without undue adverse physiological effects in the subject caused by the immunogenic trimer). If the composition is non-vectored, the polypeptide composition administered may include between approximately 1 μg and 1 mg of stabilized Env trimers, and more preferably between 50 μg and 300 μg of stabilized Env trimers of the invention. Alternatively, the subject may be administered, in the form of a viral vector, at least about $1x10^3$ viral particles (vp)/dose or between $1x10^1$ and $1x10^{14}$ vp/dose, preferably between $1x10^3$ and $1x10^{12}$ vp/dose. and more preferably between $1x10^5$ and $1x10^{11}$ vp/dose.

Following administration of the composition of the invention in a prime-boost regimen, the patient can be assessed for changes in one or more symptoms or, in particular, the level of HIV titer in the treated subject, and the regimen can be repeated as necessary as described herein above.

Example 5. Combination of mEnv trimer with cEnv trimer elicits superior neutralizing antibody responses relative to either trimer alone

Introduction

The generation of HIV-1 Env glycoprotein immunogens that can elicit both binding and nAbs against diverse, circulating HIV-1 strains is a major goal of HIV-1 vaccine development (Stephenson et al. (2013) Immunol. Rev. 254:295; Srivastava et al. (2005) Hum. Vaccin. 1:45; Barouch (2008) Nature. 455:613; Karlsson Hedestam et al. (2008) Nat. Rev. Microbiol. 6:143; Mascola et al. (2010) Annu. Rev. Immunol. 28:413). The surface Env glycoprotein, which is the primary target of neutralizing antibodies, comprises the gp120 receptor-binding subunit and the gp41 fusion subunit associated by non-covalent interactions and is present as a trimeric spike $(gp120/gp41)_3$ on the viral surface. During the course of natural HIV-1 infection, most individuals induce anti-Env antibody responses with limited neutralization capacity, and variability in the *Env* gene can be as high as 30% (Louwagie et al. (1995) J. Virol. 69:263; Kalish et al. (1995) Aids. 9:851; Korber et al. (2001) Br. Med. Bull. 58:19), posing major challenges in the development of globally relevant Env-based immunogens. It has been reported, however, that approximately 10–25% of HIV-1 infected individuals have the ability to produce broadly neutralizing antibodies (bnAbs) (Stamatatos et al. (2009) Nat. Med. 15:866) providing the rationale that similar responses may be achievable with appropriate Env immunogens. Recent studies have highlighted the therapeutic efficacy such bnAbs have in rhesus monkeys chronically infected with the pathogenic simian-human immunodeficiency virus SHIV-SF162P3 Barouch et al. (2013) Nature. 503:224).

One strategy to address HIV-1 sequence diversity has utilized bioinformatically optimized 'mosaic' proteins (Fischer et al. (2007) Nat. Med. 13:100), which are *in silico* recombined HIV-1 sequences designed for improved coverage of global HIV-1 diversity. Several proof-of-concept immunogenicity studies in non-human primates have demonstrated that vector-encoded mosaic antigens can augment the depth and breadth of cellular immune responses and also improve antibody responses when compared to consensus and/or natural sequence antigens (Stephenson et al. (2012) J. Virol. 86:11434; Barouch et al. (2010) Nat. Med. 16:319; Santra et al. (2010) Nat. Med. 16:324; Santra et al. (2012) Virology. 428:121). We have also recently reported the protective efficacy of vector-based HIV-1 mosaic antigens against SHIV challenges in rhesus monkeys (Barouch et al. (2013) Cell. 155:531). However, the generation and evaluation of HIV-1 mosaic Env protein immunogens have not previously been described.

In this study, we report the production and characterization of a mosaic M gp140 trimer (mEnv). By surface plasmon resonance, mEnv bound CD4 as well as several bnAbs including VRC01, PGT121, PGT126, PG9, PG16, and 3BNC117, demonstrating that the trimer was intact and presented these relevant epitopes. mEnv also exhibited functional capacity to infect target cells in the TZM.bl assay. Immunogenicity studies in guinea pig showed that mEnv elicited high binding antibody titers, cross-clade tier 1 TZM.bl nAbs and detectable tier 2 A3R5 nAbs that were a different spectrum than those elicited by our clade C gp140 trimer (cEnv). The nAb response elicited by a mixture of mEnv and cEnv proved additive and was superior to either trimer alone.

Methods

Production & expression of HIV-1 Env proteins

Three synthetic gp140 mosaic M Env gene sequences designated mEnv, mEnv2 (Barouch et al. (2010) Nat. Med. 16:319) and mEnv3, all containing point mutations to eliminate cleavage and fusion activity, were derived from a previously described computational optimization method (Fischer et al. (2007) Nat. Med. 13:100; Barouch et al. (2010) Nat. Med. 16:319). Human codon optimized versions were synthesized by GeneArt (Life Technologies). Each construct was engineered to express a C-terminal T4 bacteriophage fibritin 'fold-on' trimerization domain and polyhistidine motif by PCR amplification with custom primers. Genes were cloned into the *SalI-BamHI* restriction sites of a pCMV eukaryotic expression vector, inserts were verified by diagnostic restriction digests, DNA was sequenced, and expression testing was performed using 10μg of DNA with Lipofectamine (Life Technologies) in 293T cells. Stable cell lines for clade C (C97ZA.012) (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111) and mosaic M gp140 Env trimers were generated by Codex Biosolutions. For protein production, the stable cell lines were grown in Dulbeco's Modified Eagle Medium (DMEM) (supplemented with 10% FBS, penicillin/streptomycin and puromycin) to confluence and then were changed to Freestyle 293 expression medium (Invitrogen) supplemented with the same antibiotics. Cell supernatants were harvested at 96–108 hours after medium change and the His-tagged gp140 proteins purified by Ni-NTA (Qiagen) and size-exclusion chromatography as previously described (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111; Nkolola et al. (2010) J. Virol. 84:3270). The synthetic gene for full-length mosaic M gp120 with a C-terminal His-tag was generated from the mEnv construct by PCR amplification with custom primers, produced in 293T cells utilizing transient transfections with polyethylenimine and subsequently purified by Ni-NTA (Qiagen) and size-exclusion chromatography on Superdex 200 (GE Healthcare). The synthetic gene for full-length mosaic M gp160 used in the TZM.bl assay was synthesized by GeneArt (Life Technologies) and cloned into a pcDNA^{TM}3.1/V5-His-TOPO vector (Invitrogen).

Western blot immunodetection

Supernatants (20 μl) obtained 48-hours post transient transfection of 293T cells with pCMV-mEnv, mEnv2, or mEnv3 gp140 expression constructs were separately mixed with reducing sample buffer (Pierce), heated for 5 minutes at 100°C and run on a precast 4-15% SDS-PAGE gel (Biorad). Protein was transferred to a PVDF membrane using the iBlot dry blotting system (Invitrogen) and membrane blocking performed overnight at 4°C in PBS-T [Dulbeco's Phosphate Buffered Saline + 0.2%

V/V Tween 20 (Sigma) + 5% W/V non-fat milk powder]. Following overnight blocking, the PVDF membrane was incubated for 1 hour with PBS-T containing a 1:2000 dilution of monoclonal antibody penta His-HRP (Qiagen), washed 5 times with PBS-T and developed using the Amersham ECL plus Western blotting detection system (GE Healthcare). For western blot immunodetection using 2F5 and 4E10 monoclonal antibodies, clade A (92UG037.8) gp140 (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111; Nkolola et al. (2010) J. Virol. 84:3270) and mosaic M gp140 proteins were processed as above.

Surface plasmon resonance

Surface plasmon resonance (SPR) was conducted on a Biacore 3000 (GE Healthcare) at 25°C utilizing HBS-EP running buffer (GE Healthcare). Immobilization of soluble two-domain CD4 (Freeman et al. (2010) Structure. 18:1632) (1,500 RU) or protein A (ThermoScientific) to CM5 chips was performed following the manufacturer (GE Healthcare) recommendations. Immobilized IgGs were captured at 300-750 RU. Binding experiments were conducted at a flow rate of 50µl/min with a 2-minute association phase and 5-minute dissociation phase. Regeneration was conducted with a single injection of 35 mM NaOH and 1.3 M NaCl at 100µl/min followed by a 3-minute equilibration phase in HBS-EP buffer. Injections over blank surfaces were subtracted from the binding data for analyses. Binding kinetics were determined using BIAevaluation software (GE Healthcare) and the Langmuir 1:1 binding model with exception of PG16 which was determined using the bivalent analyte model. All samples were run in duplicate and yielded similar kinetic results. Soluble two-domain CD4 was produced as described previously (Freeman et al. (2010) Structure. 18:1632) and generously provided by Bing Chen (Children's Hospital Boston). 17b hybridoma was kindly provided by James Robinson (Tulane University, New Orleans, LA) and purified as previously described (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111). VRC01 was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp120 mAb (VRC01), from John Mascola (Wu et al. (2010) Science. 329:856). 3BNC117 was kindly provided by Michel Nussenzweig (Rockefeller University, New York, NY). PGT121 and PGT126 were generously provided by Dennis Burton (The Scripps Research Institute, La Jolla, CA). 2F5, 4E10, PG9, PG16 were obtained commercially (Polymun Scientific). GCN gp41-Inter (Frey et al. (2010) Nat. Struct. Mol. Biol. 17:1486) used as a positive control in 4E10 and 2F5 SPR analyses was kindly provided by Bing Chen (Children's Hospital Boston).

Animals and immunizations

Outbred female Hartley guinea pigs (Elm Hill) (n=5/group) were housed at the Animal Research Facility of Beth Israel Deaconess Medical Center under approved Institutional Animal Care and Use Committee (IACUC) protocols. Guinea pigs were immunized intramuscularly (i.m.) with either mosaic M or clade C gp140 Env protein trimers (100 µg/animal) at weeks 0, 4, 8 in 500 µl injection volumes divided between the right and left quadriceps. Adjuvants co-administered with the proteins were a previously described combination comprising 15% (vol/vol) oil-in-water Emulsigen (MVP Laboratories)/PBS and 50 µg of immunostimulatory di-nucleotide type B oCpG DNA (5'-TCGTCGTTGTCGTTTTGTCGTT-3') (Midland Reagent Company) (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111) or the ISCOM-based Matrix M (Isconova, Sweden). The groups of animals receiving the mixture of clade C and

mosaic M gp140s were such that 50μg of each was mixed for a total of 100 μg administered/animal. Serum samples were obtained from the vena cava of anesthetized animals 4 weeks after each immunization.

ELISA binding assays

Serum binding antibody titers against mosaic M or clade C gp140 Env trimers were determined by endpoint enzyme-linked immunosorbent assays (ELISAs) as previously described (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111; Nkolola et al. (2010) J. Virol. 84:3270). ELISA endpoint titers were defined as the highest reciprocal serum dilution that yielded an absorbance >2-fold over background values. For the detection of MPER epitopes in the mosaic trimer, ELISA plates were coated with either 2F5 or 4E10 IgG, antigen added, and detected utilizing anti-his tag HRP mAb (Abcam). 2F5 and 4E10 were obtained commercially (Polymun Scientific) and GCN gp41-Inter (Frey et al. (2010) Nat. Struct. Mol. Biol. 17:1486) used as a positive control in 4E10 and 2F5 ELISAs was kindly provided by Bing Chen (Children's Hospital Boston).

TZM.bl neutralization assay

Neutralizing antibody responses against tier 1 HIV-1 Env pseudoviruses were measured using luciferase-based virus neutralization assays with TZM.bl cells as previously described (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111; Nkolola et al. (2010) J. Virol. 84:3270; Mascola et al. (2005) J. Virol. 79:10103). These assays measure the reduction in luciferase reporter gene expression levels in TZM-bl cells following a single round of virus infection. The 50% inhibitory concentration (IC50) was calculated as the serum dilution that resulted in a 50% reduction in relative luminescence units compared with the virus control wells after the subtraction of cell control relative luminescence units. The panel of tier 1 viruses analyzed included easy-to-neutralize tier 1A viruses (SF162.LS, MW965.26) and an extended panel of tier 1B viruses (DJ263.8, Bal.26, TV1.21, MS208, Q23.17, SS1196.1, 6535.3, ZM109.F, ZM197M). Murine leukemia virus (MuLV) negative controls were included in all assays.

A3R5 Neutralization Assay

Tier 2 nAb responses were evaluated using the A3R5 assay as previously described (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111). Briefly, serial dilutions of serum samples were performed in 10% RPMI growth medium (100 μL per well) in 96-well flat-bottomed plates. IMC HIV-1 expressing Renilla luciferase (Edmonds et al. (2010) Virology. 408:1) was added to each well in a volume of 50μl, and plates were incubated for 1 hour at 37°C. A3R5 cells were then added (9×10^4 cells per well in a volume of 100μl) in 10% RPMI growth medium containing diethylaminoethyl-dextran (11μg/ml). Assay controls included replicate wells of A3R5 cells alone (cell control) and A3R5 cells with virus (virus control). After incubation for 4 days at 37°C, 90μl of medium was removed from each assay well, and 75μl of cell suspension was transferred to a 96-well white, solid plate. Diluted ViviRen Renilla luciferase substrate (Promega) was added to each well (30μl), and after 4 minutes the plates were read on a Victor 3 luminometer. The A3R5 cell line was generously provided by R. McLinden and J. Kim (US Military HIV Research Program, Rockville, MD). Tier 2 clade B IMC Renilla luciferase viruses included SC22.3C2.LucR, SUMA.LucR and REJO.LucR. Tier 2 clade C IMC Renilla luciferase viruses included

Du422.1.LucR.T2A.ecto, Ce2010_F5.LucR.T2A.ecto, and e1086_B2.LucR.T2A.ecto and were generously provided by C. Ochsenbauer (University of Alabama at Birmingham, Birmingham, AL). Viral stocks were prepared in 293T/17 cells as previously described (Edmonds et al. (2010) Virology. 408:1).

Results

Expression and stability of the mosaic M gp140 trimer

Eukaryotic pCMV DNA vectors expressing HIV-1 mEnv, mEnv2, and mEnv3 were transfected into 293T cells, and protein expression and stability were assessed after 48 hours. Western blot analysis revealed a band size of the appropriate size for mEnv gp140 but not for mEnv2 or mEnv3 (Figure 9A), suggesting that the mEnv was a more stable and intact protein than the others. Size exclusion chromatography (SEC) using material generated from a stable 293T cell line expressing mEnv demonstrated a mono-disperse peak corresponding to the expected size for a gp140 trimer and confirmed the homogeneity of the trimer (Figure 9B). To evaluate the stability of this trimer, 100 µg protein underwent a freeze-thaw cycle or was stored for 7 days at 4°C and re-evaluated by SEC and showed no signs of aggregation, dissociation or degradation (Figure 9C).

Antigenicity of the mosaic M gp140 trimer

Surface plasmon resonance (SPR) studies were performed to determine whether the mEnv trimer exhibited CD4 binding and presented key epitopes targeted by several broadly nAbs. The trimer bound CD4 at a high affinity of 34.7 nM, showing that the CD4 binding site (CD4bs) is present and exposed in mEnv (Table 2; Figure 10A). We next evaluated the intrinsic structural flexibility of the mEnv trimer by measuring its ability to bind 17b immunoglobulin (IgG) in the presence and absence of bound CD4. 17b recognizes a CD4-induced (CD4i) epitope exposed by CD4 binding and the formation of the bridging sheet and co-receptor binding site in gp120 (Kwong et al. (1998) Nature. 393:648; Chen et al. (2005) Nature. 433:834). The mEnv trimer showed low 17b binding in the absence of CD4, but there was a substantial increase in this binding following CD4 binding as expected (Figure 10B).

Table 2. Binding rate constants derived from surface plasmon resonance (SPR) analyses. Binding kinetics were fitted with the Langmuir 1:1 binding model.

Immobilized ligand	Flowing analyte	k_a (1/Ms)	k_d (1/s)	K_D (M)
CD4	Mosaic M gp140	1.52×10^{4}	1.73×10^{-4}	1.14×10^{-8}
17b IgG	Mosaic M gp140	2.42×10^{4}	5.00×10^{-5}	2.07×10^{-9}
VRC01 IgG	Mosaic M gp140	3.84×10^{3}	4.90×10^{-6}	1.28×10^{-9}
3BNC117 IgG	Mosaic M gp140	7.65×10^{3}	1.81×10^{-5}	2.12×10^{-9}
PGT121 IgG	Mosaic M gp140	8.63×10^{3}	2.44×10^{-4}	2.82×10^{-8}
PGT126 IgG	Mosaic M gp140	2.43×10^{4}	1.29×10^{-4}	5.39×10^{-9}
PG9 IgG	Mosaic M gp140	9.61×10^{3}	5.55×10^{-4}	5.78×10^{-8}
	Mosaic M gp120	1.86×10^{3}	2.37×10^{-3}	1.28×10^{-6}

We next assessed the ability of the CD4bs bnAbs VRC01 and 3BNC117 (Wu et al. (2010) Science. 329:856; Scheid et al. (2011) Science. 333:1633) to bind to mEnv. VRC01 and 3BNC117 bound the mEnv trimer with high affinities of 1.28 nM and 2.12 nM, respectively, showing that these epitopes are present in the mEnv trimer (Table 2; Figure 10C – 10D).

Recent studies have highlighted the prominent role of N-linked glycans in the recognition of epitopes targeted by several bnAbs (Pejchal et al. (2011) Science. 334:1097; Walker et al. (2011) Nature. 477:466). In particular, the PGT class of bnAbs interact with N-linked glycans (such N332) and variable loop 3 (V3). Both PGT121 and PGT126 bound the trimer with high affinities of 28.2 nM and 5.39 nM (Table 2; Figure 11A – 11B), respectively, indicating that the key N-linked glycan epitopes recognized by these bnAbs were present on the trimer.

PG9 and PG16 bind preferentially to intact Env trimers and target N-linked glycans and variable loops 1 and 2 (V1/V2) and N-linked glycans in this region (Walker et al. (2009) Science. 326:285; McLellan et al. (2011) Nature. 480:336). The mEnv trimer bound to PG9 with a remarkably high affinity (57.8 nM; Figure 11C), whereas the mosaic gp120 monomer bound with a 22-fold lower affinity and substantially lower magnitude (1,280 nM) (Table 2; Figure 11E). The observed binding for PG9 was comparable to that observed with the recently described soluble, fully glycosylated cleaved BG505 SOSIP.664 gp140 trimer (Julien et al. (2013) Science. 342:1477; Sanders et al. (2013) PLoS Pathog. 9:e1003618). PG16 binding was also substantially greater to the mEnv trimer than the corresponding gp120 monomer (Table 3; Figures 11D, 11F), although the off-rate was faster for PG16 compared with PG9. These data show that the mEnv trimer, but not the corresponding monomer, efficiently binds these conformation-dependent bnAbs.

Table 3. Binding rate constants derived from surface plasmon resonance (SPR) analyses. Binding kinetics were fitted with the bivalent analyte model.

Immobilized ligand	Flowing analyte	k_{a1} (1/Ms)	k_{d1} (1/s)	k_{a2} (1/Ms)	k_{d2} (1/s)	K_{D1} (M)	K_{D2}(M)
PG16 IgG	Mosaic M gp140	2.34×10^{3}	7.67×10^{-3}	1.31×10^{5}	8.57×10^{-4}	3.27×10^{-6}	6.54×10^{-9}
	Mosaic M gp120	7.14×10^{3}	1.79×10^{-2}	1.69×10^{4}	8.42×10^{-4}	2.56×10^{-6}	1.51×10^{-8}

Finally, we assessed 2F5 and 4E10 binding to the membrane proximal external region (MPER) epitopes. 2F5 and 4E10 bnAbs bind to linear epitopes (Cardoso et al. (2007) J. Mol. Biol. 365:1533; Ofek et al. (2004) J. Virol. 78:10724) and at least the 2F5 epitope was present in the linear mEnv sequence as confirmed by sequence alignment (Figure 12A) and Western blot analyses (Figure 12B). However, by SPR and ELISA, the intact mEnv trimer was unable to bind 2F5, suggesting that the MPER epitope is buried in the trimer (Ofek et al. (2004) J. Virol. 78:10724) and not accessible in the context of the stable, folded mEnv trimer (Figure 12C – 12D), similar to our findings with our previously characterized stable clade C trimer (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111). Taken together, these antigenicity data suggest that the mEnv trimer exhibits many features of a properly folded trimer.

Functionality of the mEnv trimer

We next evaluated whether the mEnv protein was functional. Full-length mosaic M gp160 was used to generate pseudovirions to assess infectivity in TZM.bl cells (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111; Nkolola et al. (2010) J. Virol. 84:3270; Mascola et al. (2005) J. Virol. 79:10103; Montefiori (2009) Methods. Mol. Biol. 485:395). We observed that, over a broad titration range,

mEnv readily infected TZM.bl target cells over-expressing CD4 and co-receptors CCR5/CXCR4 (Figure 13). These data indicated that the synthetic mEnv has the functional ability to infect TZM.bl target cells.

Immunogenicity of the mEnv trimer

Guinea pigs (n=5/group) were immunized three times at monthly intervals with 100 µg mEnv trimer, 100 µg of our previously reported clade C gp140 trimer (hereafter referred to as "cEnv"; Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111; Nkolola et al. (2010) J. Virol. 84:3270) or a mixture of 50 µg of both trimers with ISCOM-based Matrix M or Emulsigen/CpG adjuvants (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111; Ioannou et al. (2002) J. Virol. 76:9002). High titer, binding antibodies by ELISA were elicited by all the vaccination regimens with comparable kinetics (Figure 14A – 14B). These responses were detectable after a single immunization and increased after the second and third immunization. Peak immunogenicity titers ranged from 5.0-7.0 logs. Binding antibody titers elicited by cEnv and mEnv trimers were higher against their respective homologous antigens ($P<0.05$), whereas the bivalent mEnv and cEnv mixture induced comparable responses to both antigens (Figure 14A – 14B).

We next assessed nAb responses elicited in these animals pre-vaccination and post-3rd vaccination serum samples using the TZM.bl nAb assay (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111; Nkolola et al. (2010) J. Virol. 84:3270; Mascola et al. (2005) J. Virol. 79:10103; Montefiori (2009) Methods. Mol. Biol. 485:395). We included a multi-clade panel of tier 1 psuedoviruses comprising easy-to-neutralize tier 1A viruses (SF162.LS, MW965.26) and an extended panel of intermediate tier 1B viruses (DJ263.8, Bal.26, TV1.21, MS208, Q23.17, SS1196.1, 6535.3, ZM109.F, ZM197M). Background pre-vaccination titers were observed as negative to low (<20). mEnv administered in Matrix M adjuvant was observed to induce high titer TZM.bl nAb against tier 1A (SF162.LS, MW965.26) and several tier 1B (DJ263.8, Bal.26, SS1196.1) viruses (Figure 15A – 15B). When compared to cEnv administered in the same adjuvant, mEnv elicited higher magnitude nAb titers against tier 1B clade B viruses Bal.26 and SS1196.1 ($P<0.05$; unpaired t-tests) (Figure 15A – 15B). In contrast, cEnv induced higher responses than did mEnv against clade A and clade C viruses (MW965.26, DJ263.8, TV1.21, MS208.1, Q23.17 and ZM109F; $P<0.05$, unpaired t-tests) (Figure 15A – 15B).

Importantly, the nAb responses elicited by the bivalent mEnv and cEnv mixture appeared additive (Figure 15A). These data suggest that the combination induced increased breadth than either mEnv or cEnv alone, and nAb responses to each isolate induced by the combination were typically comparable to the higher of the two individual trimers (Figure 15A – 15B). These data indicate that the mEnv and cEnv mixture conferred an immunologic advantage compared with either trimer alone. Comparable results were observed in guinea pigs receiving mEnv, cEnv, or bivalent mEnv and cEnv mixture in CpG/Emulsigen adjuvants (Figure 15C – 15D). Overall, these data suggest that the mEnv and cEnv trimers were complementary and could be effectively combined as a mixture that was superior to either trimer alone in terms of nAb coverage.

Previous studies with cEnv demonstrated sporadic and low level tier 2 nAb responses by TZM.bl assay (Kovacs et al. (2012) Proc. Natl. Acad. Sci. U.S.A. 109:12111; Nkolola et al. (2010) J. Virol. 84:3270). We therefore opted to utilize the more sensitive A3R5 assay to compare tier 2 nAb activity in these groups of animals. Both cEnv and mEnv induced detectable tier 2 nAbs against C viruses (Ce1086_B2, Ce2010, Du422) (Figure 16A) and clade B viruses (SUMA, SC22.3C2) (Figure 16B). The

bivalent mEnv and cEnv mixture elicited responses that were typically comparable to the better of the two individual trimers (Figure 16A – 16B). In addition, low tier 2 nAb activity was detected against clade B virus REJO.LucR with the combined regimen but not either trimer alone (*P<0.05; Mann Whitney test) (Figure 16B). These data demonstrate that the mixture of immunologically complementary mEnv and cEnv trimers were superior to either trimer alone.

We further developed an optimized version of the mEnv polypeptide, referred to herein as "Mosaic 1 Env gp140 version 2" or "mEnv+." As shown in Figure 2, mEnv+ (lane 4) is expressed at higher levels than cEnv (lane 1) or mEnv (lane 2). mEnv+ also demonstrates robust purity and stability, as shown by size exclusion chromatography (Figures 17A – 17B) and by SDS-PAGE profile (Figure 17C). The immunogenicity of mEnv+ was comparable to that of mEnv, regardless of adjuvant used (Figures 18A – B). Neutralization assays further demonstrated the comparable immunogenicity of mEnv+ as compared to mEnv (Figure 19).

Other Embodiments

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure that come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

US Provisional Application 61/749,737 is hereby incorporated by reference in its entirety. All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference in their entirety.

What is claimed is:

CLAIMS

1. A stabilized trimer comprising three gp140 polypeptides, wherein at least one of said gp140 polypeptides comprises an amino acid sequence having at least 90% identity to SEQ ID NO: 2.

2. The stabilized trimer of claim 1, wherein said at least one gp140 polypeptides comprises an amino acid sequence having at least 95% identity to, or the sequence of, SEQ ID NO: 2.

3. The stabilized trimer of claim 1 or 2, wherein two, or each, of said gp140 polypeptides comprises an amino acid sequence having at least 90% identity to, for example at least 95% identity to, or the sequence of, SEQ ID NO: 2.

4. A stabilized trimer comprising three gp140 polypeptides, wherein at least one of said gp140 polypeptides comprises an amino acid sequence having substantially the sequence of SEQ ID NO: 1, 3, 4, or 5.

5. The stabilized trimer of claim 4, wherein two, or each, of said gp140 polypeptides comprises an amino acid sequence having substantially the sequence of SEQ ID NO: 1, 3, 4, or 5.

6. A composition comprising the stabilized trimer of any one of claims 1 to 5.

7. The composition of claim 6, further comprising one or more different stabilized trimers.

8. The composition of claim 7, wherein said one or more different stabilized trimers comprises three gp140 polypeptides, wherein at least one, preferably two or each, of said gp140 polypeptides comprises an amino acid sequence having at least 90% identity to the sequence of any one of SEQ ID NOs: 1-5.

9. The composition of claim 8, wherein said one or more different stabilized trimers comprises three gp140 polypeptides, wherein at least one, preferably two or each, of said gp140 polypeptides comprises an amino acid sequence having at least 95% identity to, or the sequence of, any one of SEQ ID NOs: 1-5.

10. The composition of any one of claims 6 to 9, further comprising a pharmaceutically acceptable carrier, excipient, or diluent.

11. The composition of any one of claims 6 or 10, further comprising an adjuvant.

12. A vaccine comprising the composition of any one of claims 6 to 11.

13. The vaccine of claim 12, wherein said vaccine is capable of treating or reducing the risk of a human immunodeficiency virus (HIV) infection in a subject in need thereof.

14. The vaccine of claim 13, wherein said vaccine elicits production of neutralizing anti-HIV antisera after administration to said subject.

15. The vaccine of claim 14, wherein said anti-HIV antisera neutralizes HIV selected from any one or more of clade A, clade B, and clade C.

16. The vaccine of claim 15, wherein said subject is a human.

17. A nucleic acid molecule comprising a nucleotide sequence that encodes at least one gp140 polypeptide, wherein said gp140 polypeptide comprises:
    (a) an amino acid sequence having at least 95% identity to SEQ ID NO: 1; and/or
    (b) an amino acid sequence having at least 95% identity to SEQ ID NO: 2; and/or
    (c) an amino acid sequence having substantially the sequence of SEQ ID NO: 3; and/or
    (d) an amino acid sequence having at least 95% identity to SEQ ID NO: 4; and/or
    (e) an amino acid sequence having at least 95% identity to SEQ ID NO: 5, and/or combinations thereof.

18. A vector comprising one or more nucleic acid molecules of claim 17.

19. The vector of claim 18, wherein said vector is an adenovirus vector or a poxvirus vector.

20. The vector of claim 19, wherein said adenovirus is a recombinant adenovirus serotype 11 (Ad11), adenovirus serotype 15 (Ad15), adenovirus serotype 24 (Ad24), adenovirus serotype 26 (Ad26), adenovirus serotype 34 (Ad34), adenovirus serotype 35 (Ad35), adenovirus serotype 48 (Ad48), adenovirus serotype 49 (Ad49), adenovirus serotype 50 (Ad50), Pan9 (AdC68), or a chimeric variant thereof.

21. The vector of claim 19, wherein said poxvirus is a modified vaccinia virus Ankara (MVA).

22. A method of treating or reducing the risk of an HIV infection in a subject in need thereof comprising administering a therapeutically effective amount of the stabilized trimer of any one of claims 1-5, the composition of any one of claims 6-11, the vaccine of any one of claims 12-16, the nucleic acid molecule of claim 17, and/or the vector of any one of claims 18-21 to said subject.

23. A method of reducing an HIV-mediated activity in a subject infected with HIV comprising administering a therapeutically effective amount of the stabilized trimer of any one of claims 1-5, the composition of any one of claims 6-11, the vaccine of any one of claims 12-16, the nucleic acid molecule of claim 17, and/or the vector of any one of claims 18-21 to said subject.

24. The method of claim 23, wherein said HIV-mediated activity is viral spread, infection, or cell fusion.

25. The method of claim 24, wherein said cell fusion is target cell entry or syncytial formation.

26. The method of claim 23, wherein HIV titer in said subject infected with HIV is decreased after administration of said vaccine to said subject.

27. The method of any one of claims 22 to 26, wherein said vaccine is administered intramuscularly, intravenously, intradermally, percutaneously, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, topically, intratumorally, peritoneally, subcutaneously, subconjunctivally, intravesicularlly, mucosally, intrapericardially, intraumbilically, intraocularly, orally, topically, locally, by inhalation, by injection, by infusion, by continuous infusion, by localized perfusion bathing target cells directly, by catheter, by lavage, by gavage, in cremes, or in lipid compositions.

28. The method of any one of claims 22 to 27, wherein said subject is administered at least one dose of said vaccine.

29. The method of claim 28, wherein said subject is administered at least two doses of said vaccine.

30. The method of claim 28 or 29, wherein said vaccine is administered to said subject as a prime, a boost, or as a prime-boost.

31. The method of claim 30, wherein said vaccine is administered to said subject as said boost.

32. The method of claim 31, comprising, prior to administering a therapeutically effective amount of the stabilized trimer of any one of claims 1-5, the composition of any one of claims 6-11, the vaccine of any one of claims 12-16, the nucleic acid molecule of claim 17, and/or the vector of any one of claims 18-21 to said subject, administering a prime vaccine comprising at least a first vector comprising a first nucleic acid molecule that encodes a polypeptide having at least 85% amino acid sequence identity to SEQ ID NO: 6 and optionally a second vector comprising a second nucleic acid molecule that encodes a polypeptide having at least 85% identity to SEQ ID NO: 7.

33. A method of manufacturing a vaccine for treating or reducing the risk of an HIV infection in a subject in need thereof, said method comprising the steps of:
    (a) contacting vector of claim 18 with a cell; and
    (b) expressing said at least one gp140 polypeptide to form a stabilized trimer in said cell.

34. The method of claim 33, wherein said method is performed *in vitro* or *ex vivo*.

35. The method of claim 33 or 34, wherein said cell is a bacterial, plant, or mammalian cell.

36. The method of claim 35, wherein said mammalian cell is a 293T cell.

37. A kit comprising:

(a) the stabilized trimer of any one of claims 1-5, the composition of any one of claims 6-11, the vaccine of any one of claims 12-16, the nucleic acid molecule of claim 17, and/or the vector of any one of claims 18-21;

(b) a pharmaceutically acceptable carrier, excipient, or diluent; and

(c) instructions for use thereof,

wherein said kit optionally includes an adjuvant.