Patents

JP2020-515234

HIV immunotherapy without a prior immunization step

Abstract

translated from Japanese

The present invention relates generally to immunotherapy for the treatment or prevention of HIV. In particular, the present disclosure provides lentiviral vectors and related methods optimized for treating HIV without a prior immunization step. In one aspect of the present disclosure, a method of treating an HIV infection in a subject is disclosed. The method includes removing leukocytes from a subject and purifying peripheral blood mononuclear cells (PBMC). The method comprises contacting PBMCs ex vivo with a therapeutically effective amount of a stimulatory agent; transducing PBMCs ex vivo with a viral delivery system encoding at least one genetic element; and transduction. The method further comprises culturing the PBMCs for at least 1 day.

Classifications

A61K35/17 Lymphocytes; B-cells; T-cells; Natural killer cells; Interferon-activated or cytokine-activated lymphocytes

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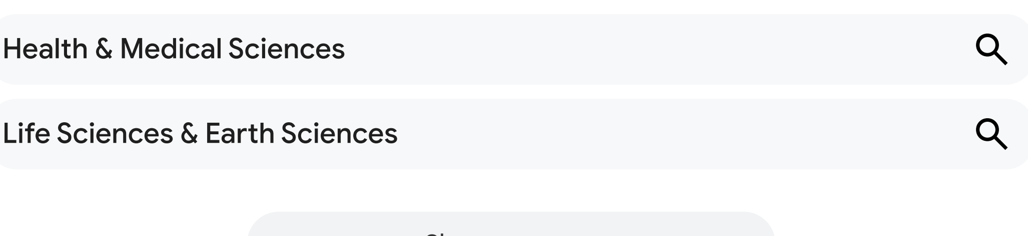

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Worldwide applications

2018 • EP JP WO KR KR CA US ~~KR~~ ~~AU~~ CN BR 2019 • IL 2022 • JP 2024 • AU

Application JP2019536901A events

- 2018-01-09 • Application filed by アメリカン ジーン テクノロジーズ インターナショナル インコーポレイテッド, アメリカン ジーン テクノロジーズ インターナショナル インコーポレイテッド
- 2020-05-28 • Publication of JP2020515234A
- 2021-10-07 • Publication of JP2020515234A5
- 2022-11-28 • Priority to JP2022189475A
- 2023-04-18 • Application granted
- 2023-04-18 • Publication of JP7260170B2
- Status • Active
- 2038-01-09 • Anticipated expiration

Info: Patent citations (11), Non-patent citations (4), Cited by (8), Legal events, Similar documents, Priority and Related Applications

Claims (73)

translated from Japanese

A method of treating cells infected with HIV, comprising:
(A) contacting peripheral blood mononuclear cells (PBMCs) isolated from an HIV-infected subject with a therapeutically effective amount of a stimulatory agent, said contacting being performed ex vivo;
(B) transducing the PBMC ex vivo with a viral delivery system encoding at least one genetic element; and (c) culturing the transduced PBMC for at least one day. .. The method of claim 1, wherein the transduced PBMC are cultured for about 1 to about 35 days. The method of claim 1, further comprising injecting the transduced PBMC into a subject. The method of claim 3, wherein the subject is a human. The method of claim 1, wherein the stimulatory agent comprises a peptide. The method of claim 5, wherein the peptide comprises the gag peptide. The method of claim 1, wherein the stimulatory agent comprises a vaccine. 8. The method of claim 7, wherein the vaccine comprises an HIV vaccine. 9. The method of claim 8, wherein the HIV vaccine comprises the MVA/HIV62B vaccine or variant thereof. The method of claim 1, wherein the viral delivery system comprises lentiviral particles. 2. The method of claim 1, wherein the at least one genetic element comprises small RNA capable of inhibiting the production of chemokine receptor CCR5, or at least one small RNA capable of targeting an HIV RNA sequence. The method of claim 1, wherein the at least one genetic element comprises a small RNA capable of inhibiting the production of the chemokine receptor CCR5 and at least one small RNA capable of targeting an HIV RNA sequence. 13. The method of claim 11 or 12, wherein the HIV RNA sequence comprises an HIV Vif sequence, an HIV Tat sequence, or variants thereof. 13. The method of claim 11 or 12, wherein the at least one genetic element comprises microRNA or shRNA. 15. The method of claim 14, wherein the at least one genetic element comprises a microRNA cluster. The at least one genetic element is
15. The method of claim 14, comprising a microRNA having a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%. The at least one genetic element is
15. The method of claim 14, comprising: The at least one genetic element is
a.
;
Has a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%, or b.
15. The method of claim 14, comprising a microRNA having a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%. The at least one genetic element is
a.
Or b.
15. The method of claim 14, comprising: The microRNA cluster is
16. The method of claim 15, comprising a sequence having a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%. The microRNA cluster is
16. The method of claim 15, comprising: A method of treating an HIV infection in a subject, comprising:
(A) removing white blood cells from the subject and purifying peripheral blood mononuclear cells (PBMC);
(B) contacting said PBMC with a therapeutically effective amount of a stimulatory agent ex vivo;
(C) transducing the PBMCs ex vivo with a viral delivery system encoding at least one genetic element; and (d) culturing the transduced PBMCs for at least one day. . 23. The method of claim 22, wherein the transduced PBMC are cultured for about 1 to about 35 days. 23. The method of claim 22, further comprising injecting the transduced PBMC into the subject. 25. The method of any one of claims 22-24, wherein the subject is a human. 23. The method of claim 22, wherein the stimulatory agent comprises a peptide. 27. The method of claim 26, wherein the stimulatory agent comprises a gag peptide. 23. The method of claim 22, wherein the stimulatory agent comprises a vaccine. 29. The method of claim 28, wherein the vaccine comprises an HIV vaccine. 30. The method of claim 29, wherein the HIV vaccine comprises the MVA/HIV62B vaccine or variant thereof. 23. The method of claim 22, wherein the viral delivery system comprises lentiviral particles. 23. The method of claim 22, wherein the at least one genetic element comprises a small RNA capable of inhibiting the production of the chemokine receptor CCR5 or at least one small RNA capable of targeting an HIV RNA sequence. 23. The method of claim 22, wherein the at least one genetic element comprises a small RNA capable of inhibiting the production of the chemokine receptor CCR5 and at least one small RNA capable of targeting an HIV RNA sequence. 34. The method of claim 32 or 33, wherein the HIV RNA sequence comprises an HIV Vif sequence, an HIV Tat sequence, or variants thereof. 34. The method of claim 32 or 33, wherein the at least one genetic element comprises microRNA or shRNA. 36. The method of claim 35, wherein the at least one genetic element comprises a microRNA cluster. The at least one genetic element is
a.
36. The method of claim 35, comprising a microRNA having a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%. The at least one genetic element is
36. The method of claim 35, including. The at least one genetic element is

a.
;
Has a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%, or b.
36. The method of claim 35, comprising a microRNA having a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%. The at least one genetic element is
a.
Or b.
36. The method of claim 35, including. The microRNA cluster is
37. The method of claim 36, comprising a sequence having a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%. The microRNA cluster is
37. The method of claim 36, including.   A lentiviral vector comprising at least one encoded genetic element, wherein said at least one encoded genetic element targets a small RNA or HIV RNA sequence capable of inhibiting the production of chemokine receptor CCR5. Lentiviral vector comprising at least one small RNA capable of being, wherein said HIV RNA sequence comprises an HIV Vif sequence, an HIV Tat sequence, or variants thereof.   A lentiviral vector comprising at least one encoded genetic element, wherein said at least one encoded genetic element targets small RNA capable of inhibiting the production of chemokine receptor CCR5, and HIV RNA sequences. Lentiviral vector comprising at least one small RNA capable of being, wherein said HIV RNA sequence comprises an HIV Vif sequence, an HIV Tat sequence, or variants thereof.   45. The lentiviral vector of claim 43 or 44, wherein the at least one encoded genetic element comprises a microRNA or shRNA.   46. The lentiviral vector of claim 45, wherein the at least one encoded genetic element comprises a microRNA cluster. The at least one encoded genetic element is
a.
46. The lentiviral vector of claim 45, which comprises a microRNA having a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%. The at least one encoded genetic element is
48. The lentiviral vector of claim 47, which comprises: Said at least one encoded genetic element is
a.
;
Has a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%, or b.
46. The lentiviral vector of claim 45, which comprises a microRNA having a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%. Said at least one encoded genetic element is
a.
Or b.
46. The lentiviral vector of claim 45, which comprises: The microRNA cluster is
47. The lentiviral vector of claim 46, comprising a sequence having a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%. The microRNA cluster is
47. The lentiviral vector of claim 46, which comprises: A lentiviral vector system for expressing a lentiviral particle, comprising:
a. 53. The lentiviral vector according to any one of claims 43 to 52;
b. An envelope plasmid for expressing an envelope protein whose infection of cells is optimized; and c. at least one helper plasmid for expressing the gag, pol, and rev genes, said lentiviral vector, said envelope plasmid, and said at least one helper plasmid being transfected into a packaging cell line, said package A lentiviral vector system, wherein lentiviral particles are produced by a S. ungula cell line, said lentiviral particles can either inhibit the production of chemokine receptor CCR5 or target HIV RNA sequences.   54. The lentiviral vector system of claim 53, comprising a first helper plasmid for expressing the gag and pol genes and a second plasmid for expressing the rev gene.   53. A lentiviral particle capable of infecting cells, comprising an envelope protein optimized for infection of cells, and a lentiviral vector according to any one of claims 43 to 52.   56. The lentiviral particle of claim 55, wherein the envelope protein is optimized for infection of T cells.   57. The lentiviral particle of claim 56, wherein the envelope protein is optimized for infection of CD4+ T cells.   58. Modified cells comprising CD4+ T cells, wherein said CD4+ T cells are infected with the lentiviral particle of any one of claims 55 to 57.   59. The modified cell of claim 58, wherein the CD4+ T cells also recognize HIV antigens.   60. The modified cell of claim 59, wherein the HIV antigen comprises a gag antigen.   59. The modified cell of claim 58, wherein the CD4+ T cells express reduced levels of CCR5 after infection with the lentiviral particles. A method of selecting a subject for therapeutic treatment, comprising:
(A) removing leukocytes from the subject, purifying peripheral blood mononuclear cells (PBMCs), and determining a first quantifiable measurement associated with at least one factor associated with the PBMCs; and b) contacting the PBMC ex vivo with a therapeutically effective amount of a second stimulatory agent and determining a second measurement associated with the at least one factor associated with the PBMC. The method wherein the subject is selected for the treatment regimen if the second quantifiable measurement is higher than the first quantifiable measurement.   63. The method of claim 62, wherein the at least one factor associated with the PBMC is T cell proliferation.   63. The method of claim 62, wherein the at least one factor is IFN gamma production.   Depleting said PBMCs for at least one subset of cells, wherein said at least one subset of cells comprises CD8+ T cells, γδ cells, NK cells, B cells, neutrophils, basophils, eosinophils The method of claim 1, comprising any one or more of: T regulatory cells, NKT cells, and red blood cells.   66. The method of claim 65, wherein the depleting step is performed after removing white blood cells.   66. The method of claim 65, wherein the depleting step occurs

concurrently with the removal of white blood cells. Depleting said PBMCs for at least one subset of cells, wherein said at least one subset of cells comprises CD8+ T cells, γδ cells, NK cells, B cells, neutrophils, basophils, eosinophils 23. The method of claim 22, comprising any one or more of:, regulatory T cells, NKT cells, and red blood cells. 69. The method of claim 68, wherein the depleting step is performed after removing the white blood cells. 69. The method of claim 68, wherein the depleting step is performed concurrently with removing the white blood cells. Depleting said PBMCs for at least one subset of cells, wherein said at least one subset of cells comprises CD8+ T cells, γδ cells, NK cells, B cells, neutrophils, basophils, eosinophils 63. The method of claim 62, comprising any one or more of:, regulatory T cells, NKT cells, and red blood cells. 72. The method of claim 71, wherein the depleting step is performed after removing the white blood cells. 72. The method of claim 71, wherein the depleting step occurs concurrently with removing the white blood cells.

Description

translated from Japanese

CROSS REFERENCE TO RELATED APPLICATIONS This application claims priority based on US Patent Application No. 62/444,147, entitled "HIV Immunotherapy With No Pre-Immunization Step," filed Jan. 9, 2017. The disclosure of which is incorporated herein by reference.

FIELD OF THE INVENTION The present invention relates generally to the field of immunotherapy for treating and preventing HIV. In particular, the disclosed methods of treatment and prevention relate to the administration of viral vectors and systems to deliver genes, as well as other therapeutic, diagnostic, or research uses, without a prior immunization step.

BACKGROUND OF THE INVENTION Combined antiretroviral therapy (cART), also known as hyperactive antiretroviral therapy or HAART, limits HIV-1 replication and delays disease progression, but is associated with drug toxicity and drug resistance. Emergence is a challenge for long-term control in HIV infected individuals. Moreover, traditional antiretroviral therapies, although successful in delaying the onset or death of AIDS, still do not provide a functional cure. Alternative treatment strategies are needed.

The emergence of data demonstrating that the immune system plays a major but usually poor role in limiting HIV replication has created a strong interest in immunotherapy against HIV infection. Virus-specific T helper cells are important and likely play a role in maintaining cytolytic T cell (CTL) function. Also, viremia is affected by neutralizing antibodies, which are generally small in size in HIV infection and lag behind evolving viral variants in vivo.

Taken together, these data indicate that increasing the strength and breadth of the HIV-specific cellular immune response may have clinical benefit through so-called HIV immunotherapy. Some studies have tested vaccines against HIV, but to date have had limited success. Further, there has been interest in enhancing HIV immunotherapy by utilizing gene therapy techniques, but with limited success, as with other immunotherapy approaches.

Because of the specific viral envelope-host cell receptor interaction and viral machinery for gene expression, viral vectors can be used to transduce genes into target cells. As a result, viral vectors are capable of transferring genes into many different cell types including whole T cells or other immune cells and embryos, fertilized eggs, isolated tissue samples, in situ tissue targets, and cultured cells. Has been used as a vehicle for. The ability to introduce and express foreign or modified genes in cells is useful for gene therapy, somatic reprogramming of induced pluripotent stem cells, and therapeutic interventions such as various types of immunotherapy.

Gene therapy is one of the most mature areas of biomedical research with the potential to create new therapies that may include the use of viral vectors. Given the wide variety of potential genes available for therapy, efficient delivery of these genes is necessary to realize the potential of gene therapy as a means of treating infectious and non-infectious diseases. Necessary means. Several viral systems have been developed as therapeutic gene transfer vectors, including the murine retrovirus, adenovirus, parvovirus (adeno-associated virus), vaccinia virus, and herpes virus.

There are many factors to consider when developing a viral vector, including tissue tropism, viral preparation stability, expression stability and control, genome packaging capacity, and construct-dependent vector stability. .. Moreover, in vivo applications of viral vectors are often limited by the host immune response to viral structural proteins and/or transgene products.

Therefore, toxicity and safety are important hurdles that must be overcome for viral vectors used in vivo for treatment of subjects. There are numerous historical examples of gene therapy applications in humans with problems associated with host immune responses to gene delivery vehicles or therapeutic gene products. Viral vectors (eg, adenoviruses) that co-transduce several viral genes with one or more therapeutic genes are of particular concern.

While lentiviral vectors generally do not induce cytotoxicity and do not elicit a strong host immune response, some lentiviral vectors, such as HIV-1, which have some immunostimulatory gene products, cause cytotoxicity. , May induce a strong immune response in vivo. However, this may not be a problem for lentivirus-derived transduction vectors that do not encode multiple viral genes after transduction. Of course, this may not always be the case, as it may be the purpose of the vector to encode a protein that will elicit a clinically useful immune response.

Another important issue with the use of lentiviral vectors is the problem of cytopathogenicity that can occur upon exposure to some cytotoxic viral proteins. Exposure to specific HIV-1 proteins may induce cell death or functional unresponsiveness in T cells. Similarly, the potential for recombination to produce a replication competent virulent virus is often problematic. Therefore, there remains a need for improved treatments for HIV.

SUMMARY OF THE INVENTION In one aspect of the present disclosure, a method of treating an HIV infection in a subject is disclosed. The method includes removing leukocytes from a subject and purifying peripheral blood mononuclear cells (PBMC). The method comprises contacting PBMCs ex vivo with a therapeutically effective amount of a stimulatory agent; transducing PBMCs ex vivo with a viral delivery system encoding at least one genetic element; and transduction. The method further comprises culturing the PBMCs for at least 1 day. The transduced PBMC may be cultured for about 1 to about 35 days. The method may further include injecting the transduced PBMC into the subject. The subject may be a human. The stimulatory agent may comprise a peptide or mixture of peptides. In a preferred embodiment, the stimulatory agent comprises the gag peptide. The stimulatory agent may include a vaccine. The vaccine may be an HIV vaccine, and in a preferred embodiment the HIV vaccine is the MVA/HIV62B vaccine or variant thereof. In a preferred embodiment, the viral delivery system comprises lentiviral particles. In one embodiment, the at least one genetic element may comprise a small RNA capable of inhibiting the production of the chemokine receptor CCR5, or at least one small RNA capable of targeting an HIV RNA sequence. In another embodiment, at least one genetic element may comprise a small RNA capable of inhibiting the production of chemokine receptor CCR5 and at least one small RNA capable of targeting an HIV RNA sequence. .. The HIV RNA sequence may include the HIV Vif sequence, the HIV Tat sequence, or variants thereof. At least one genetic element may comprise microRNA or shRNA. In a preferred embodiment, at least one genetic element comprises a microRNA cluster.

In another aspect, at least one genetic element is
With at least 80%, or at least 85%, or at least 90%, or at least 95% percent identity. In a preferred embodiment, at least one genetic element is
including.

In another aspect, the at least one genetic element is
With at least 80%, or at least 85%, or at least 90%, or at least 95% percent identity, or
With at least 80%, or at least 85%, or at least 90%, or at least 95% percent identity. In a preferred embodiment, at least one genetic element is
including.

In another aspect, the microRNA cluster is
With at least 80%, or at least 85%, or at least 90%, or at least 95% sequence identity. In a preferred embodiment, the microRNA cluster is
including.

In another aspect, a method of treating cells infected with HIV is provided. The method comprises contacting peripheral blood mononuclear cells (PBMCs) isolated from an HIV infected subject with a therapeutically effective amount of a stimulatory agent, wherein the contacting is performed ex vivo. transducing PBMCs in vivo with a viral delivery system encoding at least one genetic element; and culturing the transduced PBMCs for at least one day. The transduced PBMC may be cultured for about 1 to about 35 days. The method may further include injecting the transduced PBMC into the subject. The subject may be a human. The stimulatory agent may comprise a peptide or mixture of peptides, and in a preferred embodiment comprises a gag peptide. The stimulatory agent may include a vaccine. The vaccine may be an HIV vaccine, and in a preferred embodiment the HIV vaccine is the MVA/HIV62B vaccine or variant thereof. In a preferred embodiment, the viral delivery system comprises lentiviral particles. In one embodiment, the at least one genetic element may comprise a small RNA capable of inhibiting the production of the chemokine receptor CCR5, or at least one small RNA capable of targeting an HIV RNA sequence. In another embodiment, at least one genetic element may comprise a small RNA capable of inhibiting the production of the chemokine receptor CCR5 and at least one small RNA capable of targeting an HIV RNA sequence. .. HIV RNA sequences may include HIV Vif sequences, HIV Tat sequences, or variants thereof. At least one genetic element may comprise microRNA or shRNA. In a preferred embodiment, at least one genetic element comprises a microRNA cluster.

In another aspect, at least one genetic element is
With at least 80%, or at least 85%, or at least 90%, or at least 95% percent identity. In a preferred embodiment, at least one genetic element is

including.

In another aspect, the at least one genetic element is
With at least 80%, or at least 85%, or at least 90%, or at least 95% percent identity, or
With at least 80%, or at least 85%, or at least 90%, or at least 95% percent identity. In a preferred embodiment, at least one genetic element is
including.

In another aspect, the microRNA cluster is
With at least 80%, or at least 85%, or at least 90%, or at least 95% percent identity. In a preferred embodiment, the microRNA cluster is
including.

In another aspect, lentiviral vectors are disclosed. The lentiviral vector comprises at least one encoded genetic element, the at least one encoded genetic element targeting a small RNA or HIV RNA sequence capable of inhibiting the production of the chemokine receptor CCR5. At least one small RNA capable of In another aspect, the at least one encoded genetic element comprises a small RNA capable of inhibiting the production of the chemokine receptor CCR5 and at least one small RNA capable of targeting an HIV RNA sequence. HIV RNA sequences may include HIV Vif sequences, HIV Tat sequences, or variants thereof. The at least one encoded genetic element may comprise microRNA or shRNA. At least one encoded genetic element may comprise a microRNA cluster.

In another aspect, at least one genetic element is
With at least 80%, or at least 85%, or at least 90%, or at least 95% percent identity. In a preferred embodiment, at least one genetic element is
including.

In another aspect, at least one genetic element is
Has a percent identity with at least 80%, or at least 85%, or at least 90%, or at least 95%, or
With at least 80%, or at least 85%, or at least 90%, or at least 95% percent identity. In a preferred embodiment, at least one genetic element is
including.

In another aspect, the microRNA cluster is
With at least 80%, or at least 85%, or at least 90%, or at least 95% percent identity. In a preferred embodiment, the microRNA cluster is
including.

In another aspect, a lentiviral vector system for expressing lentiviral particles is disclosed. The system comprises a lentiviral vector as described herein; an envelope plasmid for expressing envelope proteins optimized for infection of cells; and at least one for expressing the gag, pol, and rev genes. When the packaging cell line is transfected with a lentiviral vector, an envelope plasmid, and at least one helper plasmid, the lentiviral particles are produced by the packaging cell line, and the lentiviral particles receive the chemokine receptor. The production of somatic CCR5 can be inhibited or the HIV RNA sequence can be targeted. The system may further include a first helper plasmid for expressing the gag and pol genes, and a second plasmid for expressing the rev gene.

In another aspect, lentiviral particles capable of infecting cells are disclosed. Lentiviral particles include envelope proteins that are optimized for infection of cells, and lentiviral vectors as described herein. The envelope protein may be optimized for infection of T cells. In a preferred embodiment, the envelope protein is optimized for infection of CD4+ T cells.

In another aspect, modified cells are disclosed. Modified cells include CD4+ T cells, which are infected with lentiviral particles as described herein. In a preferred embodiment, CD4+ T cells also recognize the HIV antigen. In a more preferred embodiment, the HIV antigen comprises the gag antigen. In a more preferred embodiment, CD4+ T cells express reduced levels of CCR5 after infection with lentiviral particles.

In another aspect, a method of selecting a subject for a therapeutic treatment regimen is disclosed. The method comprises removing leukocytes from a subject, purifying peripheral blood mononuclear cells (PBMCs), and determining a first quantifiable measurement associated with at least one factor associated with PBMCs; ex vivo, Contacting the PBMC with a therapeutically effective amount of a second stimulatory agent and determining a second measurement associated with at least one factor associated with PBMC, the second quantifiable measurement Is higher than the first quantifiable measurement, the subject is selected for the treatment regimen. The at least one factor may be T cell proliferation or IFNgamma production.

In another aspect, the methods disclosed herein include depleting PBMCs of at least one subset of cells. The method comprises depleting PBMCs of at least one subset of cells, wherein the at least one subset of cells comprises CD8+ T cells, γδ cells, NK cells, B cells, neutrophils, basophils, eosinophils. , Regulatory T cells, NKT cells, and red blood cells. In an embodiment, the depleting step is performed after removing the white blood cells. In an embodiment, the depleting step is performed concurrently with the removal of white blood cells.

The foregoing general description and the following brief description of the drawings and modes for carrying out the invention are exemplary and explanatory and are intended to provide a further description of the claimed invention. Other objects, advantages, and novel features will be readily apparent to those skilled in the art from the following brief description of the drawings and modes for carrying out the invention.

FIG. 1 shows a flow chart diagram of a particular clinical therapy strategy.

FIG. 2 is a diagram showing how CD4+ T cells can be altered using gene therapy to prevent other cells from infecting and/or preventing viral replication.

FIG. 3 shows an exemplary lentiviral vector system composed of a therapeutic vector, a helper plasmid, and an envelope plasmid. The therapeutic vector shown here, also referred to herein as AGT103, is a preferred therapeutic vector comprising miR30CCR5-miR21Vif-miR185-Tat.

FIG. 4 shows an exemplary 3-vector lentiviral vector system in circularized form.

FIG. 5 shows an exemplary 4-vector lentiviral vector system in circularized form.

FIG. 6 shows an exemplary vector sequence. The promoter and miR cluster positive (genomic) strand sequences were developed to inhibit the spread of CCR5-directed HIV strains. The sequences not underlined include the EF-1 alpha transcription promoter selected as the best for this miR cluster. The underlined sequences direct miR30 CCR5 (a modification of natural human miR30 that causes redirection to CCR5 mRNA), miR21 Vif (which causes redirection to Vif RNA sequence) and miR185 Tat (redirection to Tat RNA sequence). (Occurring) are shown (collectively shown in SEQ ID NO:33).

FIG. 7 shows an exemplary lentiviral vector construct according to aspects of the disclosure.

FIG. 8 shows that knockdown of CCR5 with an experimental vector prevents R5-directed HIV infection in AGTc120 cells. (A) shows CCR5 expression in AGTc120 cells with or without the AGT103 lentiviral vector. (B) shows the susceptibility of transduced AGTc120 cells to infection with an HIV BaL viral stock expressing green fluorescent protein (GFP) fused to the HIV Nef gene.

FIG. 9 shows data demonstrating that CCR5 expression was regulated by the lentiviral vector shRNA inhibitor sequences. (A) Screening data for potential candidates is shown. (B) CCR5 knockdown data after transduction with CCR5 shRNA-1 (SEQ ID NO: 16) is shown.

FIG. 10 shows data demonstrating that HIV components were regulated by the lentiviral vector shRNA inhibitor sequences. (A) Knockdown data for Rev/Tat target gene is shown. (B) Knockdown data for the Gag target gene is shown.

FIG. 11 shows data demonstrating that AGT103 reduces expression of Tat protein in cells transfected with HIV expression plasmids as described herein.

FIG. 12 shows data demonstrating that the HIV component was regulated by the synthetic microRNA sequence of the lentiviral vector. (A) Tat knockdown data is shown. (B) Vif knockdown data is shown.

FIG. 13 shows data demonstrating that CCR5 expression was regulated by the lentiviral vector synthetic microRNA sequence.

Figure 14 shows data demonstrating that CCR5 expression was regulated by synthetic microRNA sequences of lentiviral vectors containing long or short WPRE sequences.

FIG. 15 shows data demonstrating that expression of CCR5 was regulated by synthetic microRNA sequences of lentiviral vectors with or without WPRE sequences.

FIG. 16 shows data demonstrating that CCR5 expression was regulated by the CD4 promoter-regulated synthetic microRNA sequence of the lentiviral vector.

Figure 17 shows data demonstrating detection of HIV Gag-specific CD4 T cells.

FIG. 18 shows data demonstrating HIV-specific CD4 T cell proliferation and lentiviral transduction. (A) Treatment schedule is shown. (B) IFN-gamma production in CD4-gated T cells is shown as described herein. (C) IFN-gamma production and GFP expression in CD4-gated T cells is shown as described herein. (D) The frequency of HIV-specific CD4+ T cells as described herein and, importantly, before and after vaccination is shown. (E) IFN-gamma production from PBMCs after vaccination is shown as described herein.

Figure 19 shows data demonstrating a functional assay for dose response and inhibition of CCR5 expression upon increasing AGT103-GFP. (A) Dose response data for increasing amounts of AGT103-GFP are shown. (B) A population normally distributed in terms of CCR5 expression is shown. (C) Percentage inhibition of CCR5 expression with increasing dose of AGT103-GFP is shown.

FIG. 20 shows data demonstrating that AGT103 efficiently transduces primary human CD4+ T cells. (A) Frequency of transduced cells (GFP positive) is shown by FACS, as described herein. (B) Vector copy number per cell is shown as described herein.

FIG. 21 shows data demonstrating that AGT103 inhibits HIV replication in primary CD4+ T cells, as described herein.

FIG. 22 shows data demonstrating that AGT103 protects primary human CD4^{+} T cells from HIV-induced depletion.

FIG. 23 shows data demonstrating the generation of a CD4+ T cell population highly enriched for HIV-specific AGT103 transduced CD4 T cells. (A) shows CD4 and CD8 expression profiles of cell populations as described herein. (B) shows CD4 and CD8 expression profiles of cell populations as described herein. (C) shows the IFN-gamma and CD4 expression profiles of cell populations as described herein. (D) shows IFN-gamma and GFP expression profiles of cell populations as described herein. FIG. 23 shows data demonstrating the generation of a CD4+ T cell population highly enriched for HIV-specific AGT103 transduced CD4 T cells. (A) shows CD4 and CD8 expression profiles of cell populations as described herein. (B) shows CD4 and CD8 expression profiles of cell populations as described herein. (C) shows the IFN-gamma and CD4 expression profiles of cell populations as described herein. (D) shows IFN-gamma and GFP expression profiles of cell populations as described herein.

Figure 24 shows a schematic of the CD8 depletion protocol.

Figure 25 shows proliferation of Gag-specific T cells by peptide stimulation, CD8 depletion and IL-7/IL-15 incubation. (A), (B), and (C) show flow cytometry data showing that CD4+ T cell proliferation was significantly improved after depletion of CD8+ cells. In addition to improving CD4+ T cell proliferation, overgrowth of (A) Vδ1 T cells and (C) NK cells also occurred. Figure 25 shows proliferation of Gag-specific T cells by peptide stimulation, CD8 depletion and IL-7/IL-15 incubation. (A), (B), and (C) show flow cytometry data showing that CD4+ T cell proliferation was significantly improved after depletion of CD8+ cells. In addition to improving CD4+ T cell proliferation, overgrowth of (A) Vδ1 T cells and (C) NK cells also occurred. Figure 25 shows proliferation of Gag-specific T cells by peptide stimulation, CD8 depletion and IL-7/IL-15 incubation. (A), (B), and (C) show flow cytometry data showing that CD4+ T cell proliferation was significantly improved after depletion of CD8+ cells. In addition to improving CD4+ T cell proliferation, overgrowth of (A) Vδ1 T cells and (C) NK cells also occurred.

Figure 26 shows a schematic of the CD8/CD56/CD19/γδ depletion protocol.

FIG. 27 shows proliferation of Gag-specific T cells by peptide stimulation, CD8/γδ/NK/B cell depletion and IL-7/IL-15 incubation. (A)-(B) Flow cytometry showing that CD8+, γδ, or NK cell overgrowth inhibits CD4+ T cell growth or kills lentivirus-transduced antigen-specific CD4+ T cells. Show data. After depletion of CD8+, γδ, or NK cells, CD4+ T cells were expanded. FIG. 27 shows proliferation of Gag-specific T cells by peptide stimulation, CD8/γδ/NK/B cell depletion and IL-7/IL-15 incubation. (A)-(B) Flow cytometry showing that CD8+, γδ, or NK cell overgrowth inhibits CD4+ T cell growth or kills lentivirus-transduced antigen-specific CD4+ T cells. Show data. After depletion of CD8+, γδ, or NK cells, CD4+ T cells were expanded.

FIG. 28 shows Gag-specific T cell proliferation and transduction by peptide stimulation, CD8/γδ/NK/B cell depletion and IL-7/IL-15 incubation. IFN-γ positive antigen-specific CD4+ T cells produced good transduction efficiency compared to other subsets in culture.

Figure 29 shows the relationship between the percentage of transduced cells and the vector copy number. (A) shows a table showing that as the percentage of transduced cells increases, the vector copy number also increases (n=4). (B) Regression analysis of the same sample shown in the table showing a positive correlation between the percentage of transduced cells and vector copy number (n=4).

DETAILED DESCRIPTION Overview Disclosed herein are methods and compositions for treating and/or preventing human immunodeficiency virus (HIV) disease to achieve functional cure. Functional cure is defined as a condition resulting from the disclosed treatments and methods that reduce or eliminate the need for cART and

may or may not require supportive adjuvant therapy. The methods of the invention include gene delivery by integrative lentivirus, non-integrating lentivirus, and related viral vector technologies described below.

Disclosed herein are therapeutic viral vectors (eg, lentiviral vectors), immunotherapy, and methods for their use in strategies to achieve functional cure of HIV infection. As shown in FIG. 1 herein, a strategy for treating HIV was aimed at enriching a fraction of HIV-specific CD4 T cells, viremia by daily administration of HAART. Including a first therapeutic immunization with a vaccine intended to generate a strong immune response against HIV in HIV-infected patients with stable suppression of. However, as detailed herein, the initial therapeutic immunization may not be necessary. Then, (1) isolating peripheral leukocytes by leukopheresis or purifying PBMCs from venous blood, (2) restimulating CD4 T cells with HIV vaccine protein ex vivo, (3) This is followed by therapeutic lentiviral transduction, performing T cell culture ex vivo, and (4) reinjecting back into the original donor.

In view of the above description, referring to FIG. 2 herein, the method can be used to prevent new cells, such as CD4+ T cells, from becoming infected with HIV. To prevent new cells from being infected, CCR5 expression can be targeted to prevent viral attachment. Moreover, the disruption of any residual infectious viral RNA can also be targeted. In view of the above description, and with reference to Figure 2 herein, the method can also be used to arrest the HIV viral cycle in cells already infected with HIV. Viral RNA produced by latently infected cells, such as latently infected CD4+ T cells, can be targeted to arrest the HIV viral cycle.

New strategies have been developed to achieve a functional cure of HIV by providing highly effective therapeutic lentiviruses capable of inhibiting HIV.

Definitions and Interpretation Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. In general, the nomenclature and techniques used in connection with the cell and tissue culture, molecular biology, immunology, microbiology, genetics, and protein and nucleic acid chemistry, and hybridization described herein are well known. , Which are commonly used in the art. The methods and techniques of the disclosure, unless otherwise indicated, generally refer to the conventional and well-known methods in the art, and to the various general and more specialized techniques cited and discussed throughout this specification. It is carried out as described in the references. For example, Sambrook J. and Russell D. Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2000); Ausubel et al., Short Protocols in Molecular Biology: A Compendium of Methods from. Current Protocols in Molecular Biology, Wiley, John & Sons, Inc. (2002); Harlow and Lane, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1998); and Coligan et al., See Short Protocols in Protein Science, Wiley, John & Sons, Inc. (2003). Any enzymatic reaction or purification technique is performed according to manufacturer's specifications, as commonly accomplished in the art, or as described herein. The nomenclature, experimental procedures, and techniques used in connection with analytical chemistry, synthetic organic chemistry, and medical and medicinal chemistry described herein are those well known and commonly used in the art. ..

As used herein, the term "about" will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which it is used. If there are uses of the term that are not clear to those of skill in the art, given the context in which it is used, "about" will mean plus or minus 10% of the particular term.

As used herein, the term "administration" or "administering" of an active agent refers to a therapeutically effective amount of the active agent of the invention in a therapeutically useful form to a subject in need thereof. Provided in a form that can be introduced into the body.

As used herein, the term "AGT103" refers to a lentiviral vector containing a miR30-CCR5/miR21-Vif/miR185-Tat microRNA cluster sequence, as detailed herein. Embodiment of the present invention.

As used herein, the term "AGT103T" refers to cells transduced with a lentivirus or lentiviral particle containing the AGT103 lentiviral vector.

Throughout this specification and claims, the word "comprise" or its conjugations, such as "comprises" or "comprising", is used to refer to the integer or integers stated. It will be understood to indicate inclusion of the group, but not excluding any other integer or group of integers. Further, as used herein, the term "includes" means including but not limited to.

The term "transplant" refers to one of ordinary skill in the art having the ability to determine a quantitative level of transplant persistence in a subject after infusion of a cell source (eg, Rosenberg et al., N. Engl. J. Med. 323). Volume: 570-578 (1990); Dudley et al., J. Immunother. 24: 363-373 (2001); Yee et al., Curr. Opin. Immunol. 13: 141-146 (2001). Rooney et al., Blood 92: 1549-1555 (1998)).

The terms "expression", "expressed", or "encoding" refer to the process by which a polynucleotide is transcribed into mRNA and/or the transcribed mRNA is subsequently translated into a peptide, polypeptide, or protein. Point to. Expression can include splicing of mRNA in eukaryotic cells, or other forms of post-transcriptional or post-translational modification.

The term "functional cure" refers to HIV+ individuals previously in need of cART or HAART using lower or intermittent doses of cART or HAART, or discontinuing medication, resulting in low viral replication or detection. Pertaining to a situation or condition in which it is possible to survive in an impossible manner. An individual may be said to be "functionally cured" even though they still require adjunctive therapy to maintain low levels of viral replication and slow or eliminate disease progression. A possible consequence of functional cure is the eventual eradication of HIV, which prevents all possible relapses.

The term "HIV vaccine" includes immunogens, vehicles and adjuvants intended to elicit an HIV-specific immune response. An "HIV vaccine" is a purified inactivated virus particle or whole inactivated virus particle, which may be HIV, or a set capable of expressing an HIV protein, protein fragment or peptide, glycoprotein fragment or glycopeptide. Altered viral vectors may be included in addition to recombinant bacterial vectors, plasmid DNA or RNA capable of inducing cells to produce HIV proteins, glycoproteins or protein fragments capable of inducing specific immunity. Alternatively, for the purpose of enriching HIV-specific CD4 T cells prior to transduction, or for in vitro assay of lentivirus-transduced CD4 T cells, anti-CD3/CD28 beads, T cell receptor It is possible to activate dendritic, T or B cells using specific methods for immune stimulation including body-specific antibodies, mitogens, superantigens, and other chemical or biological stimuli. it can. Activators may be soluble, polymeric aggregates, liposomes or endosome-based or linked beads. Adding cytokines including interleukin-2, 6, 7, 12, 15, 23 or others to improve the cellular response to stimulation and/or improve the survival of CD4 T cells throughout the culture and transduction intervals. You can Alternatively, without limitation to any of the foregoing, the term "HIV vaccine" includes the MVA/HIV62B vaccine and variants thereof. The MVA/HIV62B vaccine is a known highly attenuated dual recombinant MVA vaccine. The MVA/HIV62B vaccine was constructed by inserting the HIV-1 gag-pol and env sequences into a known MVA vector (eg, Goepfert et al. (2014) J. Infect. Dis. 210(1): See pages 99-110, and WO 20060266667, both of which are hereby incorporated by reference). The term "HIV vaccine" also includes any one or more vaccines provided in Table 1 below.

The term "in vivo" refers to the processes that occur in an organism. The term "ex vivo" refers to a process that occurs outside an organism.

The term "miRNA" refers to microRNA and is sometimes referred to as "miR".

The term "packaging cell line" refers to any cell line that can be used to express lentiviral particles.

The term "percent identity" in the context of two or more nucleic acid or polypeptide sequences refers to the sequence comparison algorithms described below (eg, BLASTP and BLASTN, or two or more sequences with the specified percentage of the same nucleotide or amino acid residues, as determined using one of the algorithms available in the art) or by visual inspection or Refers to a partial array. "Percent identity" may, depending on the purpose, be present over the region of the sequences being compared, for example over a functional domain, or, alternatively, be present over the entire length of the two sequences being compared. Good. In the case of sequence comparison, typically one sequence acts as a reference sequence, against which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the designated program parameters.

Optimal sequence alignments for comparison are described by Needleman and Wunsch, J. Mol. Biol. 48 by the local homology algorithm of Smith and Waterman, Adv. Appl. Math. 2: 482 (1981), for example. : Pearson and Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988) by the homology alignment algorithm on page 443 (1970). , Computer implementations of these algorithms (Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis. GAP, BESTFIT, FASTA, et al. Can be carried out.

One example of a suitable algorithm for determining percent sequence identity and percent sequence similarity is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215:403-410 (1990). Have been described. Software for performing BLAST analyzes is publicly available from the National Center for Biotechnology Information website.

The percent identity between the two nucleotide sequences is NWSgapdna. CMP matrix and GCG software package (http://www.gcg.com) using 40, 50, 60, 70, or 80 gap weights and 1, 2, 3, 4, 5, or 6 length weights. GAP program (available from The percent identity between two nucleotide or amino acid sequences has also been incorporated into the ALIGN program (version 2.0) using the PAM120 weight residue table, gap length penalty of 12, and gap penalty of 4. E. Meyers. And W. Miller

(CABIOS, 4:11-17 (1989)). In addition, the percent identity between the two amino acid sequences was determined by either the Blossum62 matrix or the PAM250 matrix and the gap weights of 16, 14, 12, 10, 8, 6, or 4, and 1, 2, 3, 4,. Needleman and Wunsch (J. Mol. Biol. (Vol. 48) incorporated into the GAP program of the GCG software package (available from http://www.gcg.com) using length weights of 5 or 6. : 444-453 (1970)).

Further, the nucleic acid and protein sequences of the present disclosure can be used as a "query sequence" to perform searches of public databases, for example to identify related sequences. Such searches can be performed using the NBLAST and XBLAST programs (Version 2.0) of Altschul et al. (1990) J. Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed using the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. A BLAST protein search can be performed using the XBLAST program with score=50, wordlength=3 to obtain amino sequences homologous to protein molecules of the invention. To obtain a gapped alignment for comparison purposes, Gapped BLAST may be utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing the BLAST program and the Gapped BLAST program, the default parameters of the respective programs (eg, XBLAST and NBLAST) may be used. http://www. ncbi. nlm. nih. See gov.

As used herein, "pharmaceutically acceptable" means, within the scope of sound medical judgment, excessive toxicity, irritation, an allergic response, or other ratio that balances a reasonable benefit/risk ratio. Refers to a compound, material, composition, and/or dosage form that is suitable for use in contact with human and animal tissues, organs, and/or body fluids without causing problems or complications.

As used herein, "pharmaceutically acceptable carrier" includes any and all physiologically compatible solvents, dispersion media, coatings, antibacterial and antifungal agents, and isotonic and absorption delaying agents and the like. Point and include them. The composition can include pharmaceutically acceptable salts, such as acid addition salts or base addition salts (see, eg, Berge et al. (1977) J Pharm Sci 66: 1-19).

As used herein, the term "SEQ ID NO" is synonymous with the term "SEQ ID No."

As used herein, "small RNA" refers to a non-coding RNA that is generally less than or about 200 nucleotides in length and that has silencing or interfering functions. In other embodiments, the small RNA is about 175 nucleotides or less in length, about 150 nucleotides or less, about 125 nucleotides or less, about 100 nucleotides or less, or about 75 nucleotides or less. Such RNAs include microRNA (miRNA), small interfering RNA (siRNA), double-stranded RNA (dsRNA), and short hairpin RNA (shRNA). The “small RNA” of the present disclosure should be capable of inhibiting or knocking down gene expression of the target gene, generally through a pathway that results in the destruction of the target gene mRNA.

As used herein, the term "stimulatory agent" refers to any exogenous agent capable of stimulating white blood cells.

As used herein, the term "subject" includes not only human patients, but other mammals as well. The terms "subject", "individual", "host", and "patient" can be used interchangeably herein.

As used herein, the term "target cell" generally refers to a CD4+ T cell that responds to stimulation with a protein or peptide fragment that exhibits an HIV gene sequence, making the CD4+ T cell less sensitive to HIV. And CD4+ T cells transduced with the lentiviral vector detailed herein.

The term "therapeutically effective amount" in a composition suitable for treating or preventing the onset of symptoms, progression, or complications seen in a patient suffering from a given discomfort, injury, disease, or condition, and Refers to a sufficient amount of the active agent of the invention in a suitable dosage form. A therapeutically effective amount will vary depending on the condition of the patient or its severity, and the age, weight, etc. of the subject being treated. A therapeutically effective amount can vary depending on any of a number of factors, including, for example, the route of administration, the condition of the subject, as well as other factors understood by those of skill in the art.

As used herein, the term “therapeutic vector” is synonymous with a lentiviral vector such as the AGT103 vector.

The term "treatment" or "treating" generally refers to an intervention in an attempt to alter the natural history of the subject being treated, whether carried out prophylactically or during the course of clinical pathology. You can The desired effect is to prevent the occurrence or recurrence of the disease, alleviate the symptoms, suppress, reduce or inhibit any direct or indirect pathological consequences of the disease, improve or alleviate the disease state. And causing remission or improved prognosis, but is not limited thereto.

Description of Aspects of the Disclosure As detailed herein, in one aspect, a method of treating an HIV infection in a subject is disclosed. The method includes removing leukocytes from a subject and purifying peripheral blood mononuclear cells (PBMC). The method comprises contacting PBMCs ex vivo with a therapeutically effective amount of a stimulatory agent; transducing PBMCs ex vivo with a viral delivery system encoding at least one genetic element; and transduction. The method further comprises culturing the PBMCs for at least 1 day. The method may further comprise further enrichment of PBMCs, for example by preferably enriching PBMCs for CD4+ T cells. The transduced PBMC may be cultured for about 1 to about 35 days. The method may further include injecting the transduced PBMC into the subject. The subject may be a human. The stimulatory agent may comprise a peptide or mixture of peptides. In a preferred embodiment, the stimulatory agent comprises the gag peptide. The stimulatory agent may include a vaccine. The vaccine may be an HIV vaccine, and in a preferred embodiment the HIV vaccine is the MVA/HIV62B vaccine or variant thereof. In a preferred embodiment, the viral delivery system comprises lentiviral particles. In one embodiment, the at least one genetic element may comprise a small RNA capable of inhibiting the production of the chemokine receptor CCR5, or at least one small RNA capable of targeting an HIV RNA sequence. In another embodiment, at least one genetic element may comprise a small RNA capable of inhibiting the production of chemokine receptor CCR5 and at least one small RNA capable of targeting an HIV RNA sequence. . The HIV RNA sequence may include the HIV Vif sequence, the HIV Tat sequence, or variants thereof. At least one genetic element may comprise microRNA or shRNA. In a preferred embodiment, at least one genetic element comprises a microRNA cluster.

In another aspect, at least one genetic element is
And at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least Included are microRNAs having a percent identity of 92%, at least 93%, at least 94%, at least 95%, or higher. In a preferred embodiment, at least one genetic element is
including.

In another aspect, at least one genetic element is
With at least 80%, or at least 85%, or at least 90%, or at least 95% percent identity, or
And at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least Includes microRNAs having a percent identity of 92%, at least 93%, at least 94%, at least 95% or higher. In a preferred embodiment, at least one genetic element is including.

In another aspect, the microRNA cluster is
And at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least It includes sequences having a percent identity of 92%, at least 93%, at least 94%, at least 95% or higher. In a preferred embodiment, the microRNA cluster is including.

In another aspect, a method of treating cells infected with HIV is provided. The method comprises contacting peripheral blood mononuclear cells (PBMCs) isolated from an HIV infected subject with a therapeutically effective amount of a stimulatory agent, wherein the contacting is performed ex vivo. transducing PBMCs in vivo with a viral delivery system encoding at least one genetic element; and culturing the transduced PBMCs for at least one day. The transduced PBMC may be cultured for about 1 to about 35 days. The method may further include injecting the transduced PBMC into the subject. The subject may be a human. The stimulatory agent may comprise a peptide or mixture of peptides, and in a preferred embodiment comprises a gag peptide. The stimulatory agent may include a vaccine. The vaccine may be an HIV vaccine, and in a preferred embodiment the HIV vaccine is the MVA/HIV62B vaccine or variant thereof. In a preferred embodiment, the viral delivery system comprises lentiviral particles. In one embodiment, the at least one genetic element may comprise a small RNA capable of inhibiting the production of the chemokine receptor CCR5, or at least one small RNA capable of targeting an HIV RNA sequence. In another embodiment, at least one genetic element may comprise a small RNA capable of inhibiting the production of the chemokine receptor CCR5 and at least one small RNA capable of targeting an HIV RNA sequence. .. HIV RNA sequences may include HIV Vif sequences, HIV Tat sequences, or variants thereof. At least one genetic element may comprise microRNA or shRNA. In a preferred embodiment, at least one genetic element comprises a microRNA cluster.

In another aspect, the at least one genetic element is
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In another aspect, at least one genetic element is
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And at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least It includes microRNAs having a percent identity of 92%, at least 93%, at least 94%, at least 95%, or higher. In a preferred embodiment, at least one genetic element is including.

In another aspect, the microRNA cluster is
And at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least Includes sequences with a percent identity of 92%, at least 93%, at least 94%, at least 95%, or higher. In a preferred embodiment, the microRNA cluster is including.

In another aspect, lentiviral vectors are disclosed. The lentiviral vector comprises at least one encoded genetic element, the at least one encoded genetic element targeting a small RNA or HIV RNA sequence capable of inhibiting the production of the chemokine receptor CCR5. At least one small RNA capable of In another aspect, the at least one encoded genetic element comprises a small RNA capable of inhibiting the production of the chemokine receptor CCR5 and at least one small RNA capable of targeting an HIV RNA sequence. Viral vectors are disclosed. The HIV RNA sequence may include the HIV Vif sequence, the HIV Tat sequence, or variants thereof. The at least one encoded genetic element may comprise microRNA or shRNA. At least one encoded genetic element may comprise a microRNA cluster.

In another aspect, the at least one genetic element is
And at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least Included are microRNAs having a percent identity of 92%, at least 93%, at least 94%, at least 95%, or higher. In a preferred embodiment, at least one genetic element is
including.

In another aspect, at least one genetic element is
And at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least Has a percent identity of 92%, at least 93%, at least 94%, at least 95%, or higher, or
And at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least It includes microRNAs having a percent identity of 92%, at least 93%, at least 94%, at least 95%, or higher. In a preferred embodiment, at least one genetic element is including.

In another aspect, the microRNA cluster is
And at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least Includes sequences with a percent identity of 92%, at least 93%, at least 94%, at least 95%, or higher. In a preferred embodiment, the microRNA cluster is including.

In another aspect, a lentiviral vector system for expressing lentiviral particles is disclosed. The system comprises a lentiviral vector as described herein; an envelope plasmid for expressing envelope proteins optimized for infection of cells; and at least one for expressing the gag, pol, and rev genes. When the packaging cell line is transfected with a lentiviral vector, an envelope plasmid, and at least one helper plasmid, the lentiviral particles are produced by the packaging cell line, and the lentiviral particles are capable of accepting chemokine receptors. The production of somatic CCR5 can be inhibited or the HIV RNA sequence can be targeted.

In another aspect, lentiviral particles capable of infecting cells are disclosed. Lentiviral particles include envelope proteins that are optimized for infection of cells, and lentiviral vectors as described herein. The envelope protein may be optimized for infection of T cells. In a preferred embodiment, the envelope protein is optimized for infection of CD4+ T cells.

In another aspect, modified cells are disclosed. Modified cells include CD4+ T cells, which are infected with lentiviral particles as described herein. In a preferred embodiment, CD4+ T cells also recognize the HIV antigen. In a more preferred embodiment, the HIV antigen comprises the gag antigen. In a more preferred embodiment, CD4+ T cells express reduced levels of CCR5 after infection with lentiviral particles.

In another aspect, a method of selecting a subject for a therapeutic treatment regimen is disclosed. The method comprises removing leukocytes from a subject, purifying peripheral blood mononuclear cells (PBMCs), and determining a first quantifiable measurement associated with at least one factor associated with PBMCs; ex vivo, Contacting the PBMC with a therapeutically effective amount of a second stimulatory agent and determining a second measurement associated with at least one factor associated with PBMC, the second quantifiable measurement Is higher than the first quantifiable measurement, the subject is selected for the treatment regimen. The at least one factor may be T cell proliferation or IFNgamma production.

In another aspect, any of the methods described herein comprising treating cells infected with HIV further comprises depleting PBMCs of at least one subset of cells. In an embodiment, the method comprises depleting at least one subset of cells from PBMC, wherein the at least one subset of cells comprises CD8+ T cells, γδ cells, NK cells, B cells, neutrophils, basophils. , Eosinophils, regulatory T cells, NKT cells, and erythrocytes. In an embodiment, the depleting step is performed after removing the white blood cells. In an embodiment, the depleting step is performed concurrently with the removal of white blood cells.

In other aspects, any of the methods comprising treating HIV in a subject described herein further comprises depleting PBMCs of at least one subset of cells. In an embodiment, the method comprises depleting at least one subset of cells from PBMC, wherein the at least one subset of cells comprises CD8+ T cells, γδ cells, NK cells, B cells, neutrophils, basophils. , Eosinophils, regulatory T cells, NKT cells, and erythrocytes. In an embodiment, the depleting step is performed after removing the white blood cells. In an embodiment, the depleting step is performed concurrently with the removal of white blood cells.

In another aspect, any of the methods comprising selecting a subject for a treatment regimen described herein further comprises depleting PBMCs of at least one subset of cells. In an embodiment, the method comprises depleting at least one subset of cells from PBMC, wherein the at least one subset of cells comprises CD8+ T cells, γδ cells, NK cells, B cells, neutrophils, basophils. , Eosinophils, regulatory T cells, NKT cells, and erythrocytes. In an embodiment, the depleting step is performed after removing the white blood cells. In an embodiment, the depleting step is performed concurrently with the removal of white blood cells.

In another aspect, any of the methods described herein further comprises depleting PBMCs from at least one subset of immune cells, wherein the at least one subset of cells comprises CD8+ T cells, γδ cells. , NK cells, B cells, neutrophils, basophils, eosinophils, regulatory T cells, NKT cells, and erythrocytes. In an embodiment, the cells that are depleted from PBMC are CD8+ T cells. In embodiments, the cells that are depleted from PBMC are γδ cells. In an embodiment, the cells that are depleted from PBMC are NK cells. In an embodiment, the cells that are depleted from PBMC are B cells. In an embodiment, the cells that are depleted from PBMC are regulatory T cells. In an embodiment, the cells that are depleted from PBMC are NKT cells. In an embodiment, the cells that are depleted from PBMC are red blood cells. In an embodiment, the cells that are depleted from PBMC are CD8+ T cells and γδ cells. In embodiments, the cells that are depleted from PBMC are CD8+ T cells, γδ cells, and NK cells. In embodiments, cells that are depleted from PBMC are CD8+ T cells, γδ cells, NK cells, and B cells. In embodiments, cells that are depleted from PBMC are CD8+ T cells, γδ cells, NK cells, B cells, and regulatory T cells. In embodiments, the cells that are depleted from PBMC are CD8+ T cells, γδ cells, NK cells, B cells, regulatory T cells, and NKT cells. In embodiments, the cells that are depleted from PBMC are CD8+ T cells, γδ cells, NK cells, B cells, regulatory T cells, NKT cells, and red blood cells. In an embodiment, the cells that are depleted from PBMC are γδ cells and NK cells. In embodiments, the cells that are depleted from PBMC are γδ cells, NK cells, and B cells. In embodiments, the cells that are depleted from PBMC are γδ cells, NK cells, B cells, and regulatory T cells. In embodiments, the cells that are depleted from PBMC are γδ cells, NK cells, B cells, regulatory T cells, and NKT cells. In embodiments, the cells that are depleted from PBMC are γδ cells, NK cells, B cells, regulatory T cells, NKT cells, and red blood cells. In an embodiment, the cells that are depleted from PBMC are NK cells and B cells. In embodiments, PBMC-depleted cells are NK cells, B cells, and regulatory T cells. In embodiments, the cells that are depleted from PBMC are NK cells, B cells, regulatory T cells, and NKT cells. In embodiments, the cells that are depleted from PBMC are NK cells, B cells, regulatory T cells, NKT cells, and red blood cells. In embodiments, the cells that are depleted from PBMC are B cells and regulatory T cells. In embodiments, the cells that are depleted of PBMCs are B cells, regulatory T cells, and NKT cells. In embodiments, the cells that are depleted from PBMC are B cells, regulatory T cells, NKT cells, and red blood cells. In an embodiment, the cells that are depleted from PBMC are regulatory T cells and NKT cells. In embodiments, cells that are depleted from PBMC are regulatory T cells, NKT cells, and red blood cells. In an embodiment, the cells that are depleted from PBMC are NKT cells and red blood cells. In an embodiment, the cells that are depleted from PBMC are CD8+ T cells and NK cells. In embodiments, the cells that are depleted of PBMC are CD8+ T cells, NK cells, and B cells. In embodiments, the cells that are depleted from PBMC are CD8+ T cells, NK cells, B cells, and regulatory T cells. In embodiments, the cells that are depleted from PBMCs are CD8+ T cells, NK cells, B cells, regulatory T cells, and NKT cells. In embodiments, the cells that are depleted from PBMCs are CD8+ T cells, NK cells, B cells, regulatory T cells, NKT cells, and red blood cells. In an embodiment, the cells that are depleted from PBMC are γδ and B cells. In embodiments, the cells that are depleted from PBMC are γδ, B cells, and regulatory T cells. In an embodiment, the cells that are depleted from PBMC are γδ, B cells, regulatory T cells, and NKT cells. In embodiments, the cells that are depleted from PBMC are γδ, B cells, regulatory T cells, NKT cells, and red blood cells. In an embodiment, the cells that are depleted from PBMC are NK cells and regulatory T cells. In embodiments, the cells that are depleted from PBMC are NK cells, regulatory T cells, and NKT cells. In embodiments, the cells that are depleted from PBMC are NK cells, regulatory T cells, NKT cells, and red blood cells. In embodiments, the cells that

are depleted of PBMC are B cells and NKT cells. In embodiments, the cells that are depleted from PBMC are B cells, NKT cells, and red blood cells. In embodiments, the cells that are depleted from PBMC are regulatory T cells and red blood cells. In embodiments, the cells that are depleted of PBMC, as described herein, comprise any one or any combination of neutrophils, basophils, and eosinophils.

In another aspect, CD8+ T cells are depleted at the beginning of cell proliferation to improve CD4+ T cell proliferation. In embodiments, cell depletion is performed after peptide stimulation and before lentiviral transduction, which allows cells to better withstand mechanical stress. In embodiments, after depletion of CD8+ T cells, the cells are left in culture medium for approximately 24 hours. In embodiments, after CD8+ cell depletion, the cells are left in culture for less than 24 hours, such as less than 20 hours, less than 16 hours, less than 8 hours, or less than 4 hours. In embodiments, after CD8+ T cell depletion, the cells are longer than 24 hours, eg, longer than 30 hours, longer than 36 hours, longer than 42 hours, or longer than 48 hours. Place in culture over time. In an embodiment, the culture medium comprises IL-7. In an embodiment, the culture medium comprises IL-15. In embodiments, the culture medium comprises IL-7 and IL-15. In embodiments, cell depletion is performed prior to peptide stimulation. In an embodiment, gag protein is used to cause peptide stimulation. In embodiments, HIV vaccines are used to induce peptide stimulation. In an embodiment, the vaccine is the MVA/HIV62B vaccine used to induce peptide stimulation. In embodiments, CD8+ T cells are depleted with PE anti-human CD8 antibody and anti-PE microbeads. In embodiments, the CD8 antibody is an anti-rat antibody. In embodiments, the CD8 antibody is an anti-mouse antibody. In embodiments, the CD8 antibody is an anti-rabbit antibody. In embodiments, the CD8 antibody is an anti-goat antibody. In embodiments, cells are transduced after cell depletion and peptide stimulation. In embodiments, the cells are transduced with a lentivirus. In an embodiment, the lentivirus has GFP. In an embodiment, the lentivirus has RFP. In an embodiment, the lentivirus has EGFP. In embodiments, the cells are placed in post-transduction culture. In an embodiment, the culture medium comprises IL-7. In an embodiment, the culture medium comprises IL-15. In embodiments, the culture medium comprises IL-7 and IL-15. In embodiments, the cells are cultured for approximately 2 days to allow for CD4+ T cell expansion. In embodiments, the cells are cultured for approximately 3 days to allow for CD4+ T cell expansion. In embodiments, the cells are cultured for less than 2 days, such as less than 42 hours, less than 36 hours, less than 30 hours, less than 24 hours, less than 18 hours, less than 12 hours, or less than 6 hours. In an embodiment, the cells are more than 3 days long, eg, more than 4 days longer, 5 days longer, 6 days longer, 7 days longer, 8 days longer. , Cultured for more than 9 days, or for more than 10 days. In embodiments, the cells are cultured for 2 to 3 days, for example about 30 hours, about 36 hours, or about 42 hours.

In another aspect, CD8+, γδ, NK, or B cells are depleted to improve CD4+ T cell proliferation. In embodiments, any two or more of CD8+, γδ, NK, and B cells are depleted to improve CD4+ T cell proliferation. In embodiments, CD8+, γδ, NK, B, regulatory T, NKT, or red blood cells are depleted to improve CD4+ T cell proliferation. In embodiments, any two or more of CD8+, γδ, NK, B, regulatory T, NKT, and red blood cells are depleted to improve CD4+ T cell proliferation. In embodiments, cell depletion is performed after peptide stimulation and before lentiviral transduction. In embodiments, after cell depletion, the cells are left in culture medium for about 24 hours. In embodiments, after cell depletion, the cells are left in culture for less than 24 hours, such as less than 20 hours, less than 16 hours, less than 8 hours, or less than 4 hours. In embodiments, after CD8+ T cell depletion, the cells are longer than 24 hours, eg, longer than 30 hours, longer than 36 hours, longer than 42 hours, or longer than 48 hours. Place in culture over time. In an embodiment, the culture medium comprises IL-7. In an embodiment, the culture medium comprises IL-15. In embodiments, the culture medium comprises IL-7 and IL-15. In embodiments, cell depletion is performed prior to peptide stimulation. In an embodiment, gag protein is used to cause peptide stimulation. In embodiments, HIV vaccines are used to induce peptide stimulation. In an embodiment, the MVA/HIV62B vaccine is used to induce peptide stimulation. In embodiments, CD8+ T, γδ, NK, and/or B cells are depleted with PE-labeled specific antibody and anti-PE microbeads. In an embodiment, the antibody used is an anti-human antibody. In an embodiment, the antibody used was an anti-rat antibody. In embodiments, the antibody used is an anti-mouse antibody. In embodiments, the antibody used is an anti-goat antibody. In embodiments, cells are transduced after cell depletion and peptide stimulation. In embodiments, the cells are transduced with a lentivirus. In an embodiment, the lentivirus has GFP. In an embodiment, the lentivirus has RFP. In an embodiment, the lentivirus has EGFP. In embodiments, the cells are placed in post-transduction culture. In an embodiment, the culture medium comprises IL-7. In an embodiment, the culture medium comprises IL-15. In embodiments, the culture medium comprises IL-7 and IL-15. In embodiments, the cells are cultured for approximately 2 days to allow for CD4+ T cell expansion. In embodiments, the cells are cultured for about 3 days to allow for CD4+ T cell expansion. In embodiments, the cells are cultured for less than 2 days, such as less than 42 hours, less than 36 hours, less than 30 hours, less than 24 hours, less than 18 hours, less than 12 hours, or less than 6 hours. In an embodiment, the cells are more than 3 days long, eg, more than 4 days longer, 5 days longer, 6 days longer, 7 days longer, 8 days longer. , Cultured for more than 9 days, or for more than 10 days. In embodiments, the cells are cultured for 2 to 3 days, for example about 30 hours, about 36 hours, or about 42 hours.

In another aspect, the lentivirus comprises GFP used to measure transduction efficiency. In an embodiment, the lentivirus comprises RFP. In an embodiment, the lentivirus has EGFP. In embodiments, a cytokine capture system is used to identify antigen-specific CD4+ T cells with GFP positive cells. In embodiments, GFP is used to identify transduced cell subsets. In embodiments, RFP is used to identify transduced cell subsets. In embodiments, EGFP is used to identify transduced cell subsets. In embodiments, any of the transduction methods described herein can be used to measure transduction efficiency. In an embodiment, prior to lentiviral transduction, any of

the depletion methods described herein can be used for CD8+ T, γδ, NK, B, neutrophils, basophils, eosinophils, regulatory It can be used to deplete any one or more of T, NKT, and red blood cells.

In another aspect, transduction efficiency is measured by detecting vector copy number (VCN) by qPCR. In embodiments, the percentage of VCN-based transduced cells in the final cell product can be estimated by establishing a relationship between transduced cells and VCN. In an embodiment, lentivirus with GFP is used to determine the percentage of transduced cells. In embodiments, lentivirus with RFP is used to determine the percentage of transduced cells. In embodiments, lentivirus with EGFP is used to determine the percentage of transduced cells. In embodiments, any of the transduction methods described herein can be used to measure transduction efficiency. In embodiments, any of the depletion methods described herein is used to deplete any one or more of CD8+ T, γδ, NK, B cells prior to lentiviral transduction. obtain.

Human immunodeficiency virus (HIV)
Human immunodeficiency virus, also commonly referred to as "HIV," is a retrovirus that causes acquired immunodeficiency syndrome (AIDS) in humans. AIDS is a condition in which life-threatening opportunistic infections and cancers are rampant due to the progressive failure of the immune system. Without treatment, the average survival time after HIV infection is estimated to be 9-11 years depending on the HIV subtype. HIV infection results from the transfer of body fluids including, but not limited to, blood, semen, vaginal fluid, ejaculate semen, saliva, tears, lymph or cerebrospinal fluid, or breast milk. HIV can be present in infected individuals, both as free viral particles and in infected immune cells.

HIV infects living cells of the human immune system, such as helper T cells, but the tropism may vary within the HIV subtype. Immune cells that may be specifically susceptible to HIV infection include, but are not limited to, CD4+ T cells, macrophages, and dendritic cells. HIV infection includes a number of, including but not limited to, apoptosis of uninfected bystander cells, direct viral killing of infected cells, and killing of infected CD4+ T cells by CD8 cytotoxic lymphocytes that recognize infected cells. The mechanism results in reduced levels of CD4+ T cells. When CD4+ T cell numbers fall below a critical level, cellular immunity is lost and the body becomes more progressive and more susceptible to opportunistic infections and cancer.

Structurally, HIV differs from many other retroviruses. The RNA genome encodes 19 proteins, at least 7 structural landmarks (LTR, TAR, RRE, PE, SLIP, CRS, and INS) and at least 9 genes (gag, pol, env, tat, rev). , Nef, vif, vpr, vpu, and sometimes the fusion of tat, env, and rev, the tenth tev). The three gags, pols, and envs of these genes contain the information necessary to make new viral particle structural proteins.

HIV replicates primarily in CD4 T cells, causing cell destruction or dysregulation that compromises host immunity. HIV establishes infection as an integrated provirus, resulting in a latent infection condition in which viral expression in a particular cell is reduced below the level of cytopathology affecting that cell or detection by the host immune system. Due to the potential for migration, HIV is difficult to treat and has not been eradicated even after long-term highly active antiretroviral therapy (HAART). Although survival may be extended by HAART, in most cases HIV infection causes a fatal disease.

The main goal in the fight against HIV is to develop strategies to cure the disease. Extending HAART has not achieved this goal, so researchers are turning to alternative procedures. Initial efforts to improve host immunity by therapeutic immunization (using vaccines after infection occurred) were of modest or no impact. Similarly, strengthening the procedure had moderate or no impact.

Although some progress has been made using gene therapy, the positive result is sporadic, which is a gene encoding CCR5 (chemokine receptor) that plays an important role in virus entry into host cells. It was found only among rare humans with defects in one or both alleles. However, many researchers are optimistic that gene therapy has the highest potential to ultimately achieve HIV cure.

As disclosed herein, the methods and compositions of the present invention can achieve a functional cure with or without complete eradication of all HIV from the body. As mentioned above, functional cure is achieved when HIV+ individuals previously in need of HAART survive with low or undetectable viral replication and use lower or intermittent doses of HAART. Is defined as a situation or condition in which HAART can potentially be completely discontinued. As used herein, functional cure may still require adjunctive therapy to maintain low levels of viral replication and slow or eliminate disease progression. Absent. A possible outcome of a functional cure is the eventual eradication of HIV to prevent all possible relapses.

A major obstacle to achieving functional healing lies in the basic biology of HIV itself. Viral infection deletes CD4 T cells, which are important for almost all immune functions. Most importantly, HIV infection and depletion of CD4 T cells requires activation of individual cells. Activation is a mechanism specific to individual CD4 T cell clones that uses rearranged T cell receptors to recognize pathogens or other molecules.

In the case of HIV, infection activates and consequently depletes a population of HIV-specific T cells before other T cells that are less specific for the virus, effectively protecting the immune system against the virus. Incapacity to. The ability of HIV-specific T cell responses is reconstituted during long-term HAART; however, when HAART is interrupted, recurrent viral infection repeats the process, again depleting virus-specific cells, Reset the disease progression clock.

Apparently, functional healing is only possible if HAART is interrupted and sufficient HIV-specific CD4 T cells are protected so that the host's innate immunity can counter and control HIV. .. In one embodiment, aspects of the present disclosure provide methods and compositions for enhancing host immunity to HIV to provide functional cure without the need for prior immunization.

Gene Therapy Viral vectors are used to deliver gene constructs to host cells for the purpose of therapy or prophylaxis of disease.

A genetic construct is a regulatory gene that includes a functional gene or part of a gene that corrects or complements an existing defect, a DNA sequence encoding a regulatory protein, antisense, short homology RNA, long non-coding RNA, small interfering RNA or others. Can include, but is not limited to, a DNA sequence encoding an RNA molecule and a decoy sequence encoding either RNA or a protein designed to compete for cellular factors important for altering the disease state .. Gene therapy involves the delivery of these therapeutic gene constructs to target cells to provide treatment or alleviation of certain diseases.

There have been multiple efforts to utilize gene therapy in the treatment of HIV disease, but so far the results have been poor. A small number of successful treatments have been obtained in rare HIV patients with a spontaneous deletion of the CCR5 gene (allele known as CCR5delta32).

Lentiviral-delivered nucleases or other mechanisms for gene deletion/modification can be used to help reduce overall expression of CCR5 and/or reduce HIV replication. There is at least one study reporting successful treatment of this disease when lentivirus was administered to patients with a CCR5delta32 genetic background. However, this is only one example of success and many other patients who do not have the CCR5delta32 genotype have not been successfully treated. As a result, there is a substantial need to improve the performance of viral gene therapy against HIV, both in terms of improving the performance of individual viral vector constructs and the use of vectors by strategies to achieve functional HIV healing. ..

For example, some existing therapies rely on zinc finger nucleases to delete a portion of CCR5 in an attempt to make cells resistant to HIV infection. However, even after optimal treatment, only 30% of T cells are modified by nucleases, of which only 10% of the total CD4 T cell population prevent HIV infection. It was only modified. In contrast, the disclosed method results in CCR5 expression being reduced in virtually all cells harboring the lentiviral transgene to levels below those required to allow HIV infection. This can result in successful treatment of HIV without a prior immunization step to increase the number of initial CD4+ T cell pools.

For purposes of the disclosed methods, gene therapy includes T cell receptors with enhanced affinity, chimeric antigen receptors on CD4 T cells (or alternatively on CD8 T cells), viral proteins. Altering signaling pathways to avoid induced cell death, TREX, SAMHD1, MxA or MxB proteins, APOBEC complex, TRIM5-alpha complex, tetherin (BST2), and reducing HIV replication in mammalian cells It may include, but is not limited to, increased expression of HIV restriction elements, including similar proteins identified as capable of.

Immunotherapy Historically, vaccines have been the go-to weapon for deadly infectious diseases, including smallpox, polio, measles, and yellow fever. Unfortunately, there are currently no approved vaccines for HIV. The HIV virus has a unique means of evading the immune system, and the human body seems unable to mount an effective immune response against it. As a result, scientists have no clear understanding of what is needed to provide protection against HIV.

However, immunotherapy may offer a solution previously unattainable by conventional vaccination approaches. Immunotherapy, also called biological therapy, is a type of treatment designed to enhance the body's natural defenses to combat infections or cancer. It uses materials made either in the body or in the laboratory to improve, target, or restore the function of the immune system.

In certain aspects of the present disclosure, immunotherapeutic approaches can be used to enrich a population of HIV-specific CD4 T cells for the purpose of increasing host anti-HIV immunity. In another aspect of the disclosed invention, an integrated or non-integrated lentiviral vector can be used to transduce immune cells of the host for the purpose of increasing the host's anti-HIV immunity. Other aspects of the disclosure include killed particles, virus-like particles, HIV peptides or peptide fragments in combination with a suitable vehicle and/or biological or chemical adjuvant to increase the immune response of the host. Vaccines containing, but not limited to, HIV proteins, recombinant viral vectors, recombinant bacterial vectors, purified subunits or plasmid DNA can be used to enrich virus-specific T cell populations or antibodies. Can be further enhanced by the use of HIV targeted gene therapy with lentivirus or other viral vectors.

Methods In one aspect, the disclosure provides methods of using viral vectors to achieve functional cure of HIV disease. The method may include immunotherapy to enrich the proportion of HIV-specific CD4 T cells, followed by lentiviral transduction to optionally deliver HIV and inhibitors of CCR5 and CXCR4. Importantly, enrichment of HIV-specific CD4 T cells and lentiviral transduction can be effective even without a prior immunization step.

In an embodiment, the method comprises therapeutic immunization as a method for enriching the proportion of HIV-specific CD4 T cells, which immunization is performed concurrently with or after infusion of stimulated cells into the subject. .. Therapeutic immunization includes biological or chemical including purified proteins, inactivated viruses, viral vectored proteins, bacterial vectored proteins, peptides or peptide fragments, virus-like particles (VLPs), cytokines and/or chemokines. Adjuvants, vehicles, as well as methods for immunization can be included.

Therapeutic vaccines can include one or more HIV proteins with protein sequences that represent the predominant viral type of the geographic region being treated. Therapeutic vaccines include biological or chemical adjuvants containing purified proteins, inactivated viruses, viral vectored proteins, bacterial vectored proteins, peptides or peptide fragments, virus like particles (VLPs), cytokines and/or chemokines. , Vehicles, as well as methods for immunization. Vaccination can be administered according to standard methods known in the art, HIV patients receiving antiretroviral therapy during the period of immunization and subsequent ex vivo lymphocyte culture including lentiviral transduction. I can continue.

In certain embodiments, HIV+ patients are immunized with an HIV vaccine and the frequency of HIV-specific CD4 T cells is about 2, about 25, about 250, about 500, about 750, about 1000, about 1250, or about 1250. It can be increased 1500 times (or any amount between these values). The vaccine may be any clinically utilized or experimental HIV vaccine, including the disclosed lentiviruses, other viral vectors or other bacterial vectors used as a vaccine delivery system. In another embodiment, the vector may encode a virus-like particle (VLP) to induce higher titers of neutralizing antibodies and stronger HIV-specific T cell responses. In another embodiment, the vector comprises gag, pol, and env, tat, rev, nef, vif, vpr, vpu, and tev as well as LTR, TAR, RRE, PE, SLIP, CRS, and INS. It may encode a peptide or peptide fragment related to HIV without limitation. Alternatively, the HIV vaccine used in the disclosed methods comprises purified protein, inactivated virus, viral vectored protein, bacterial vectored protein, peptide or peptide fragment, virus-like particle (VLP), or cytokine. And/or may include biological or chemical adjuvants including chemokines.

For example, peripheral blood mononuclear cells (PBMC) can be obtained by leukocyte apheresis and treated ex vivo to obtain about 1×10^{10} CD4 T cells, of which about 0.1%, about 1%, About 5%, or about 10%, or about 30% are HIV specific in terms of antigen response and are HIV resistant by having the therapeutic transgene delivered by the disclosed lentiviral vector. Alternatively, about 1×10^{7}, about 1×10^{8}, about 1×10^{9}, about 1×10^{10}, about 1×10^{11}, or about 1×10^{12} CD4 T cells are restimulated. Can be isolated for. Importantly, any suitable amount of CD4 T cells can be isolated for ex vivo restimulation.

Isolated CD4 T cells can be cultured in a suitable medium through restimulation with HIV vaccine antigens with or without the antigens present in previous therapeutic vaccinations. Antiretroviral therapeutic agents, including inhibitors of reverse transcriptase, proteases or integrase, can be added to prevent viral re-emergence during long-term ex vivo culture. CD4 T cell restimulation can be used to enrich the proportion of HIV-specific CD4 T cells in culture. Use the same procedure also for analytical purposes to identify HIV-specific T cells and measure the frequency of this subpopulation using a small blood volume with peripheral blood mononuclear cells obtained by purification You can

The PBMC fraction can be enriched for HIV-specific CD4 T cells by contacting the cells with HIV proteins that are identical or complementary to components of vaccines previously used for in vivo immunization. Ex vivo restimulation increases the relative frequency of HIV-specific CD4 T cells by about 5, about 10, about 25, about 50, about 75, about 100, about 125, about 150, about 175, or about 200-fold. You can Ex vivo restimulation can increase the relative frequency of HIV-specific CD4 T cells with or without a prior immunization step.

The methods detailed herein can include restimulation of CD4 T cells ex vivo using lentiviral transduction and culture ex vivo. The methods detailed herein can also include restimulation of CD4 T cells ex vivo with lentiviral transduction and culture ex vivo without a prior immunization step.

Thus, in one embodiment, the restimulated PBMC fraction enriched for HIV-specific CD4 T cells is transduced with a therapeutic anti-HIV lentivirus or other vector for about 1 to about 21 days or It can be kept in culture for up to about 35 days. Alternatively, the cells can be cultured for about 1 to about 18 days, about 1 to about 15 days, about 1 to about 12 days, about 1 to about 9 days, or about 3 to about 7 days. Thus, the transduced cells can be about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14 cells. , About 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 30 It may be cultured for 31, about 32, about 33, about 34, or about 35 days.

Once the transduced cells are in sufficient culture, the transduced CD4 T cells are infused back into the original patient. Injection can be performed using various machines and methods known in the art. In some embodiments, infusion may be accompanied by pretreatment with cyclophosphamide or a similar compound to increase the efficiency of reimplantation.

In some embodiments, CCR5-targeted therapy may be added to the subject's antiretroviral therapy regimen continuously throughout the treatment process. Examples of CCR5-targeted therapy include, but are not limited to, Maraviroc (CCR5 antagonist) or Rapamycin (immunosuppressive agent that lowers CCR5). In some embodiments, antiretroviral therapy is discontinued and the subject can be tested for viral rebound. If rebound does not occur, adjuvant therapy can also be removed and the subject can be retested for viral rebound.

Continued viral suppression with or without antiretroviral therapy, including cART or HAART, with or without adjuvant therapy for about 26 weeks was associated with functional cure of HIV. I can think. Other definitions of functional cure are provided herein.

Lentiviral vectors and other vectors used in the disclosed methods include at least one, at least two, at least three, at least four, or at least five genes of interest, or at least six genes of interest, or At least 7 genes of interest, or at least 8 genes of interest, or at least 9 genes of interest, or at least 10 genes of interest, or at least 11 genes of interest, or at least 12 genes of interest, Can be coded. Given the diversity and therapeutic potential of HIV-targeted gene therapy, the viral vectors of the present invention comprise: (i) an antibody to an antigen associated with an infectious disease or a toxin produced by an infectious agent; (ii) an immune cell. Including a cytokine, including interleukins, (iii) CD8 inhibitor, which is required for the growth or function of EGF and may be a therapy for immune dysregulation encountered with HIV and other chronic or acute human viral or bacterial pathogens. Factors suppressing HIV proliferation in vivo, (iv) mutation or deletion of chemokine receptor CCR5, mutation or deletion of chemokine receptor CXCR4, or mutation or deletion of chemokine receptor CXCR5, (v) related to HIV Antisense DNA or RNA to a specific receptor or peptide or host protein related to HIV, (vi) a small interfering RNA to a specific receptor or peptide related to HIV or host protein related to HIV, or (vii 3.) It may encode a gene or nucleic acid sequence that includes, but is not limited to, various other therapeutically useful sequences that may be used to treat HIV or AIDS.

Further examples of HIV targeted gene therapy that can be used in the disclosed methods are T cell receptors with enhanced affinity, chimeric antigens on CD4 T cells (or alternatively on CD8 T cells). Receptors, modification of signaling pathways to avoid cell death caused by viral proteins, TREX, SAMHD1, MxA or MxB proteins, APOBEC complex, TRIM5-alpha complex, tetherin (BST2), and HIV in mammalian cells It can include, but is not limited to, increased expression of HIV restriction elements, including similar proteins identified as capable of reducing replication.

In some embodiments, the patient may be concurrently receiving cART or HAART while being treated according to the methods of the invention. In other embodiments, the patient may undergo cART or HAART before or after being treated according to the methods of the invention. In some embodiments, cART or HAART is maintained throughout treatment according to the methods of the invention and the patient is monitored for HIV viral load in the blood and frequency of lentivirus-transduced CD4 T cells in the blood. Good. Preferably, a patient undergoing cART or HAART before being treated according to the method of the present invention is able to discontinue or reduce cART or HAART after treatment according to the method of the present invention.

For purposes of assessing efficacy, the frequency of transduced HIV-specific CD4 T cells, a novel surrogate marker for gene therapy efficacy, may also be determined as discussed in more detail herein. Good.

Compositions In one aspect, the disclosed invention provides lentiviral vectors capable of delivering genetic constructs to inhibit HIV entry of susceptible cells. For example, one mechanism of action is to reduce CCR5 and/or CXCR4 chemokine receptor mRNA levels, thereby reducing the rate of viral entry into susceptible cells.

Alternatively, the disclosed lentiviral vector may be able to inhibit the formation of HIV infected cells by reducing the stability of incoming HIV genomic RNA. In yet another embodiment, the disclosed lentiviral vectors are capable of preventing HIV production from latently infected cells, the mechanism of action of which is short homology, small interfering, or other regulatory RNA species. The action of the inhibitory RNA contained therein causes the instability of the viral RNA sequence.

Therapeutic lentiviruses disclosed in the present application generally include at least one of two types of genetic cargo. First, the lentivirus may encode genetic elements that direct the expression of small RNAs that can inhibit the production of chemokine receptors CCR5 and/or CXCR4, which are important for HIV entry of susceptible cells. A second type of gene cargo targets HIV RNA sequences for the purpose of preventing reverse transcription, RNA splicing, protein producing RNA translation, or packaging of viral genomic RNA for particle production and infection spread. Includes constructs capable of expressing small RNA molecules. An exemplary structure is shown in FIG.

As shown in FIG. 3 (top panel), the exemplary construct can include multiple sections or components. For example, in one embodiment, an exemplary LV construct may include the following sections or components:
RSV-Rous Sarcoma virus terminal repeats;
5'LTR-part of the HIV terminal repeat that can be cleaved to prevent replication of the vector after chromosomal integration;
Psi-a packaging signal that allows the vector RNA genome to be incorporated into viral particles during packaging;
RRE-Rev responsive element can be added to improve expression from the transgene by translocating RNA from the nucleus to the cytoplasm of the cell;
CPPT-a polypurine tract that promotes second-strand DNA synthesis before the transgene is integrated into the chromosome of the host cell;
-Promoter-A promoter is one that initiates RNA transcription from an integrated transgene to express a microRNA cluster (or other genetic element of the construct), and in some embodiments, the vector is EF. -1 promoter may be used;
Anti-CCR5-microRNA that targets messenger RNA of host cell factor CCR5 and reduces expression on the cell surface;
Anti-Rev/Tat-a microRNA that targets HIV genomic RNA or messenger RNA at the junction between the HIV Rev and Tat coding regions, sometimes referred to as miRNA Tat, or in this application A similar statement is given;
A microRNA targeting HIV genomic RNA or messenger RNA within the Anti-Vif-Vif coding region;
WPRE-Woodchuck hepatitis virus post-transcriptional regulatory element is an additional vector component that can be used to promote nuclear RNA trafficking; and-delta U3 3'LTR. -A modified version of the HIV 3'terminal repeat in which a part of the U3 region has been deleted to improve the safety of the vector.

Those skilled in the art will appreciate that the above components are merely examples, such components may be rearranged and replaced with other elements, as long as the construct can prevent HIV gene expression and reduce spread of infection, Or, it will be appreciated that it may be altered in other ways, including, but not limited to, by substituting, deleting, adding, or mutating nucleotides.

The vectors of the present invention may contain one or both of the types of gene cargo discussed above (ie, the genetic elements that direct gene expression or small RNAs such as siRNA, shRNA or miRNA that can block translation or transcription). The vectors of the invention may also be included and may encode additional useful products for purposes of treating or diagnosing HIV. For example, in some embodiments, these vectors also encode green fluorescent protein (GFP) for the purpose of selectively maintaining genetically modified cells in vivo, and for tracking vector or antibiotic resistance genes. You may.

The combination of genetic elements incorporated into the disclosed vector is not particularly limited. For example, the vector may be 1 small RNA, 2 small RNAs, 3 small RNAs, 4 small RNAs, 5 small RNAs, 6 small RNAs, 7 small RNAs, 8 small RNAs. It may encode small RNA, 9 small RNAs, or 10 small RNAs, or 11 small RNAs, or 12 small RNAs. Such vectors may additionally encode other genetic elements that function in concert with small RNAs to prevent HIV expression and infection.

Those skilled in the art understand that therapeutic lentiviruses may use alternative sequences in place of promoter regions, targeting of regulatory RNAs, and types of regulatory RNAs. In addition, the therapeutic lentiviruses of the present disclosure may include alterations in the plasmid used to package the lentiviral particles; these alterations are required to increase the level of production in vitro. Become.

Lentiviral Vector System Lentiviral virions (particles) are expressed by a vector system that encodes viral proteins required for the production of virions (viral particles). There is at least one vector that contains the nucleic acid sequence encoding the lentiviral pol protein that is required for reverse transcription and integration and is operably linked to the promoter. In another embodiment, the pol protein is expressed by multiple vectors. There is also a vector that encodes the lentiviral gag protein required to form the viral capsid and contains a nucleic acid sequence operably linked to a promoter. In certain embodiments, the gag nucleic acid sequence is in a vector that differs from at least some of the pol nucleic acid sequences. In another embodiment, the gag nucleic acid is in a vector that differs from all the pol nucleic acid sequences that encode the pol protein.

Many modifications can be made to the vector. Such modifications are used to create particles with a further reduced chance of obtaining a wild-type revertant. These include, but are not limited to, deletions of the U3 region of the LTR, tat deletions, and matrix (MA) deletions.

The gag, pol, and env vectors do not contain the lentiviral genome-derived nucleotides that package the lentiviral RNA, called lentiviral packaging sequences.

The particle-forming vector preferably does not contain a lentivirus genome-derived nucleic acid sequence expressing an envelope protein. Preferably, another vector containing a nucleic acid sequence encoding an envelope protein operably linked to a promoter is used. This env vector also does not contain lentiviral packaging sequences. In one embodiment, the env nucleic acid sequence encodes a lentivirus envelope protein.

In another embodiment, the envelope protein is from a different virus than the lentivirus. The resulting particles are called pseudotyped particles. With the proper choice of envelope, virtually any cell can be "infected". For example, influenza virus, VSV-G, alphavirus (semliki forest virus, sindbis virus), arenavirus (lymphocytic choriomeningitis virus), flavivirus (tick-borne encephalitis virus, dengue virus, hepatitis C virus) , GB virus), rhabdovirus (vesicular stomatitis virus, rabies virus), paramyxovirus (mumps or measles), and orthomyxovirus (influenza virus). An env gene can be used that encodes an envelope protein that targets the endocytic compartment. Other envelopes that can be preferably used include those derived from Moloney leukemia virus, such as MLV-E, MLV-A, and GALV. These latter envelopes are particularly preferred when the host cell is a primary cell. Other envelope proteins may be selected depending on the desired host cell. For example, for brain delivery, targeting specific receptors such as dopamine receptors can be used. Another target may be the vascular endothelium. These cells can be targeted using the filovirus envelope. For example, GP of Ebola become GP and GP_2 glycoproteins by post-translational modifications. In another embodiment, various lentiviral capsids with pseudotyped envelopes can be used. For example, FIV or SHIV [US Pat. No. 5,654,195]. The SHIV pseudotyped vector can be easily used in animal models such as monkeys.

As detailed herein, lentiviral vector systems typically include at least one helper plasmid containing at least one of the gag, pol, or rev genes. Each of the gag, pol, rev genes may be provided on a separate plasmid, or one or more genes may be provided together on the same plasmid. In one embodiment, the gag, pol, and rev genes are provided on the same plasmid (eg, Figure 4). In another embodiment, the gag and pol genes are provided on the first plasmid and the rev gene is provided on the second plasmid (eg, FIG. 5). Thus, both the 3-vector system and the 4-vector system can be used to produce lentiviruses as described in the Examples section and elsewhere herein. The therapeutic vector, the envelope plasmid, and at least one helper plasmid are transfected into the packaging cell line. A non-limiting example of a packaging cell line is the 293T/17 HEK cell line. The therapeutic vector, the envelope plasmid, and at least one helper plasmid are transfected into the packaging cell line to ultimately produce lentiviral particles.

In another aspect, a lentiviral vector system for expressing lentiviral particles is disclosed. The system comprises a lentiviral vector as described herein; an envelope plasmid for expressing an envelope protein optimized to infect cells; and at least for expressing gag, pol, and rev genes. The lentivirus vector, the envelope plasmid, and the at least one helper plasmid containing one helper plasmid are transfected into the packaging cell line, whereby the packaging cell line produces a lentivirus particle, the lentivirus particle comprising: The production of the chemokine receptor CCR5 can be inhibited or the HIV RNA sequence can be targeted.

In another aspect, as detailed herein, a lentiviral vector, also referred to herein as a therapeutic vector, can include the following elements: hybrid 5'terminal repeat (RSV/5). 'LTR) (SEQ ID NOS: 34-35), Psi sequence (RNA packaging site) (SEQ ID NO: 36), RRE (Rev response element) (SEQ ID NO: 37), cPPT (polyprint tract) (SEQ ID NO: 38), EF -1α promoter (SEQ ID NO: 4), miR30CCR5 (SEQ ID NO: 1), miR21Vif (SEQ ID NO: 2), miR185Tat (SEQ ID NO: 3), woodchuck post-transcriptional regulatory element (WPRE) (SEQ ID NO: 32 or 80), and ΔU3 3. 'LTR (SEQ ID NO:39). In another aspect, sequence mutations by substitutions, deletions, or additions can be used to modify the sequences referenced above.

In another aspect, as detailed herein, the helper plasmid is designed to include the following elements: CAG promoter (SEQ ID NO:41); HIV component gag (SEQ ID NO:43); HIV components pol (SEQ ID NO:44); HIV Int (SEQ ID NO:45); HIV RRE (SEQ ID NO:46); and HIV Rev (SEQ ID NO:47). In another aspect, the helper plasmid may be modified to include a first helper plasmid for expressing the gag and pol genes, and a second separate plasmid for expressing the rev gene. In another aspect, sequence mutations by substitutions, deletions, or additions can be used to modify the sequences referenced above.

In another aspect, an envelope plasmid, as detailed herein, is designed from left to right to include the following elements: RNA polymerase II promoter (CMV) (SEQ ID NO:60). And vesicular stomatitis virus G glycoprotein (VSV-G) (SEQ ID NO:62). In another aspect, sequence mutations by substitutions, deletions, or additions can be used to modify the sequences referenced above.

In another aspect, the plasmids used for lentiviral packaging can be modified with similar elements without loss of vector function and intron sequences can potentially be removed. For example, the following elements can be used in place of similar elements in plasmids containing the packaging system: elongation factor-1 (EF-1), phosphoglycerate kinase (PGK), and ubiquitin C (UbC) promoter. Can be used in place of the CMV or CAG promoters. SV40 poly A and bGH poly A can be used instead of rabbit beta globin poly A. The HIV sequence of the helper plasmid may be constructed from different HIV strains or strains. The VSV-G glycoprotein is used for feline endogenous virus (RD114), gibbon ape leukemia virus (GALV), rabies (FUG), lymphocytic choriomeningitis virus (LCMV), influenza A poultry plague virus (FPV), and loss. It can be replaced with a membrane glycoprotein derived from river alphavirus (RRV), murine leukemia virus 10A1 (MLV), or Ebola virus (EboV).

Notably, lentiviral packaging systems are commercially available (eg, Lenti-vpak packaging kit from OriGene Technologies, Inc., Rockville, MD), as described herein. It can also be designed. Furthermore, it is within the skill of one in the art to replace or modify aspects of the lentiviral packaging system to improve any number of relevant

factors, including the efficiency of production of lentiviral particles.

Bioassays In one aspect, the invention includes bioassays for determining the success of HIV treatment to achieve functional cure. These assays provide a method for measuring the efficacy of the disclosed method by measuring the frequency of transduced HIV-specific CD4 T cells in a patient. HIV-specific CD4 T cells proliferate, modify the composition of cell surface markers, induce signal transduction pathways including phosphorylation, or induce cytokines, chemokines, caspases, phosphorylation signaling molecules or other cytoplasmic and/or It recognizes because it expresses a specific marker protein that can be a nuclear component. Specific responsive CD4 T cells can be screened for HIV specific cells using, for example, flow cytometry sorting, magnetic bead separation or other recognized methods for antigen specific CD4 T cell isolation. Recognized using specific in situ amplification of labeled monoclonal antibodies or mRNA sequences, which allows. The isolated CD4 T cells are tested to determine the frequency of cells with integrated therapeutic lentivirus. Single cell assays, including microfluidic separation of individual cells in combination with mass spectrometry, PCR, ELISA, or antibody staining to confirm responsiveness to HIV and the presence of integrated therapeutic lentivirus are also provided. It may also be used.

Thus, in certain embodiments, treatment according to the invention (eg, (a) without immunization, (b) ex vivo lymphocyte culture; (c) purified protein, inactivated virus, viral vectored protein, bacterial. Determine the efficacy of treatment after application of biological or chemical adjuvants containing vectored proteins, cytokines and/or chemokines, restimulation with vehicle; and (d) injection of enriched and transduced T cells) The patient may then be assayed to do so. The threshold of target T cells within the cell product for infusion can be measured, for example, by measuring about 1×10^{8} HIV-specific CD4 T cells with therapeutic lentiviral genetic modifications to measure functional healing. May be established. Alternatively, the threshold value is about 1×10^{5}, about 1×10^{6}, about 1×10^{7}, about 1×10^{8}, about 1×10^{9} or about 1×10^{10} in the patient's body. Of CD4 T cells.

HIV-specific CD4 T cells with therapeutic lentivirus genetic modifications are, for example, without limitation, flow cytometry, cell sorting, FACS analysis, DNA cloning, PCR, RT-PCR or Q-PCR, It may be determined using any suitable method such as ELISA, FISH, Western blotting, Southern blotting, high throughput sequencing, RNA sequencing, oligonucleotide primer extension, or other methods known in the art. it can.

Methods for defining antigen-specific T cells with genetic modifications are known in the art. However, utilizing such a method that combines the identification of HIV-specific T cells with integrated or non-integrated gene therapy constructs as a standard measure of efficacy is in the field of HIV treatment. It is a new concept.

Dosages and Dosage Forms The disclosed methods and compositions can be used to treat HIV+ patients during various stages of the disease. Accordingly, the dosing regimen may vary based on the condition of the patient and the method of administration.

In one aspect, the HIV-specific vaccine may be administered to the subject at the same time as or after the infusion of stimulated cells. In one embodiment, the HIV-specific vaccine may be administered to the subject in need thereof at various doses. Generally, vaccines delivered by intramuscular injection will either be inactivated viral particles, whole viral proteins prepared from virus-like particles, or purified viral proteins from recombinant systems or purified from viral preparations. Or about 100 μg to about 300 μg, about 25 μg to about 275 μg, about 50 μg to about 250 μg, about 75 μg to about 225, or about 100 μg to about 200 μg HIV protein. The recombinant viral or bacterial vector may be administered by any of the routes described. Intramuscular vaccines include about 1 μg to about 100 μg, about 10 μg to about 90 μg, about 20 μg to about 80 μg, about 30 μg to about 70 μg, about 40 μg to about 60 μg, or about 50 μg of an appropriate adjuvant molecule, and 0 per injection dose. Suspended in a volume of 1-5 ml of oil, saline, buffer or water, which may be a soluble or emulsion preparation. Vaccines delivered to the oral, rectal, buccal, genital mucosa or nasal cavity, including some viral or bacterial vectored vaccines, fusion proteins, liposomal formulations or similar preparations will produce higher amounts of viral protein and An adjuvant may be included. Transdermal, subdermal or subcutaneous vaccines utilize amounts of proteins and adjuvants more similar to oral, rectal or intranasal delivery vaccines. Depending on the response to the initial immunization, vaccination may be repeated 1-5 times using the same or alternative routes for delivery. The interval may be between 2 and 24 weeks between immunizations. The immune response to vaccination is measured by testing HIV-specific antibodies in serum, plasma, vaginal secretions, rectal secretions, saliva or bronchoalveolar lavage fluid using an ELISA or similar method. The cellular immune response is stimulated in vitro with a vaccine antigen, followed by staining for intracellular cytokine accumulation, followed by flow cytometry, or changes in lymphocyte proliferation, expression of phosphorylated signaling proteins or cell surface activation markers. Is tested by a similar method including. The upper limit of dosing may be determined on an individual patient basis, depending on the toxicity/safety profile of each individual product or product lot.

Immunization may be performed once, twice, three times, or repeatedly. For example, a drug for HIV immunization may be administered once a week, once every two weeks, once every three weeks, once a month, once every two months, once every three months, once every six months, 9 times a week. It may be administered to a subject in need once a month, once a year, once every 18 months, once every two years, once every 36 months, or once every three years.

After ex vivo expansion and enrichment of CD4 T cells, immunizations are performed once, twice, three times or more after ex vivo lymphocyte culturing/restimulation and infusion. May be.

In one embodiment, the HIV vaccine for immunization is administered as a pharmaceutical composition. In one embodiment, the pharmaceutical composition comprising the HIV vaccine may be formulated in a wide variety of nasal, pulmonary, oral, topical, or parenteral dosage forms for clinical application. Each dosage form may contain various disintegrants, surfactants, fillers, thickeners, binders, diluents such as wetting agents or other pharmaceutically acceptable excipients. Pharmaceutical compositions containing HIV vaccines can also be formulated for injection.

HIV vaccine compositions for immunization purposes include intranasal, buccal, sublingual, oral, rectal, ocular, parenteral (intravenous, intradermal, intramuscular, subcutaneous, intracisternal, intraperitoneal), Any pharmaceutically acceptable method, such as intrapulmonary, vaginal, topical, topical, post-challenge topical, mucosal, via aerosol, or via a buccal or nasal spray formulation. Can be used and administered.

Additionally, HIV vaccine compositions include solid dosage forms, tablets, pills, lozenges, capsules, liquid dispersions, gels, aerosols, lung aerosols, nasal aerosols, ointments, creams, semisolid dosage forms, and suspensions, and the like. It can be formulated into any pharmaceutically acceptable dosage form. Further, the composition may be a controlled release formulation, a sustained release formulation, an immediate release formulation, or any combination thereof. Further, the composition may be a transdermal delivery system.

In another embodiment, the pharmaceutical composition comprising the HIV vaccine may be formulated in a solid dosage form for oral administration, which may be a powder, granules, capsules, tablets or pills. In yet another embodiment, the solid dosage form may include one or more excipients such as calcium carbonate, starch, sucrose, lactose, microcrystalline cellulose or gelatin. In addition, solid dosage forms can contain, in addition to excipients, lubricants such as talc or magnesium stearate. In some embodiments, oral dosage forms can be immediate release or modified release forms. Modified release dosage forms include controlled or sustained release, enteric release and the like. Excipients used in modified release dosage forms are generally known to those of skill in the art.

In a further embodiment, the pharmaceutical composition comprising the HIV vaccine may be formulated as a sublingual or buccal dosage form. Such dosage forms include sublingual tablets or solution compositions administered sublingually and buccal tablets placed between the cheek and gums.

In yet a further embodiment, the pharmaceutical composition comprising the HIV vaccine may be formulated as a nasal dosage form. Such dosage forms of the invention include solution, suspension and gel compositions for nasal delivery.

In one embodiment, the pharmaceutical composition may be formulated in liquid dosage forms for oral administration such as suspensions, emulsions or syrups. In other embodiments, liquid dosage forms include, in addition to commonly used simple diluents such as water and liquid paraffin, various excipients such as humectants, sweeteners, fragrances or preservatives. Can be included. In certain embodiments, compositions containing the HIV vaccine or pharmaceutically acceptable salts thereof may be formulated for administration to pediatric patients.

In one embodiment, the pharmaceutical composition may be formulated in a dosage form for parenteral administration such as sterile aqueous solution, suspension, emulsion, non-aqueous solution or suppository. In other embodiments, non-aqueous solutions or suspensions may include propylene glycol, polyethylene glycol, vegetable oils such as olive oil, or injectable esters such as ethyl oleate. As a suppository base, witepsol, macrogol, tween 61, cocoa oil, lauric oil or glycerinated gelatin can be used.

The dosage of the pharmaceutical composition may vary depending on the patient's weight, age, sex, time and form of administration, excretion rate, and severity of the disease.

For purposes of restimulation, lymphocytes, PBMCs, and/or CD4 T cells are removed from the patient and isolated for stimulation and culture. The isolated cells may be contacted with the same HIV vaccine or activator used for immunization or with a different HIV vaccine or activator. In one embodiment, the isolated cells are contacted with about 10 ng to 5 μg (or any other suitable amount) of HIV vaccine or activator per about 10^{6} cells in culture. More specifically, the isolated cells are about 50 ng, about 100 ng, about 200 ng, about 300 ng, about 400 ng, about 500 ng, about 600 ng, about 700 ng, about 700 ng per about 10^{6} cells in culture. Contacted with 800 ng, about 900 ng, about 1 μg, about 1.5 μg, about 2 μg, about 2.5 μg, about 3 μg, about 3.5 μg, about 4 μg, about 4.5 μg, or about 5 μg HIV vaccine or activator You may.

The activator or vaccine is generally used once for each in vitro cell culture, but may be repeated after an interval of about 15 to about 35 days. For example, repeated doses are about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about. It may be done in 29, about 30, about 31, about 32, about 33, about 34, or about 35 days.

For the transduction of enriched and restimulated cells, the cells may be transduced with a lentiviral vector or other known vector system disclosed herein. Transduced cells are about 1 to 1,000 (or any other suitable amount) (as measured by RT-PCR assay of culture medium containing the lentiviral vector) per target cell in culture. It may be contacted with the viral genome. Lentivirus transduction may be repeated 1-5 times using the same range of 1-1000 viral genomes per target cell in culture.

Cell Enrichment In one approach, cells such as T cells may be obtained from HIV infected patients and cultured in multi-well plates in culture medium containing conditioned medium (“CM”). Levels of supernatant p24 ^{gag} (“p24”) and viral RNA levels can be assessed by standard means. Patients whose CM cultures have peak p24 supernatant levels of less than 1 ng/ml are suitable patients for the large expansion of T cells in CM with or without additional antiviral agents. You may. In addition, different drugs of interest or combinations of drugs of interest may be added to different wells and the effect on virus levels in the sample assessed by standard means. Drug combinations that provide adequate viral suppression are therapeutically useful combinations. It is within the competence of qualified professionals to determine what constitutes adequate virus suppression for a particular subject. To test the effectiveness of the drug of interest in limiting virus growth, additional factors such as anti-CD3 antibodies may be added to the culture to stimulate virus production. Unlike methods known in the art for culturing HIV-infected cell samples, CM allows culturing of T cells for longer than 2 months, thereby increasing long-term drug efficacy. Provide an effective system for assaying.

This approach allows inhibition of gene expression driven by the HIV LTR promoter region in cell populations by culturing cells in medium containing CM. Culturing in CM4 is likely to inhibit HIV LTR-driven gene expression by altering one or more interactions between transcription-mediated proteins and HIV gene expression regulatory elements. Transcription-mediated proteins of interest include host cell-encoded proteins such as AP-1, NFkappaB, NF-AT, IRF, LEF-1, and Sp1, as well as the HIV-encoded protein Tat. . HIV gene expression regulatory elements of interest include AP-1, NFkappaB, NF-AT, IRF, LEF-1, and Sp1 binding sites, and a trans-acting response element (“TAR”) that interacts with Tat. Be done.

In a preferred embodiment, HIV infected cells are obtained from a subject having sensitive transcriptional mediator protein sequences and sensitive HIV regulatory element sequences. In a more preferred embodiment, HIV infected cells are obtained from a subject having wild type transcription-mediating protein sequences and wild type HIV regulatory sequences.

Another method of enriching T cells utilizes immunoaffinity-based selection. This approach may involve the simultaneous enrichment or selection of first and second cell populations such as CD4+ and CD8+ cell populations. Immuno-affinity of cells, including primary human T cells, in a incubation composition with a first immunoaffinity reagent that specifically binds to CD4 and a second immunoaffinity reagent that specifically binds to CD8 The reagents are contacted under conditions that specifically bind to the CD4 and CD8 molecules, respectively, on the surface of the cells in the sample. The cells bound with the first and/or second immunoaffinity reagent are recovered, thereby producing a concentrated composition comprising CD4+ cells and CD8+ cells. This approach may involve incubating the composition with a concentration of the first and/or second immunoaffinity reagent that is a suboptimal yield concentration. Notably, in some embodiments, the transduced cells are a mixed T cell population, and in other embodiments, the transduced cells are not a mixed T cell population.

In some embodiments, immunoaffinity-based selection is used, where the solid support is a sphere such as a bead, such as a microbead or a nanobead. In other embodiments, the beads may be magnetic beads. In another embodiment, the antibody is capable of forming a reversible bond with a binding reagent that is immobilized on a solid surface, such as a sphere or a chromatographic matrix, and reversibly immobilizing the antibody on the solid surface. Or includes multiple binding partners. In some embodiments, cells expressing a cell surface marker to which the solid surface antibody binds can be recovered from the matrix by disrupting the reversible binding between the binding reagent and the binding partner. In some embodiments, the binding reagent is streptavidin, or a streptavidin analog or variant.

Stable transduction of hematopoietic and/or hematopoietic stem cell primary cells can be obtained by contacting both the lentiviral vector and at least one molecule that binds to the cell surface, in vitro or ex vivo. You can The cells may be cultivated in a ventilated vessel containing two or more layers under conditions that promote growth and/or proliferation. In some embodiments, this approach can be used with non-CD4+ T cell depletion and/or broad polyclonal expansion.

In another approach to T cell enrichment, PBMCs are stimulated with peptides and enriched for cells that secrete cytokines such as interferon-gamma. This approach generally involves stimulating a mixture of cells, including T cells, with an antigen, depending on the degree to which the product is labeled, achieving separation of the antigen-stimulated cells. Antigen stimulation is achieved by exposing cells to at least one antigen under conditions effective to induce antigen-specific stimulation of at

least one T cell. Labeling with a product modifies the surface of the cell to include at least one capture moiety such that the product is secreted, released and specifically binds to said capture moiety ("captured" or "captured").)) by culturing the cells under conditions and labeling the captured product with a labeling moiety, where the labeled cells are not lysed as part of the labeling procedure or part of the separation procedure .. The capture moiety may incorporate detection of the cell surface glycoprotein CD3 or CD4 in order to refine the enrichment step and increase the proportion of antigen-specific T cells in general, and in particular CD4+ T cells.

The following examples are presented to illustrate aspects of the invention. However, it should be understood that the invention is not limited to the particular conditions or details described in these examples. All publications referenced herein are specifically incorporated by reference.

(Example 1: Development of lentivirus vector system)
The lentiviral vector system was developed as summarized in Figure 3 (linear form) and Figure 4 (circularized form). Referring first to the upper portion of Figure 3, a representative therapeutic vector was designed and generated to have the following elements from left to right: Hybrid 5'terminal repeat (RSV/5'LTR) (SEQ ID NOs: 34 to 35), Psi sequence (RNA packaging site) (SEQ ID NO: 36), RRE (Rev response element) (SEQ ID NO: 37), cPPT (polyprint tract) (SEQ ID NO: 38), EF-1α promoter (SEQ ID NO: 4), miR30CCR5 (SEQ ID NO: 1), miR21Vif (SEQ ID NO: 2), miR185Tat (SEQ ID NO: 3), woodchuck post-transcriptional regulatory element (WPRE) (SEQ ID NO: 32 or 80), and ΔU3 3'LTR (sequence). No. 39). The therapeutic vector detailed in Figure 3 is also referred to herein as AGT103.

Next, referring to the middle part of FIG. 3, a helper plasmid was designed and produced from left to right having the following elements: CAG promoter (SEQ ID NO:41); HIV component gag (SEQ ID NO:43). HIV component pol (SEQ ID NO:44); HIV Int (SEQ ID NO:45); HIV RRE (SEQ ID NO:46); and HIV Rev (SEQ ID NO:47).

Next, referring to the lower part of FIG. 3, an envelope plasmid was designed and produced from left to right with the following elements: RNA polymerase II promoter (CMV) (SEQ ID NO: 60) and vesicular stomatitis. Viral G glycoprotein (VSV-G) (SEQ ID NO:62).

Lentiviral particles were produced in 293T/17 HEK cells (purchased from American Type Culture Collection, Manassas, VA), followed by therapeutic vector, envelope plasmid, and helper plasmid (as shown in Figure 3). Transfected. For transfection of 293T/17 HEK cells that produced functional viral particles, the reagent poly(ethyleneimine) (PEI) was used to increase the efficiency of plasmid DNA uptake. First, plasmid and DNA were added separately to the serum-free culture medium in a 3:1 ratio (PEI to DNA mass ratio). After 2-3 days, cell culture medium was collected and lentiviral particles were purified by high speed centrifugation and/or filtration before anion exchange chromatography. The concentration of lentiviral particles can be expressed in transducing units/ml (TU/ml). TU determination was carried out by measuring HIV p24 levels in culture (p24 protein is incorporated into lentiviral particles), measuring viral DNA copy number per cell by quantitative PCR, or infecting cells. And using light (when the vector encodes a luciferase or fluorescent protein marker).

As mentioned above, a 3 vector system (ie, a 2 vector lentiviral packaging system) was designed for the production of lentiviral particles. A schematic diagram of the 3-vector system is shown in FIG. The schematic diagram of FIG. 4 is a circularized version of the linear system described above in FIG. Briefly, referring to Figure 4, the top vector is a helper plasmid, which in this case contains Rev. The vector appearing in the middle of FIG. 4 is an envelope plasmid. The bottom vector is the therapeutic vector described above.

Referring to FIG. 4 in more detail, the helper+Rev plasmid contains CAG enhancer (SEQ ID NO:40); CAG promoter (SEQ ID NO:41); chicken beta actin intron (SEQ ID NO:42); HIV gag (SEQ ID NO:43); HIV Pol. (SEQ ID NO:44); HIV Int (SEQ ID NO:45); HIV RRE (SEQ ID NO:46); HIV Rev (SEQ ID NO:47); and rabbit beta globin poly A (SEQ ID NO:48).

The envelope plasmid contains the CMV promoter (SEQ ID NO:60); beta globin intron (SEQ ID NO:61); VSV-G (SEQ ID NO:62); and rabbit beta globin poly A (SEQ ID NO:63).

Synthesis of a two-vector lentiviral packaging system containing helper (+Rev) and envelope plasmids.
Materials and methods:
Construction of helper plasmids: Helper plasmids were constructed by initial PCR amplification of DNA fragments derived from the pNL4-3 HIV plasmid (NIH AIDS reagent program) containing the Gag, Pol, and integrase genes. The primers were designed to amplify a fragment with EcoRI and NotI restriction sites that can be used to insert at the same sites in the pCDNA3 plasmid (Invitrogen). The forward primer was (5'-TAAGCAGAATTCATGAATTTTGCCAGGAAGAT-3') (SEQ ID NO: 81) and the reverse primer was (5'-CCATACAAATGAATGGACACTAGGCGGCCGCACGAAT-3') (SEQ ID NO: 82). The sequences of Gag, Pol and integrase fragments were as follows:

Next, a DNA fragment containing the Rev, RRE, and rabbit beta globin poly A sequences and having XbaI and XmaI flanking restriction sites was synthesized by MWG Operon. The DNA fragment was then inserted into the XbaI and XmaI restriction sites of the plasmid. The DNA sequence was as follows:

Finally, the CMV promoter of pCDNA3.1 was replaced with the CAG enhancer/promoter+chicken beta actin intron sequence. A DNA fragment containing the CAG enhancer/promoter/intron sequence and having MluI and EcoRI flanking restriction sites was synthesized by MWG Operon. The DNA fragment was then inserted into the MluI and EcoRI restriction sites of the plasmid. The DNA sequence was as follows:

Construction of VSV-G envelope plasmid:
A vesicular stomatitis Indianavirus glycoprotein (VSV-G) sequence with flanking EcoRI restriction sites was synthesized by MWG Operon. The correct orientation was then determined by inserting the DNA fragment into the EcoRI restriction site of pCDNA3.1 plasmid (Invitrogen) and sequencing using CMV specific primers. The DNA sequence was as follows:

Also, a 4-vector system (ie, a 3-vector lentivirus packaging system) was designed and produced using the methods and materials described herein. A schematic diagram of the 4-vector system is shown in FIG. Briefly referring to FIG. 5, the top vector is a helper plasmid, which in this case does not contain Rev. The second vector from the top is another Rev plasmid. The second vector from the bottom is an envelope plasmid. The bottom vector is the therapeutic vector described above.

Referring in part to FIG. 5, the helper plasmids are CAG enhancer (SEQ ID NO:49); CAG promoter (SEQ ID NO:50); chicken beta actin intron (SEQ ID NO:51); HIV gag (SEQ ID NO:52); HIV Pol. (SEQ ID NO:53); HIV Int (SEQ ID NO:54); HIV RRE (SEQ ID NO:55); and rabbit beta globin poly A (SEQ ID NO:56).

The Rev plasmid contains the RSV promoter (SEQ ID NO:57); HIV Rev (SEQ ID NO:58); and rabbit beta globin poly A (SEQ ID NO:59).

The envelope plasmid contains the CMV promoter (SEQ ID NO:60); beta globin intron (SEQ ID NO:61); VSV-G (SEQ ID NO:62); and rabbit beta globin poly A (SEQ ID NO:63).

Synthesis of a 3-vector lentivirus packaging system containing helper, Rev, and envelope plasmids.
Materials and methods:
Construction of Rev-free helper plasmid:
A Rev-free helper plasmid was constructed by inserting a DNA fragment containing the RRE and rabbit beta globin poly A sequences. This sequence with flanking XbaI and XmaI restriction sites was synthesized by MWG Operon. The RRE/rabbit poly A beta globin sequence was then inserted into the XbaI and XmaI restriction sites of the helper plasmid. The DNA sequence is as follows:

Construction of Rev plasmid:
The RSV promoter and HIV Rev sequences were synthesized by MWG Operon as a single DNA fragment with flanking MfeI and XbaI restriction sites. Then, the DNA fragment was inserted into the MfeI and XbaI restriction sites of pCDNA3.1 plasmid (Invitrogen) in which the CMV promoter was replaced with the RSV promoter. The DNA sequence was as follows:

The plasmids of the 2 and 3 vector packaging systems can be modified with similar elements to potentially remove intron sequences without loss of vector function. For example, the following elements could potentially be used in place of similar elements in the 2-Vector and 3-Vector packaging systems.

Promoters: elongation factor-1 (EF-1) (SEQ ID NO: 64), phosphoglycerate kinase (PGK) (SEQ ID NO: 65), and ubiquitin C (UbC) (SEQ ID NO: 66), CMV (SEQ ID NO: 60) or It can be used instead of the CAG promoter (SEQ ID NO: 100).

Poly A sequences: SV40 poly A (SEQ ID NO:67) and bGH poly A (SEQ ID NO:68) can be used in place of rabbit beta globin poly A (SEQ ID NO:48).

HIV Gag, Pol, and integrase sequences: HIV sequences in helper plasmids can be constructed from different HIV strains or clades. For example, HIV Gag (SEQ ID NO:69); HIV Pol (SEQ ID NO:70); and HIV Int (SEQ ID NO:71) from the Bal strain are added to a helper/helper+Rev plasmid as outlined herein. It can be replaced with the included gag, pol, and int sequences.

Envelope: VSV-G glycoproteins are feline endogenous virus (RD114) (SEQ ID NO: 72), gibbon ape leukemia virus (GALV) (SEQ ID NO: 73), rabies (FUG) (SEQ ID NO: 74), lymphocyte choroid meninges. Inflammatory virus (LCMV) (SEQ ID NO:75), influenza A poultry plague virus (FPV) (SEQ ID NO:76), Ross River alphavirus (RRV) (SEQ ID

NO:77), murine leukemia virus 10A1 (MLV) (SEQ ID NO:78).), or a membrane glycoprotein derived from Ebola virus (EboV) (SEQ ID NO:79). The sequences of these envelopes are identified in the sequence section herein.

In summary, the 3 vs. 4 vector systems can be compared and contrasted as follows. The three-vector lentiviral vector system includes: Helper plasmids: HIV Gag, Pol, integrase, and Rev/Tat;2. 2. Envelope plasmid: VSV-G/FUG envelope; and 3. Therapeutic vectors: RSV 5'LTR, Psi packaging signal, Gag fragment, RRE, Env fragment, cPPT, WPRE, and 3'delta LTR. The 4-vector lentiviral vector system includes: Helper plasmids: HIV Gag, Pol, and integrase;2. Rev plasmid: Rev;3. 3. Envelope plasmid: VSV-G/FUG envelope; and 4. Therapeutic vectors: RSV 5'LTR, Psi packaging signal, Gag fragment, RRE, Env fragment, cPPT, WPRE, and 3'delta LTR. Sequences corresponding to the above elements are identified in the Sequence Listing portion of this specification.

(Example 2: Development of anti-HIV lentivirus vector)
The purpose of this example was to develop an anti-HIV lentiviral vector.

Inhibitory RNA design. The sequence of the Homo sapiens chemokine CC motif receptor 5 (CCR5) (GC03P046377) mRNA was used to search for potential siRNA or shRNA candidates that knocked down CCR5 levels in human cells. Potential RNA interfering sequences were chosen from candidates selected by siRNA or shRNA design programs such as from the Broad Institute or BLOCK-iT RNAi Designer from Thermo Scientific. To control shRNA expression, individual selected shRNA sequences were inserted into the lentiviral vector immediately 3'to the RNA polymerase III promoter such as Hl, U6, or 7SK. These lentivirus-shRNA constructs were used to transduce cells and measure changes in specific mRNA levels. The most potent shRNAs to reduce mRNA levels were individually embedded within the microRNA backbone to allow expression by either the CMV or EF-1 alpha RNA polymerase II promoters. The microRNA backbone was selected from mirbase.org/. RNA sequences were also synthesized as synthetic siRNA oligonucleotides and introduced directly into cells without the use of lentiviral vectors.

The genomic sequence of the human immunodeficiency virus type 1 BaL strain (HIV-185US_BaL, accession number AY713409) was used to search for potential siRNA or shRNA candidates that knockdown HIV replication levels in human cells. Based on sequence homology and experience, we have focused our search on regions of the HIV Tat and Vif genes, but those skilled in the art are not limited to the use of these regions and other potential targets have been selected. Understand what you get. Importantly, highly conserved regions of Gag or the polymerase gene could not be targeted by shRNA because these same sequences were present in the packaging system complementing plasmid required for vector production. Like CCR5 (NM 05709.3, NM 001100168.1-specific) RNA, potential HIV-specific RNA interfering sequences have been identified from the Gene-E Software Suite hosted by the Broad Institute (broadinstitute.org/mai/public). SiRNA or shRNA design programs such as or BLOCK-iT RNAi Designer from Thermo Scientific (rnadesigner.thermophisher.com/rnaipress/setOption.do=designated60?6067, selected from 60; To control shRNA expression, individual selected shRNA sequences were inserted into the lentiviral vector immediately 3'to the RNA polymerase III promoter such as Hl, U6, or 7SK. These lentivirus-shRNA constructs were used to transduce cells and measure changes in specific mRNA levels. The most potent shRNAs to reduce mRNA levels were individually embedded within the microRNA backbone to allow expression by either the CMV or EF-1 alpha RNA polymerase II promoters.

Vector construction. Oligonucleotide sequences containing BamHI and EcoRI restriction sites for CCR5, Tat or Vif shRNA were synthesized by Eurofins MWG Operon, LLC. Overlapping sense and antisense oligonucleotide sequences were mixed and annealed while cooling from 70°C to room temperature. The lentiviral vector was digested with the restriction enzymes BamHI and EcoRI at 37° C. for 1 hour. The digested lentiviral vector was purified by agarose gel electrophoresis and extracted from the gel using the Invitrogen DNA gel extraction kit. The DNA concentration was determined and the vector to oligo (3:1 ratio) was mixed, annealed and ligated. The ligation reaction was carried out for 30 minutes at room temperature using T4 DNA ligase. 2.5 microliters of ligation mix was added to 25 microliters of STBL3 competent bacterial cells. Transformation was achieved after heat shock at 42°C. Bacterial cells were spread on agar plates containing ampicillin and drug resistant colonies (indicating the presence of ampicillin resistant plasmid) were harvested, purified and grown in LB broth. Plasmid DNA was extracted from harvested bacterial cultures using the Invitrogen DNA miniprep kit to check the insertion of oligo sequences. Insertion of the shRNA sequence in the lentiviral vector was confirmed by DNA sequencing using specific primers for the promoter used to regulate shRNA expression. Exemplary vector sequences determined to limit HIV replication can be found in FIG. Then, for example, the shRNA sequence with the highest activity on CCR5, Tat, or Vif gene expression was assembled into a microRNA (miR) cluster under the control of the EF-1 alpha promoter. The promoter and miR sequences are shown in FIG.

Furthermore, using standard molecular biology techniques (eg Sambrook; Molecular Cloning: A Laboratory Manual, 4th Edition) as well as the techniques described herein, it is illustrated in Figure 7 herein. As described above, a series of lentiviral vectors was developed.

Vector 1 was developed. Vector 1 comprises, from left to right, the following: terminal repeat sequence (LTR) portion (SEQ ID NO:35); H1 element (SEQ ID NO:101); shCCR5 (SEQ ID NO:16, 18, 20, 22, or 24); Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (SEQ ID NOS: 32, 80); and terminal repeat sequence

portion (SEQ ID NO: 102).

Vector 2 was developed. Vector 2 contains, from left to right, the following: terminal repeat sequence (LTR) portion (SEQ ID NO: 35); H1 element (SEQ ID NO: 101); shRev/Tat (SEQ ID NO: 10); H1 element (sequence No. 101); shCCR5 (SEQ ID NO: 16, 18, 20, 22, or 24); Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (SEQ ID NO: 32, 80); and terminal repeat sequence portion (SEQ ID NO: 102). .

Vector 3 was developed. Vector 3 contains, from left to right, the following: terminal repeat sequence (LTR) portion (SEQ ID NO:35); H1 element (SEQ ID NO:101); shGag (SEQ ID NO:12); H1 element (SEQ ID NO:101).); shCCR5 (SEQ ID NO: 16, 18, 20, 22, or 24); woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (SEQ ID NO: 32, 80); and terminal repeat sequence portion (SEQ ID NO: 102).

Vector 4 was developed. Vector 4 contains, from left to right, the following: terminal repeat sequence (LTR) portion (SEQ ID NO: 35); 7SK element (SEQ ID NO: 103); shRev/Tat (SEQ ID NO: 10); H1 element (sequence No. 101); shCCR5 (SEQ ID NO: 16, 18, 20, 22, or 24); Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (SEQ ID NO: 32, 80); and terminal repeat sequence portion (SEQ ID NO: 102). .

Vector 5 was developed. Vector 5 comprises, from left to right, the following: terminal repeat sequence (LTR) portion (SEQ ID NO:35); EF1 element (SEQ ID NO:4); miR30CCR5 (SEQ ID NO:1); MiR21Vif (SEQ ID NO:2). MiR185Tat (SEQ ID NO: 3); woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (SEQ ID NOs: 32, 80); and terminal repeat sequence portion (SEQ ID NO: 102).

Vector 6 was developed. Vector 6 comprises, from left to right, the following: terminal repeat sequence (LTR) portion (SEQ ID NO: 35); EF1 element (SEQ ID NO: 4); miR30CCR5 (SEQ ID NO: 1); MiR21Vif (SEQ ID NO: 2). MiR155Tat (SEQ ID NO: 104); woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (SEQ ID NOs: 32, 80); and terminal repeat sequence portion (SEQ ID NO: 102).

Vector 7 was developed. Vector 7 comprises, from left to right, the following: terminal repeat sequence (LTR) portion (SEQ ID NO:35); EF1 element (SEQ ID NO:4); miR30CCR5 (SEQ ID NO:1); MiR21Vif (SEQ ID NO:2). MiR185Tat (SEQ ID NO: 3); woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (SEQ ID NOs: 32, 80); and terminal repeat sequence portion (SEQ ID NO: 102).

Vector 8 was developed. Vector 8 comprises, from left to right, the following: terminal repeat sequence (LTR) portion (SEQ ID NO:35); EF1 element (SEQ ID NO:4); miR30CCR5 (SEQ ID NO:1); MiR21Vif (SEQ ID NO:2). MiR185Tat (SEQ ID NO: 3); and terminal repeat sequence portion (SEQ ID NO: 102);

Vector 9 was developed. Vector 9 comprises, from left to right, the following: terminal repeat sequence (LTR) portion (SEQ ID NO:35); CD4 element (SEQ ID NO:30); miR30CCR5 (SEQ ID NO:1); miR21Vif (SEQ ID NO:2). MiR185Tat (SEQ ID NO: 3); woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (SEQ ID NOs: 32, 80); and terminal repeat sequence portion (SEQ ID NO: 102).

Vector Development It should be noted that not all the vectors developed for these experiments performed as planned. More specifically, a lentiviral vector for HIV may contain three major components: 1) reduce the level of target cell surface HIV binding protein (receptor) to promote early viral attachment and entry. Inhibitory RNA to block; 2) overexpression of the HIV TAR sequence that would sequester the viral Tat protein and diminish its ability to transactivate viral gene expression; and 3) critical conservation within the HIV genome. Inhibitory RNA that attacks sequences.

With respect to the first point above, an important cell surface HIV binding protein is the chemokine receptor CCR5. HIV particles attach to sensitive T cells by binding to CD4 and CCR5 cell surface proteins. Since CD4 is an essential cell surface glycoprotein important for T cell immunological function, it was not chosen as a target to manipulate its expression level. However, people who are naturally homozygous for a null mutation in the CCR5 gene and who lack complete receptor expression have increased susceptibility to a few infectious diseases and, rarely, autoimmunity. Live a normal life except that it can develop. Since a relatively small number of systemic CD4+ T cells were genetically modified to reduce CCR5 expression, it is unlikely that CD4+ T cells required for the control of pathogen immunity or autoimmunity will be present in the modified cells. The safety is enhanced in. Therefore, modulation of CCR5 was determined to be a relatively safe approach and made it a major target in the development of anti-HIV lentiviral vectors.

With respect to the second point above, the viral TAR sequence is a highly structured region of HIV genomic RNA that binds tightly to the viral Tat protein. The Tat:TAR complex is important for efficient production of viral RNA. Overexpression of the TAR region is contemplated as a decoy molecule that sequesters the Tat protein and reduces the level of viral RNA. However, TAR has proven to be toxic to most mammalian cells, including the cells used to make lentiviral particles. Furthermore, TAR is inefficient at inhibiting viral gene expression in other laboratories and has been abandoned as a viable component in HIV gene therapy.

With respect to the third point above, viral gene sequences were identified that meet the following three criteria: i) The sequences are reasonably conserved across a range of HIV isolates and represent epidemics of the geographical region of interest. Ii) reducing the RNA level due to the activity of inhibitory RNA in the viral vector will reduce the corresponding protein level by an amount sufficient to significantly reduce HIV replication; And iii) the viral gene sequences targeted by the inhibitory RNA are not present in the genes required for packaging and incorporating viral vector particles during manufacture. The last point is important because it is completely disadvantageous to have inhibitory RNAs that target the genes required for the efficient functioning of the viral particle itself. In this embodiment, the sequence at the junction between the HIV Tat gene and the Rev gene, and the second sequence within the HIV Vif gene were targeted by the inhibitory RNA. Tat/Rev targeting has the additional benefit of reducing HIV envelope glycoprotein expression as this region overlaps with the envelope gene of the HIV genome.

Strategies for developing and testing vectors first identify suitable targets (as described herein), and then express individual or multiple inhibitory RNA species for testing in a cell model. To construct a lentiviral vector containing inhibitory RNA with proven anti-HIV function. Lentiviral vectors are tested for toxicity, yield during in vitro production, and efficacy against HIV in reducing CCR5 expression levels or reducing viral gene products to inhibit viral replication.

Table 2 below shows the history of arriving at clinical candidates via multiple versions of inhibitory constructs. First, a shRNA (short homologous RNA) molecule was designed and expressed from a plasmid DNA construct.

Plasmids 1-4 detailed in Table 2 below were tested for shRNA sequences against the HIV Gag, Pol, and RT genes. Each shRNA was active in suppressing viral protein expression in a cellular model, but there were two key issues that prevented further development. First, the sequences targeted laboratory isolates of HIV that are not representative of the Clade B HIV strains currently circulating in North America and Europe. Second, these shRNAs targeted a key component of the lentiviral vector packaging system, which would significantly reduce vector yield during production. Plasmid 5, detailed in Table 2, was selected to target CCR5 and provided the lead candidate sequence. Plasmids 6, 7, 8, 9, 10, and 11 detailed in Table 2 incorporate the TAR sequence and have unacceptable toxicity to mammalian cells, including cells used to make lentiviral vectors. It was found that In plasmid 2 detailed in Table 2, a lead shRNA sequence was identified that could reduce Tat RNA expression. Plasmid 12 detailed in Table 2 demonstrated that shCCR5 expressed as a lentiviral vector microRNA (miR) was effective, confirming that it should be in the final product. . Plasmid 13, detailed in Table 2, demonstrated that shVif expressed as a microRNA (miR) in a lentiviral vector was effective, confirming that it should be in the final product. . Plasmid 14, detailed in Table 2, demonstrated that shTat expressed as a lentiviral vector microRNA (miR) was effective, confirming that it should be in the final product. . Plasmid 15 detailed in Table 2 contained miR CCR5, miR Tat, and miR Vif in the form of miR clusters expressed from a single promoter. These miRs did not target a key component of the lentiviral vector packaging system, demonstrating negligible toxicity to mammalian cells. The miRs within the cluster were as effective as the individual miRs tested previously. The overall effect was a significant reduction in replication of the CCR5-tropic HIV BaL strain.

Functional assay. It contains a CCR5, Tat or Vif shRNA sequence and expresses green fluorescent protein (GFP) under the control of the CMV Immediate Early Promoter for experimental purposes and was named AGT103/CMV-GFP. Individual lentiviral vectors were tested for their ability to knockdown CCR5, Tat or Vif expression. Lentiviral particles were transduced into mammalian cells in the presence or absence of polybrene. Cells were harvested after 2-4 days; proteins and RNA were analyzed for CCR5, Tat or Vif expression. Analytical flow sites comparing the fluorescence of modified and unmodified cells using a Western blot assay or labeling cells with a specific fluorescent antibody (CCR5 assay), followed by either using a CCR5-specific antibody or an isotype control antibody Protein levels were tested by metric.

Start of lentivirus test. RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin was used to make T cell culture medium. IL2 10,000 units/ml, IL-12 1 μg/ml, IL-7 1 μg/ml, IL-15 1 μg/ml cytokine stocks were also prepared in advance.

Prior to lentivirus transduction, infectious virus titers were determined and used to calculate the amount of virus added for the appropriate multiplicity of infection (MOI).

Day 0-12: Antigen-specific enrichment: On day 0, thaw cryopreserved PBMC, wash with 10 ml of 37° C. medium for 10 minutes at 1200 rpm, 2×10^{6} cells/37° C. medium/ Resuspended at a concentration of ml. Cells were cultured at 0.5 ml/well in 24-well plates at 37° C. in 5% CO_2. To define optimal stimulation conditions, cells were stimulated with the combination of reagents listed in Table 3 below:

Final concentration: IL-2=20 units/ml, IL-12=10 ng/ml, IL-7=10 ng/ml, IL-15=10 ng/ml, peptide=5 μg/ml individual peptides, MVA MOI=1.

On days 4 and 8, 0.5 ml of fresh medium and cytokines at the listed concentrations (all concentrations represent final concentrations in culture) were added to the stimulated cells.

Days 12-24: Nonspecific growth and lentiviral transduction. On day 12, stimulated cells were pipetted off and resuspended in fresh T cell medium at a concentration of 1×10^{6} cells/ml. The resuspended cells were transferred to T25 culture flasks and stimulated with DYNABEADS® Human T-Activator CD3/CD28 and the cytokines listed above according to the manufacturer's instructions; flasks were incubated in vertical position.

On day 14, AGT103/CMV-GFP was added at MOI 20 and the cultures were returned to the incubator for 2 days. At this point, cells were harvested by pipetting, collected by centrifugation at 1300 rpm for 10 minutes, resuspended in the same volume of fresh medium and centrifuged again to form a loose cell pellet. The cell pellet was resuspended in fresh medium containing the same cytokines used in the previous step at 0.5×10^{6} viable cells per ml.

From day 14 to day 23, the number of cells was evaluated every 2 days and the cells were diluted with fresh medium to 0.5×10^{6} cells/ml. Cytokines were added each time.

Cells were harvested on day 24 and beads were removed from the cells. To remove the beads, the cells were transferred to a suitable tube and placed in a sorting magnet for 2 minutes. The supernatant containing the cells was transferred to a new tube. Then, the cells were cultured in fresh medium for 1 day at 1×10^{6} cells/ml. Assays were performed to determine the frequency of antigen-specific T cells and lentivirus transduced cells.

Amprenavir (0.5 ng/ml) or saquinavir (0.5 ng/ml) or another suitable protease or integrase inhibitor to prevent possible viral growth on the first day of stimulation and every other day in culture. Was added to the culture.

Antigen-specific T cells are examined by intracellular cytokine staining for IFN-gamma. Cultured cells after peptide stimulation or lentiviral transduction at 1×10^{6} cells/ml were treated with medium alone (negative control), Gag peptide (5 μg/ml individual peptides), or PHA (5 μg/ml, positive control). I was stimulated. After 4 hours, BD GolgiPlug™ (1:1000, BD Biosciences) was added to block Golgi transport. After 8 hours, cells are washed and extracellular (CD3, CD4 or CD8; BD Biosciences) and intracellular (IFN-gamma; BD) using the BD Cytofix/Cytoperm™ kit according to the manufacturer's instructions. Biosciences) antibody was used for staining. Samples were analyzed on a BD FACSCalibur™ flow cytometer. A control sample labeled with the appropriate isotype-matched antibody was included in each experiment. Data were analyzed using Flowjo software.

The lentivirus transduction rate was determined by the frequency of GFP+ cells. Transduced antigen-specific T cells are determined by the frequency of CD3+CD4+GFP+IFNgamma+ cells; a test of CD3+CD8+GFP+IFNgamma+ cells is included as a control.

These results demonstrate that target T cell populations, CD4 T cells, can be transduced with a lentivirus designed to specifically knock down the expression of HIV-specific proteins, thus rendering the virus It is shown to generate a proliferative population of T cells that are immune to. This example is a proof of concept showing that the disclosed lentiviral constructs can be used to produce a functional cure in HIV patients.

(Example 4: CCR5 knockdown with experimental vector)
AGTc120 is a Hela cell line that stably expresses high amounts of CD4 and CCR5. Transduction with AGTc120 with or without LV-CMV-mCherry (red fluorescent protein mCherry expressed under control of CMV immediate early promoter) or AGT103/CMV-mCherry. Gene expression of the mCherry fluorescent protein was controlled by the CMV (cytomegalovirus immediate early promoter) expression cassette. The AGT103/CMV-mCherry expressed therapeutic miRNAs against CCR5, Vif and Tat, while the LV-CMV-mCherry vector lacked microRNA clusters.

As shown in FIG. 8A, transduction efficiency was >90%. After 7 days, cells were harvested, stained with a fluorescent monoclonal antibody against CCR5 and subjected to analytical flow cytometry. Isotype controls are shown in gray in these histograms in which the number of cells normalized to mode (y-axis) is plotted against the mean fluorescence intensity of CCR5 APC (x-axis). After staining of cell surface CCR5, cells treated with no lentivirus or control lentivirus (expressing only mCherry marker) showed no change in CCR5 density, whereas AGT103 (right section) showed CCR5 staining intensity. It was reduced to approximately the level of the isotype control. Seven days later, cells were infected with or without the R5-tropic HIV reporter virus Bal-GFP. After 3 days, cells were collected and analyzed by flow cytometry. Over 90% of cells were transduced. AGT103-CMV/CMVmCherry reduced CCR5 expression in transduced AGTc120 cells and blocked R5-directed HIV infection compared to cells treated with control vector.

FIG. 8B shows the relative insensitivity of transfected AGTc120 cells to infection with HIV. As above, the lentiviral vector expresses mCherry protein and HIV infected transduced cells (expressing GFP) appear as double positive cells in the upper right quadrant of the pseudocolor flow cytometry dot plot. In the absence of HIV (top

panel), there were no GFP+ cells under any conditions. After HIV infection (lower panel), 56% of cells were infected in the absence of lentiviral transduction, and 53.6% of AGTc120 cells transduced with LV-CMV-mCherry were infected. When cells were transduced with the therapeutic AGT103/CMV-mCherry vector, only 0.83% of the cells appeared in the double positive quadrant, indicating they were transduced and infected. .

Dividing 53.62 (percentage of double-positive cells with control vector) by 0.83 (percentage of double-positive cells with therapeutic vector), AGT103 exceeds 65-fold over HIV in this experimental system It is shown to have provided protection.

(Example 5: Regulation of CCR5 expression by shRNA inhibitor sequence of lentivirus vector)
Inhibitory RNA design. The sequence of the Homo sapiens chemokine receptor CCR5 (CCR5, NC000003.12.) was used to search for potential siRNA or shRNA candidates for knocking down CCR5 levels in human cells. Potential RNA interfering sequences were selected from candidates selected by siRNA or shRNA design programs such as from Broad Institute or BLOCK-IT RNA iDesigner from Thermo Scientific. The shRNA sequence may be inserted immediately after the RNA polymerase III promoter of the plasmid, such as H1, U6, or 7SK, to regulate shRNA expression. Alternatively, the shRNA sequence may be inserted into a lentiviral vector in which a similar promoter is used, or within the microRNA backbone to allow expression by an RNA polymerase II promoter such as CMV or EF-1alpha. It may be embedded in. RNA sequences may also be synthesized as siRNA oligonucleotides and utilized independently of plasmid or lentiviral vectors.

Plasmid construction. For CCR5 shRNA, oligonucleotide sequences containing BamHI and EcoRI restriction sites were synthesized by MWG Operon. The oligonucleotide sequences were annealed by incubation at 70°C and then cooled to room temperature. The annealed oligonucleotides were digested with the restriction enzymes BamHI and EcoRI for 1 hour at 37°C, after which the enzyme was inactivated at 70°C for 20 minutes. In parallel, plasmid DNA was digested with the restriction enzymes BamHI and EcoRI for 1 hour at 37°C. The digested plasmid DNA was purified by agarose gel electrophoresis and extracted from the gel using the Invitrogen DNA gel extraction kit. The DNA concentration was determined and plasma was ligated to the oligonucleotide sequences with a 3:1 insert to vector ratio. The ligation reaction was carried out for 30 minutes at room temperature using T4 DNA ligase. 2.5 μL of ligation mix was added to 25 μL of STBL3 competent bacterial cells. Heat shock at 42°C was required for transformation. Bacterial cells were spread on agar plates containing ampicillin and colonies were grown in L broth. To check the insertion of oligo sequences, the Invitrogen DNA miniprep kit was used to extract plasmid DNA from harvested bacterial cultures and tested by restriction enzyme digestion. Insertion of the shRNA sequence into the plasmid was confirmed by DNA sequencing using primers specific for the promoter used to regulate shRNA expression.

Functional assay for CCR5 mRNA reduction: The assay for inhibition of CCR5 expression required co-transfection of two plasmids. The first plasmid contains one of five different shRNA sequences for CCR5 mRNA. The second plasmid contains the cDNA sequence of the human CCR5 gene. The plasmid was co-transfected into 293T cells. After 48 hours, cells were lysed and RNA was extracted using Qiagen's RNeasy kit. CDNA was synthesized from RNA using the Superscript kit from Invitrogen. Samples were then analyzed by quantitative RT-PCR using an Applied Biosystems Step One PCR machine. CCR5 expression uses forward primer (5'-AGGAATTGATGGCGAGAAGG-3') (SEQ ID NO:93) and reverse primer (5'-CCCCAAAAGAAGGTCAAGGTAATCA-3') (SEQ ID NO:94) under standard conditions for polymerase chain reaction analysis. And detected using SYBR green from Invitrogen. The sample was standardized for polymerase chain reaction analysis using a forward primer (5'-AGCGCGGCTACAGCTTCA-3') (SEQ ID NO:95) and a reverse primer (5'-GGCGGACGTAGCACAGCTTTCT-3') (SEQ ID NO:96). , B-actin gene expression was normalized to mRNA. Relative expression of CCR5 mRNA was determined by its Ct value normalized to the level of actin messenger RNA in each sample. Results are shown in FIG.

CCR5 knockdown was tested in 293T cells by co-transfection of CCR5 shRNA construct and CCR5 expression plasmid as shown in FIG. 9A. Control samples were transfected with a scrambled shRNA sequence and a CCR5 expression plasmid that did not target any human gene. Samples were harvested 60 hours after transfection and CCR5 mRNA levels were measured by quantitative PCR. Furthermore, as shown in FIG. 9B, CCR5 was knocked down after transduction with a lentivirus expressing CCR5 shRNA-1 (SEQ ID NO: 16).

(Example 6: Regulation of HIV component by shRNA inhibitor sequence of lentivirus vector)
Inhibitory RNA design.
Using the sequences of HIV type 1 Rev/Tat (5'-GCGGAGACAGCGACGAAGAGC-3') (SEQ ID NO: 9) and Gag (5'-GAAGAAATGATGGACAGCAT-3') (SEQ ID NO: 11), shRNAs were designed, they were synthesized and cloned into a plasmid as described above.

Plasmid construction. Rev/Tat or Gag target sequences were inserted into the 3'UTR (untranslated region) of the firefly luciferase gene, which is commonly used as a reporter of gene expression in cells or tissues. In addition, one plasmid was constructed to express Rev/Tat shRNA and a second plasmid was constructed to express Gag shRNA. Plasmid construction was as described above.

Functional assay of shRNA targeting of Rev/Tat or Gag mRNA: Using plasmid co-transfection, we have determined whether the shRNA plasmid is capable of degrading luciferase messenger RNA and co-transfected. It was tested whether it was possible to reduce the intensity of luminescence of the cells. A shRNA control (scrambled sequence) was used to establish the maximum light output from cells transfected with luciferase. Co-transfection of a luciferase construct containing a Rev/Tat target sequence inserted in the 3'-UTR (untranslated region of mRNA) with a Rev/Tat shRNA sequence resulted in an approximately 90% reduction in luminescence, resulting in a reduction in shRNA. The function of the sequence was shown to be powerful. Similar results were obtained when the luciferase construct containing the Gag target sequence in the 3'-UTR was cotransfected with the Gag shRNA sequence. These results indicate that the activity of the shRNA sequence is strong.

As shown in FIG. 10A, knockdown of Rev/Tat target gene was measured by transient transfection of 293T cells by reducing the activity of luciferase fused to the target mRNA sequence of 3'UTR. . As shown in FIG. 10B, the Gag target gene sequence fused to the luciferase gene was knocked down. Results are expressed as the mean ± SD of 3 independent transfection experiments, each in triplicate.

(Example 7: AGT103 decreases Tat and Vif expression)
Cells were transfected with the exemplary vector AGT103/CMV-GFP. AGT103 and other exemplary vectors are defined in Table 3 below.

A T lymphoblastoid cell line (CEM; CCRF-CEM; United States Cultured Cell Line Preservation Organization Catalog Number CCL119) was transduced with AGT103/CMV-GFP. After 48 hours, cells were transfected with an HIV expression plasmid encoding the entire viral sequence. After 24 hours, RNA was extracted from cells and tested for levels of intact Tat sequences using reverse transcriptase polymerase chain reaction. Relative expression levels of intact Tat RNA decreased from approximately 850 in the presence of control lentiviral vector to approximately 200 in the presence of AGT103/CMV-GFP, >4 fold reduction, as shown in FIG. 11. did.

(Example 8: Regulation of HIV components by synthetic microRNA sequence of lentivirus vector)
Inhibitory RNA design. The sequences of the HIV-1 Tat and Vif genes were used to search for potential siRNA or shRNA candidates that knockdown Tat or Vif levels in human cells. Potential RNA interference sequences were selected from candidates selected by siRNA or shRNA design programs such as from the Broad Institute or BLOCK-IT RNA iDesigner from Thermo Scientific. Selected shRNA sequences with the strongest Tat or Vif knockdown were embedded within the microRNA backbone to allow expression by RNA polymerase II promoters such as CMV or EF-Ialpha. Alternatively, the RNA sequences may be synthesized as siRNA oligonucleotides and used independently of the plasmid or lentiviral vector.

Plasmid construction. The Tat target sequence (5'-TCCGCTTCTCTCGCGCCATAG-3') (SEQ ID NO: 7) was incorporated into the miR185 backbone to generate a Tat miRNA. Was inserted into a lentiviral vector and expressed under the control of the EF-1 alpha promoter. Similarly, the Vif target sequence (5'-GGGATGGTGTACTTTCTGAACTT-3') (SEQ ID NO: 6) was incorporated into the miR21 backbone to produce Vif miRNA.
Was inserted into a lentiviral vector and expressed under the control of the EF-1 alpha promoter. The resulting Vif/Tat miRNA expressing lentiviral vector was produced in 293T cells using the lentiviral vector packaging system. Vif and Tat miRNAs were all embedded in a microRNA cluster composed of miR CCR5, miR Vif, and miR Tat expressed under the control of the EF-1 promoter.

Functional assay of miR185 Tat inhibition of Tat mRNA accumulation. Lentiviral vector expressing miR185 Tat (LV-EF1-miR-CCR5-Vif-Tat) was used to transduce 293T cells using a multiplicity of infection equal to 5. Twenty-four hours after transduction, cells were transfected with a plasmid expressing HIV strain NL4-3 (pNL4-3) using Lipofectamine 2000 under standard conditions. After 24 hours, RNA was extracted and the level of Tat messenger RNA was tested by RT-PCR using Tat specific primers and compared to control actin mRNA levels.

Functional assay of miR21 Vif inhibition of Vif protein accumulation. A lentiviral vector expressing the miR21 Vif (LV-EF1-miR-CCR5-Vif-Tat) was used at a multiplicity of infection equal to 5 to transduce 293T cells. Twenty-four hours after transduction, cells were transfected with a plasmid expressing HIV strain NL4-3 (pNL4-3) using Lipofectamine2000. After 24 hours, cells were lysed and total soluble protein was tested to determine Vif protein content. Cell lysates were separated by SDS-PAGE according to established techniques. Separated proteins were transferred to nylon membrane and probed with Vif-specific monoclonal antibody or actin control antibody.

As shown in FIG. 12A, Tat knockdown was tested in 293T cells transduced with either a control lentiviral vector or a lentiviral vector expressing either a synthetic miR185 Tat or miR155 Tat microRNA. did. Twenty-four hours later, the HIV vector pNL4-3 was transfected with Lipofectamine2000 for 24 hours, after which RNA was extracted for qPCR analysis using primers for Tat. As shown in FIG. 12B, Vif knockdown was tested in 293T cells transduced with either a control lentiviral vector or a lentiviral vector expressing synthetic miR21 Vif microRNA. Twenty-four hours later, the HIV vector pNL4-3 was transfected with Lipofectamine2000 for 24 hours, after which the protein was extracted for immunoblot analysis with an antibody against HIV Vif.

Example 9: Regulation of CCR5 Expression by Lentiviral Vector Synthetic MicroRNA Sequences
The CEM-CCR5 cells, lentiviral vectors containing the synthetic miR30 sequences CCR5 (AGT103: TGTAAACTGAGCTTGCTCTA (SEQ ID NO: 97), AGT103-R5-1: TGTAAACTGAGCTTGCTCGC (SEQ ID NO: 98), or AGT103-R5-2: CATAGATTGGACTTGACAC (SEQ ID NO: 99)) was transduced. After 6 days, CCR5 expression was determined by FACS analysis with APC-conjugated CCR5 antibody and quantified by mean fluorescence intensity (MFI). CCR5 levels were expressed as% of CCR5 relative to LV-control as 100%. The target sequences for AGT103 and AGT103-R5-1 are in the same region as CCR5 target sequence #5. The target sequence of AGT103-R5-2 is the same as CCR5 target sequence #1. AGT103 (2% of total CCR5) is most effective in reducing CCR5 levels compared to AGT103-R5-1 (39% of total CCR5) and AGT103-R5-2 which did not reduce CCR5 levels. . The data is validated in Figure 13 herein.

Example 10: Regulation of CCR5 expression by synthetic microRNA sequences of lentiviral vectors containing either long or short WPRE sequences.
Vector construction. Lentiviral vectors often require RNA regulatory elements for optimal expression of the therapeutic gene or gene construct. A common choice is to use the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). We have a full length WPRE:
A shortened WPRE element containing AGT103
And a modified AGT103 vector containing

Functional assay of cell surface CCR5 expression modulation as a function of long versus short WPRE elements within the vector sequence. AGT103 containing long or short WPRE elements was used to transduce CEM-CCR5 T cells with a multiplicity of infection equal to 5. Six days after transduction, cells were harvested and stained with a monoclonal antibody capable of detecting cell surface CCR5 protein. The antibody was conjugated to a fluorescent marker. The staining intensity is directly proportional to the level of CCR5 on the cell surface. The control lentivirus did not affect cell surface CCR5 levels, resulting in a single population with a mean fluorescence intensity of 73.6 units. Conventional AGT103 with a long WPRE element reduced CCR5 expression to an average fluorescence intensity level of 11 units. AGT103 modified to incorporate a short WPRE element resulted in a single population of cells with an average fluorescence intensity of 13 units. Thus, substitution of the short WPRE element had little or no effect on AGT103's ability to reduce cell surface CCR5 expression.

As shown in FIG. 14, CEM-CCR5 cells were transduced with AGT103 containing either long or short WPRE sequences. After 6 days, CCR5 expression was determined by FACS analysis with APC-conjugated CCR5 antibody and quantified as mean fluorescence intensity (MFI). CCR5 levels were expressed as% of CCR5 relative to 100% LV-control. The reduction in CCR5 levels was similar for AGT103 with either a short chain (5.5% of total CCR5) or a long chain (2.3% of total CCR5) WPRE sequences.

Example 11: Regulation of CCR5 Expression by Synthetic MicroRNA Sequences of Lentiviral Vectors With or Without WPRE Sequences
Vector construction. To test whether WPRE is required for AGT103 downregulation of CCR5 expression, we constructed a modified vector that did not contain the WPRE element sequence.

Functional assay of cell surface CCR5 expression modulation as a function of the AGT103 vector with or without the long WPRE element. To test whether WPRE is required for AGT103 modulation of CCR5 expression levels, we used a multiplicity of infection equal to 5 and used CEM with modified vectors lacking AGT103 or WPRE. -Transduced into CCR5 T cells. Six days after transduction, cells were harvested and stained with a monoclonal antibody capable of recognizing cell surface CCR5 protein. The monoclonal antibody was directly conjugated to a fluorescent marker. The staining intensity is directly proportional to the number of CCR5 molecules per cell surface. The lentivirus control vector did not affect cell surface CCR5 levels and resulted in a homogeneous population with a mean fluorescence intensity of 164. A lentiviral vector (AGT103 having a long WPRE and also expressing a GFP marker protein), AGT103 lacking GFP but containing a long WPRE element, or AGT103 lacking both GFP and WPRE are all cell surface It was equally effective in modulating CCR5 expression. After removal of GFP, AGT103 with or without the WPRE element was indistinguishable in terms of their ability to modulate cell surface CCR5 expression.

CEM-CCR5 cells were transduced with AGT103 with or without GFP and WPRE. After 6 days, CCR5 expression was determined by FACS analysis with APC-conjugated CCR5 antibody and quantified as mean fluorescence intensity (MFI). CCR5 levels were expressed as% of CCR5 relative to LV-control as 100%. The reduction in CCR5 levels was similar for AGT103 with or without WPRE sequences (0% of total CCR5) or without (0% of total CCR5). The data is validated in Figure 15.

Example 12: Regulation of CCR5 Expression by CD4 Promoter-Regulated Synthetic MicroRNA Sequences of Lentiviral Vectors
Vector construction. A modified version of AGT103 was constructed to test the effect of substituting an alternative promoter to express a microRNA cluster that suppresses CCR5, Vif, and Tat gene expression. We used the following sequence to replace the normal EF-1 promoter with a T cell specific promoter for expressing the CD4 glycoprotein:

A functional assay comparing the EF-1 and CD4 gene promoters in terms of potency to reduce cell surface CCR5 protein expression. AGT103 modified by using the CD4 gene promoter instead of the regular EF-1 promoter was used for transduction of CEM-CCR5 T cells. Six days after transduction, cells were harvested and stained with a monoclonal antibody capable of recognizing cell surface CCR5 protein. The monoclonal antibody was conjugated to a fluorescent marker. The staining intensity is directly proportional to the level of cell surface CCR5 protein. Control lentivirus transduction resulted in a population of CEM-CCR5 T cells stained with a CCR5-specific monoclonal antibody and showing a mean fluorescence intensity of 81.7 units. The modified AGT103, in which the CD4 gene promoter was used in place of the EF-1 promoter to express microRNAs, showed a broad staining distribution with a mean fluorescence intensity equal to approximately 17.3 units. Based on this result, the EF-1 promoter is at least similar to, and may be superior to, the CD4 gene promoter for microRNA expression. Depending on the desired target cell population, the EF-1 promoter is universally active in all cell types and the CD4 promoter is only active in T lymphocytes.

CEM-CCR5 cells were transduced with a lentiviral vector (AGT103) containing a CD4 promoter that regulates synthetic microRNA sequences for CCR5, Vif, and Tat. After 6 days, CCR5 expression was determined by FACS analysis with APC-conjugated CCR5 antibody and quantified as mean fluorescence intensity (MFI). CCR5 levels were expressed as% of CCR5 relative to LV-control as 100%. In cells transduced with LV-CD4-AGT103, CCR5 levels were 11% of total CCR5. This is equivalent to that observed with LV-AGT103 containing the EF1 promoter. This data is validated in FIG.

(Example 13: Detection of HIV Gag-specific CD4 T cells)
Cells and reagents. Viable frozen peripheral blood mononuclear cells (PBMC) were obtained from a vaccine company. The data were obtained using a representative specimen from an HIV+ individual who was enrolled in an early stage clinical trial (clinical trials. gov NCT01378156) testing a candidate HIV therapeutic vaccine. Two specimens were obtained for the "pre-vaccination" and "post-vaccination" studies. Cell culture products, supplements, and cytokines were from commercial suppliers. Cells were Thompson, M., SL Heath, B. Sweeton, K. Williams, P. Cunningham, BF Keele, S. Sen, BE Palmer, N. Chomont, Y. Xu, R. Basu, MS Hellerstein, S. Kwa and HL Robinson (2016). "DNA/MVA Vaccination of HIV-1 Infected Participants with Viral Suppression on Antiretroviral Therapy, followed by Treatment Interruption: Elicitation of Immune Responses without Control of Re-Emergent Virus." PLoS One 11 (No. 10): e0163164. Were tested for response to the recombinant modified Vaccinia Ankara 63B of Geovax Corporation. A synthetic peptide representing the entire HIV-1 Gag polyprotein was obtained from GeoVax. Alternatively, the HIV(GAG) Ultra peptide set was obtained from JPT Peptide Technologies GmbH (www.jpt.com), Berlin, Germany. HIV(GAG) Ultra contains 150 peptides, each 15 amino acids in length and overlapping by 11 amino acids. They had been chemically synthesized, then purified and analyzed by liquid chromatography-mass spectrometry. Taken together, these peptides represent the major immunogenic regions of the HIV Gag polyprotein and are designed for 57.8% average coverage among known HIV strains. Peptide sequences are based on the HIV sequence database of Los Alamos National Laboratory (http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html). Peptides are provided as 25 micrograms of dry trifluoroacetate salt per peptide. It is dissolved in approximately 40 microliters DMSO and diluted to final concentration with PBS. Monoclonal antibodies for detecting CD4 and cytoplasmic IFN-gamma were obtained from commercial sources. Intracellular staining was performed with the BD Pharmingen intracellular staining kit for interferon-gamma. The peptide was resuspended in DMSO. We include DMSO only control conditions.

Functional assay for detecting HIV-specific CD4+ T cells. Frozen PBMC were thawed, washed and resuspended in RPMI medium containing 10% fetal bovine serum, supplements and cytokines. Cultured PBMCs collected before or after vaccination were treated with DMSO control, MVA GeoVax (multiplicity of infection equal to 1 plaque forming unit per cell), peptide GeoVax (1 microgram/ml), or HIV (GAG) Ultra peptide mixture (1 microgram/ml) for 20 hours in the presence of Golgi Stop reagent. Cells were collected, washed, fixed, permeabilized and stained with monoclonal antibodies specific for cell surface CD4 or intracellular interferon-gamma. The stained cells were analyzed on a FACSCalibur analytical flow cytometer. Data were gated on CD4+ T cell subsets. Highlighted cells within the boxed area were double positive and designated as HIV-specific CD4 T cells based on interferon-gamma expression following MVA or peptide stimulation. Numbers in the boxed area indicate the percentage of total CD4 identified as HIV-specific. We did not detect a strong response to DMSO or MVA. The GeoVax peptide induced fewer responsive cells compared to the JPT HIV(GAG) Ultra peptide mixture, although the difference was small and not significant.

As shown in FIG. 17, PBMCs derived from HIV-positive patients before or after vaccination were treated with DMSO (control), recombinant MVA expressing HIV Gag of GeoVax (MVA GeoVax), Gag peptide of GeoVax. (Pep GeoVax, also referred to herein as Gag peptide pool 1) or JPT Gag peptide (HIV(GAG) Ultra peptide mixture, also referred to herein as Gag peptide pool 2) was stimulated for 20 hours. IFNg production was detected by intracellular staining and flow cytometry using standard protocols. Flow cytometry data are gated on CD4 T cells. The numbers displayed within the boxes are the percentage of total CD4 T cells designated as "HIV-specific" based on the cytokine response to antigen-specific stimulation.

Example 14: HIV-specific CD4 T cell proliferation and lentiviral transduction.
Design and testing of methods for enriching PBMCs to increase the proportion of HIV-specific CD4 T cells and transducing those cells with AGT103 to produce the cell product AGT103T. A protocol was designed for ex vivo culture of PBMC (peripheral blood mononuclear cells) from HIV positive patients who received the therapeutic HIV

vaccine. In this example, the therapeutic vaccine comprises 3 doses of plasmid DNA expressing the HIV Gag, Pol, and Env genes, followed by 2 doses of MVA 62-B(expressing the same HIV Gag, Pol, and Env genes. It was composed of a modified vaccinia Ankara number 62-B). The protocol is not specific to the vaccine product and only requires sufficient levels of HIV-specific CD4+ T cells after immunization. Venous blood was collected and PBMCs were purified by Ficoll-Paque density gradient centrifugation. Alternatively, PBMC or defined cell traction may be prepared by positive or negative selection methods using antibody cocktail and fluorescence activated or magnetic bead sorting. Purified PBMCs are washed and cultured in standard medium containing supplements, antibiotics, and fetal bovine serum. To these cultures was added a pool of synthetic peptides representing possible T cell epitopes within the HIV Gag polyprotein. Interleukin-2 and interleukin-12, interleukin-2 and interleukin-7, interleukin-2 and interleukin-15 are tested, followed by addition of selected cytokines, interleukin-2 and interleukin-12. To supplement the culture. Peptide stimulation is performed, followed by culture for about 12 days. Fresh media and fresh cytokine supplements were added approximately every 4 days during the 12 day culture.

Peptide stimulation intervals are designed to increase the frequency of HIV-specific CD4 T cells in PBMC cultures. These HIV-specific CD4 T cells were activated by previous therapeutic immunizations. They can be restimulated and expanded by exposure to synthetic peptides. Our goal is to achieve by the end of the peptide-stimulated culture period that the total CD4 T cells specific for HIV will be greater than or equal to 1%.

At approximately 12 days in culture, cells are washed to remove residual material and then stimulated with synthetic beads that have been modified with antibodies to CD4 T cell surface proteins CD3 and CD28. This well established method for polyclonal stimulation of T cells will reactivate the cells and render them more susceptible to AGT103 lentiviral transduction. Approximately 13 days in culture, lentiviral transduction is performed using a multiplicity of infection of 1-5. After transduction, the cells were washed to remove residual lentivirus vector, cultured in medium containing interleukin-2 and interleukin-12, and freshly cultured approximately every 4 days until approximately 24 days of culture. Add medium and cytokines.

Throughout the culture interval, the antiretroviral drug saquinavir was added at a concentration of approximately 100 nM to suppress any possible HIV growth.

Approximately 24 days after culture, cells were harvested, washed, sampled for potency and release assays, and the remaining cells were suspended in cryopreservation medium before being transfected with AGT103. Freeze as a single aliquot of approximately 1×10^{10} cells per dose that will contain 1×10^{8} HIV-specific CD4 T cells.

The potency of the cell product (AGT103T) is tested in one of two alternative potency assays. In potency assay 1, the average genomic copy number per CD4 T cell (integrated AGT103 vector sequence) is tested. The minimum potency for releasing product is approximately 0.5 genomic copies per CD4 T cell. This assay uses magnetic bead labeled monoclonal antibodies to positively select for CD3+/CD4+ T cells, extract total cellular DNA, and use a quantitative PCR reaction to detect sequences unique to the AGT103 vector. Carried out by. Potency Assay 2 tests the average genomic copy number of integrated AGT103 within a subpopulation of HIV-specific CD4 T cells. This essay is accomplished by first stimulating PBMCs with a pool of synthetic peptides representing HIV Gag proteins. The cells are then stained with a specific antibody reagent capable of binding to CD4 T cells and also trapping secreted interferon-gamma cytokine. CD4 positive/interferon-gamma positive cells are captured by magnetic bead selection, total cellular DNA is prepared and the genomic copy number of AGT103 per cell is determined by quantitative PCR reaction. Efficacy-based release criteria using Assay 2 requires that more than or equal to 0.5 genomic copies per HIV-specific CD4 T cell be present in the AGT103 cell product.

Functional test of enrichment and transduction of HIV-specific CD4 T cells derived from PBMCs of HIV-positive patients who received therapeutic HIV vaccine. The effect of therapeutic vaccination on the frequency of HIV-specific CD4 T cells was tested in a peptide stimulation assay (Figure 14 panel B). The frequency of HIV-specific CD4 T cells before vaccination was 0.036% in this representative individual. The frequency of HIV-specific CD4 T cells after vaccination increased to approximately twice the value, 0.076%. Responsive cells (HIV specific) identified by accumulation of cytoplasmic interferon-gamma were detected only after specific peptide stimulation.

We also show that by peptide stimulation to enrich HIV-specific CD4 T cells, followed by transduction with AGT103, approximately 1% of all CD4 T cells in culture are HIV-specific. , And whether our goal of generating CD4 T cells transduced with AGT103 was achieved. In this case, we used an experimental version of AGT103 expressing green fluorescent protein (see GFP). In panel C of FIG. 14, post-vaccination cultures after peptide stimulation (HIV(GAG)Ultra) and AGT103 transduction, 1.11% of total CD4 T cells were HIV-specific (responsive to peptide stimulation). , Based on the expression of interferon-gamma), and that AGT103 was transduced (based on the expression of GFP).

Several patients in a therapeutic HIV vaccine study were tested to assess the extent of response to peptide stimulation and to establish eligibility criteria for inclusion in gene therapy populations in future human clinical trials. Panel D of FIG. 18 shows the frequency of HIV-specific CD4 T cells in four vaccine trial participants, comparing pre-vaccination and post-vaccination specimens. Importantly, in three cases, the post-vaccination specimens show HIV-specific CD4 T cell levels higher than or equal to 0.076% of total CD4 T cells. The ability to reach this value was not predicted by pre-vaccinated specimens. Because both patient 001-004 and patient 001-006 had a pre-

vaccination value of HIV-specific CD4 T cells of 0.02% at the beginning, but one patient eventually received 0.12%. This is because the post-vaccination value of HIV-specific CD4 T cells was reached, whereas the other individual was unable to increase this value after vaccination. The same three patients who responded well to the vaccine also showed a substantial enrichment of HIV-specific CD4 T cells after peptide stimulation and culture in that they increased the frequency of HIV-specific CD4 T cells. In the three cases shown in FIG. 18, panel E, 2.07%, 0.72%, or 1.54% of total CD4 T cells, respectively, were HIV-specific by peptide stimulation and subsequent culture. Was produced. These values enable our goal to make HIV-specific and AGT103-transduced CD4 T cells reach approximately 1% of total CD4 T cells in the final cell product. It is shown that most individuals who respond to the therapeutic HIV vaccine will have an ex vivo response to peptide stimulation large enough to do so.

As shown in FIG. 18, panel A illustrates the treatment schedule. Panel B demonstrates that PBMCs were stimulated with Gag peptide or DMSO control for 20 hours. IFN-gamma production was detected by FACS by intracellular staining. $CD4^+$ T cells were gated for analysis. Panel C demonstrates that $CD4^+$ T cells were expanded and transduced with AGT103-GFP using the method as shown in panel A. Expanded $CD4^+$ T cells were rested in fresh medium without any cytokines for 2 days and restimulated with Gag peptide or DMSO control for 20 hours. IFN-gamma production and GFP expression were detected by FACS. $CD4^+$ T cells were gated for analysis. Panel D demonstrates that the frequency of HIV-specific $CD4^+$ T cells (IFN-gamma positive, pre-vaccination and post-vaccination) was detected in 4 patients, as discussed herein. Panel E demonstrates that PBMCs were expanded after vaccination from 4 patients and tested for HIV-specific $CD4^+$ T cells.

(Example 15: Dose response)
Vector construction. A modified version of AGT103 was constructed to test the dose response at increasing AGT103 and its effect on cell surface CCR5 levels. AGT103 was modified to contain a green fluorescent protein (GFP) expression cassette under the control of the CMV promoter. Transduced cells express the miR30CCR5 miR21Vif miR185Tat microRNA cluster and emit green light due to GFP expression.

Functional assay for dose response and inhibition of CCR5 expression upon increasing AGT103-GFP. CEM-CCR5 T cells were transduced with AGT103-GFP using a multiplicity of infection of 0 to 5 per cell. Transduced cells were stained with a fluorescent conjugate (APC) monoclonal antibody specific for cell surface CCR5. The staining intensity is proportional to the number of CCR5 molecules per cell surface. The intensity of green fluorescence is proportional to the incorporated AGT103-GFP copy number per cell.

As shown in Figure 19, panel A demonstrates the dose response upon increasing AGT103-GFP and its effect on cell surface CCR5 expression. At a multiplicity of infection equal to 0.4, only 1.04% of the cells are green (indicating transduction) and show a significant reduction in CCR5 expression. At a multiplicity of infection equal to 1, the number of low CCR5, GFP+ cells increased to 68.1%, and at a multiplicity of infection equal to 5, the number of low CCR5, GFP+ cells increased to 95.7%. These data, presented in histogram form in FIG. 19 panel B, show that the population normally distributed in terms of CCR5 staining migrated towards lower mean fluorescence intensities with increasing AGT103-GFP dose. .. The potency of AGT103-GFP is presented in graphical form in Panel C of Figure 19, showing the percentage inhibition of CCR5 expression with increasing doses of AGT103-GFP. At a multiplicity of infection equal to 5, there was a greater than 99% reduction in CCR5 expression levels.

(Example 16: AGT103 efficiently transduces primary human $CD4^+$ T cells)
Transduction of primary AGT103 lentiviral vector into primary CD4 T cells. A modified AGT103 vector containing a green fluorescent protein marker (GFP) was used at a multiplicity of infection of 0.2-5 to transduce purified primary human CD4 T cells.

Functional assay of transduction efficiency of AGT103 into primary human CD4 T cells. CD4 T cells were isolated from human PBMCs (HIV negative donors) using magnetic bead labeled antibodies and standard procedures. Purified CD4 T cells were stimulated ex vivo with CD3/CD28 beads and cultured in medium containing interleukin-2 for 1 day before AGT103 transduction. The relationship between lentiviral vector dose (multiplicity of infection) and transduction efficiency is demonstrated in FIG. 20, panel A, with a multiplicity of infection equal to 0.2 transducing 9.27% AGT103. It has been shown that at multiplicity of infection equal to 5 resulting in CD4+ T cells, this value of AGT103 transduced CD4+ T cells increased to 63.1%. In addition to achieving efficient transduction of primary CD4-positive T cells, it is also necessary to quantify the genomic copy number per cell. In panel B of Figure 20, total cellular DNA from primary human CD4 T cells transduced at several multiplicities of infection was tested by quantitative PCR to determine the genomic copy number per cell. At a multiplicity of infection equal to 0.2, we measured 0.096 genomic copies per cell. This was in good agreement with 9.27% of GFP-positive CD4 T cells in panel A. A multiplicity of infection equal to 1 produced 0.691 genomic copies per cell, and a multiplicity of infection equal to 5 produced 1.245 genomic copies per cell.

As shown in FIG. 20, $CD4^+$ T cells isolated from PBMC were stimulated with CD3/CD28 beads and IL-2 for 1 day to transduce various concentrations of AGT103. After 2 days, the beads were removed and the $CD4^+$ T cells were collected. As shown in panel A, the frequency of transduced cells (GFP positive) was detected by FACS. Vector

copy number per cell was determined by qPCR, as shown in panel B. At a multiplicity of infection (MOI) of 5, 63% of $CD4^+$ T cells were transduced with an average of 1 vector copy per cell.

(Example 17: AGT103 inhibits HIV replication in primary $CD4^+$ T cells)
Protection of primary human CD4-positive T cells from HIV infection by transducing the cells with AGT103. Therapeutic lentivirus AGT103 was used at a multiplicity of infection of 0.2-5 per cell to transduce primary human CD4-positive T cells. The transduced cells were then challenged with the CXCR4 tropic HIV strain NL4.3, which does not require cell surface CCR5 for entry. In this assay, the potency of microRNAs against the HIV Vif and Tat genes is tested in terms of preventing proliferative infection in primary CD4+ T cells, but the amount of HIV released from infected primary human CD4 T cells. An indirect method of detecting is used.

Functional assay of AGT103 protection against CXCR4-directed HIV infection of primary human CD4-positive T cells. CD4 T cells were isolated from human PBMCs (HIV negative donors) using magnetic bead labeled antibodies and standard procedures. Purified CD4 T cells were stimulated ex vivo with CD3/CD28 beads and cultured for 1 day in medium containing interleukin-2 before transducing AGT103 using a multiplicity of infection of 0.2-5. Two days after transduction, CD4-positive T cell cultures were challenged with HIV strain NL4.3, which was engineered to express green fluorescent protein (GFP). Transduced HIV-exposed primary CD4 T cell cultures were maintained for 7 days before collecting HIV-free cell-free cultures. The cell-free medium was used to infect the highly permissive T cell line C8166 for 2 days. The percentage of C8166 cells infected with HIV was determined by flow cytometry detecting GFP fluorescence. In the case of mock lentivirus infection, a multiplicity of infection of 0.1 for NL4.3 HIV was released into the culture medium in an amount that made it possible to establish a productive infection with 15.4% C8166 T cells. Brought HIV. At a dose of AGT103 with a multiplicity of infection of 0.2, this value for HIV infection of C8166 cells was reduced to 5.3%, with AGT103 with a multiplicity of infection equal to 1, only 3.19% of C8166 T cells became HIV. I was not infected. C8166 infection was further reduced to 0.62% after AGT103 transduction using a multiplicity of infection equal to 5. There is a clear dose-response relationship between the amount of AGT103 used for transduction and the amount of HIV released into the culture medium.

As shown in FIG. 21, $CD4^+$ T cells isolated from PBMC were stimulated with CD3/CD28 beads and IL-2 for 1 day to transduce various concentrations (MOI) of AGT103. Two days later, the beads were removed and $CD4^+$ T cells were infected with 0.1 MOI of HIV NL4.3-GFP. After 24 hours, cells were washed 3 times with PBS and cultured with IL-2 (30 U/ml) for 7 days. At the end of the culture, the supernatant was collected and infected with the HIV-permissive cell line C8166 for 2 days. HIV-infected C8166 cells (GFP positive) were detected by FACS. Viable HIV decreased with increasing multiplicity of infection of AGT103 (MOI 0.2=65.6%, MOI 1=79.3%, and as observed with less infection of C8166 cells). MOI 5=96%).

(Example 18: AGT103 protects primary human $CD4^+$ T cells from HIV-induced depletion)
AGT103 transduction of primary human CD4 T cells to protect against HIV-mediated cytopathology and cell depletion. PBMCs were obtained from healthy HIV negative donors, stimulated with CD3/CD28 beads and then cultured in medium containing interleukin-2 for 1 day before using a multiplicity of infection of 0.2-5. AGT103 was transduced.

Functional assay of AGT103 protection of primary human CD4 T cells from HIV-mediated cytopathology. Primary human CD4 T cells transduced with AGT103 were infected with HIV NL4.3 strain (CXCR4 tropic) that does not require CCR5 for cell entry. When using CXCR4 tropic NL4.3, only the effect of Vif and Tat microRNAs on HIV replication is tested. The HIV NL4.3 dose had a multiplicity of infection of 0.1. One day after HIV infection, cells were washed to remove residual virus and cultured in medium and interleukin-2. Cells were harvested every 3 days during 14 days of culture and then stained with a monoclonal antibody that is specific for CD4 and is directly conjugated to a fluorescent marker to determine the percentage of CD4+ T cells in PBMC. Made possible Untreated CD4 T cells, or CD4 T cells transduced with a control lentiviral vector, are highly susceptible to HIV challenge, and the percentage of CD4 positive T cells in PBMC is 10% by day 14 of culture. Fell below. In contrast, AGT103 showed a dose-dependent effect on preventing cell depletion by HIV challenge. At a multiplicity of infection AGT103 dose of 0.2, more than 20% of PBMC were CD4 T cells by day 14 of culture, and at a multiplicity of infection AGT103 dose equal to 5, by day 14 of culture. The PBMC that became CD4-positive T cells increased to a value higher than 50%. Again, AGT103 showed a clear dose response effect on HIV cell pathogenicity of human PBMC.

As shown in FIG. 22, PBMCs were stimulated with CD3/CD28 beads and IL-2 for 1 day to transduce various concentrations (MOI) of AGT103. Two days later, the beads were removed and the cells were infected with 0.1 MOI of HIV NL4.3. After 24 hours, cells were washed 3 times with PBS and incubated with IL-2 (30 U/ml). Cells were collected every 3 days and the frequency of $CD4^+$ T cells was analyzed by FACS. After 14 days of exposure to HIV, LV-control transduced $CD4^+$ T cells were reduced by 87%, AGT103 MOI0.2 was reduced by 60%, AGT103 MOI1 was reduced by 37%, and AGT103 MOI5 was reduced by 17%. It was

Example 19: Generation of a population of CD4+ T cells enriched for HIV specificity and transduced with AGT103/CMV-GFP.
Therapeutic vaccination against HIV had minimal effects on the distribution of CD4+, CD8+, and CD4+/CD8+ T cells. As shown in FIG. 23A, the CD4 T cell population is shown in the upper left quadrant of the analytical flow cytometry dot plot and varies from 52% to 57% of total T cells after the vaccination series. These are representative data.

Peripheral blood mononuclear cells from participants in the HIV therapeutic vaccine trial were cultured for 12 days in medium +/-interleukin-2/interleukin-12 or +/-interleukin-7/interleukin-15. Several cultures were stimulated with overlapping peptides representing the entire p55 Gag protein of HIV-1 (HIV(GAG) Ultra peptide mixture) as a source of epitope peptides for T cell stimulation. These peptides are 10-20 amino acids in length, overlapping 20-50% of their length and represent the entire Gag precursor protein (p55) from the HIV-1 BaL strain. The composition and sequence of individual peptides can be adjusted to compensate for regional variations in the major circulating HIV sequences, or when detailed sequence information is available to individual patients undergoing this therapy. it can. At the end of culture, cells were harvested, stained with anti-CD4 or anti-CD8 monoclonal antibodies, gated on the CD3+ population and displayed here. HIV (GAG) Ultra peptide mixture stimulation for samples either pre-vaccination or post-vaccination was similar to the media control, which indicates that the HIV (GAG) Ultra peptide mixture was not toxic to cells and It shows that it did not act as a mitogen. The results of this analysis can be found in Figure 23B.

HIV(GAG) Ultra peptide mixture and interleukin-2/interleukin-12 were provided for optimal expansion of antigen-specific CD4 T cells. As shown in the upper panel of FIG. 23C, there was an increase in cytokine (interferon-gamma) secreting cells in post-vaccination specimens exposed to the HIV(GAG) Ultra peptide mixture. In pre-vaccinated samples, cytokine-secreting cells increased from 0.43 to 0.69% as a result of exposure to antigenic peptides. In contrast, post-vaccination samples showed an increase in cytokine secreting cells from 0.62 to 1.76% of total CD4 T cells as a result of peptide stimulation. These data demonstrate the strong impact of vaccination on CD4 T cell responses to HIV antigens.

Finally, AGT103/CMV-GFP transduction of antigen-expanded CD4 T cells produces HIV-specific and HIV-resistant helper CD4 T cells required for infusion into patients as part of a functional cure for HIV. (According to various other aspects and embodiments, AGT103 is used alone, eg, clinical embodiments may not include a CMV-GFP segment). The upper panel of FIG. 23C shows the results of analyzing the CD4+ T cell population in culture. The x-axis in FIG. 23C shows green fluorescent protein (GFP) release, indicating that individual cells were transduced with AGT103/CMV-GFP. In post-vaccination samples, 1.11% of the total CD4 T cells that secrete cytokines were recovered, which meant that the cells responded specifically to the HIV antigen and to these cells AGT103/CMV-GFP. Are transduced. It is a target cell population and clinical product intended for HIV infusion and functional cure. With the efficiency of cell proliferation during antigen stimulation of ex vivo culture and subsequent polyclonal growth phase, it is possible to produce 4×10^{8} antigen-specific, lentivirus-transduced CD4 T cells. It exceeds the target of cell production four-fold and achieves a number of antigen-specific and HIV-resistant CD4 T cells of approximately 40 cells/microliter of blood or approximately 5.7% of total circulating CD4 T cells. Could be

Table 4 below shows the results of ex vivo production of HIV specific and HIV resistant CD4 T cells using the disclosed vectors and methods.

(Example 20)

Clinical study of treatment of HIV positive subjects without immunization AGT103T is a genetically modified autologous PBMC containing $\geqq5\times10^{7}$ HIV-specific CD4 T cells that have been further transduced with AGT103 lentiviral vector. ..

Phase I clinical trial confirmed HIV infection, adult study participation with CD4+ T cell counts >600 cells/mm^{3} blood and stable viral suppression of <200 copies/ml plasma while undergoing cART. The safety and feasibility of ex vivo modified autologous CD4 T cell (AGT103T) injection into individuals will be tested. All study participants will continue to receive standard antiretroviral drug therapy throughout Phase I clinical trials. Study participants will be screened by submitting blood for in vitro studies to determine the frequency of CD4+ T cells in response to stimulation by pools of overlapping synthetic peptides representing the HIV-1 Gag polyprotein. Subjects with ≧0.065% of all CD4 T cells designated as Gag-specific CD4 T cells will be enrolled in a gene therapy study and undergo leukocyte apheresis, followed by purification of PBMC (by Ficoll density gradient centrifugation or antibody). (Using negative selection), PBMCs were cultured ex vivo, stimulated with HIV Gag peptide and interleukin-2 and interleukin-12 for 12 days, then modified with CD3/CD28 bispecific antibody. It is stimulated again by the beads. The antiretroviral drug saquinavir was included at 100 nM to prevent the emergence of autologous HIV during ex vivo culture. One day after CD3/CD28 stimulation, cells are transduced with AGT103 at a multiplicity of infection of 1-10. The transduced cells are cultured for an additional 7-14 days, during which time the transduced cells grow by polyclonal expansion. The culture period is terminated by harvesting, the cells are washed, sorted for potency and release safety assays, and the remaining cells are resuspended in cryopreservation medium. A single dose is $\leqq1\times10^{10}$ autologous PBMCs. In potency assays, the frequency of CD4 T cells in response to peptide stimulation is measured by interferon-gamma expression. Other release criteria include that the product must contain $\geqq0.5\times10^{7}$ HIV-specific CD4 T cells that were further transduced with

AGT103. Another release criterion is that the AGT103 genome copy number per cell should not exceed 3. Five days prior to infusion of AGT103T, subjects received a dose of busulfuram (or cytoxan or fludarabine or an appropriate drug combination) conditioning regimen, followed by ≦$1×10^{10}$ cells containing genetically modified CD4 T cells. PBMC are injected.

A Phase II study will assess the efficacy of AGT103 T cell therapy. Participants in Phase II studies were successfully enrolled in our Phase I study previously, with successful stable engraftment of genetically modified autologous HIV-specific CD4 T cells, and efficacy evaluation (1.3). Individuals determined to have a clinical response defined as a positive change in the monitored parameter as described in. Study participants will be required to add maraviroc to their existing regimens of antiretroviral drug therapy. Maraviroc is a CCR5 antagonist that enhances the efficacy of gene therapy aimed at reducing CCR5 levels. Upon completion of maraviro-crezimen, subjects were discontinued from the previous antiretroviral drug regimen and treated with maraviroc monotherapy alone for 28 days or with two consecutive weekly blood samplings of plasma viral RNA levels per ml. It will be required to maintain until over 10,000. In the case of persistently high viremia, participants should return to their original antiretroviral drug regimen with or without maraviroc, as recommended by their HIV clinician.

If a participant's HIV remained suppressed (<2,000 vRNA copies per ml plasma) with maraviroc monotherapy for >28 days, participants were gradually reduced their maraviroc dose over a 4-week period. Then, intensive monitoring will be required for another 28 days. Subjects who maintain HIV suppression with maraviroc monotherapy are considered to have a functional cure. Subjects who maintain HIV suppression after Maraviroc withdrawal are also considered to have functional cures. Monthly monitoring for 6 months followed by less intensive monitoring will establish the persistence of functional healing.

1.1 Patient Selection Selection Criteria:
-Age of 18 to 60 years.
-The HIV infection is known before the study registration.
They must be willing to respond to the assessments required by the study, including not changing their antiretroviral regimens (unless medically indicated) during the study period.

· CD4 + T cell count per cubic millimeter> 600 (cells / $mm^{3)}$ It · CD4 + T cells nadir is found> 400 It · HIV viral load is a cell / mm^3 is, 1 ml (mL >) per 1,000> Exclusion criteria:
• Any viral hepatitis • Acute HIV infection • HIV viral load is >1,000,000 copies/mL • Active or recent (past 6 months) AIDS-defining complications • Entering study Any change in HIV drug therapy within 12 weeks • Cancer or malignancy that has not been in remission for at least 5 years, except successfully treated basal cell carcinoma of the skin • NYHA Grade 3 or 4 congestive heart failure or poor management Current diagnosis of angina or arrhythmia ・History of bleeding disorder ・Use of chronic steroids for the past 30 days ・Pregnant women or breastfeeding ・Active drug or alcohol abuse ・Severe illness for the past 30 days ・Currently in another clinical trial Participating or any previous gene therapy

1.2 Safety evaluation/acute infusion reaction/safety follow-up survey after infusion

1.3 Efficacy Evaluation-Phase I-Number and frequency of modified CD4 T cells.
-Persistence of modified CD4 T cells.
-In vitro response to Gag peptide restimulation as a measure of memory T cell function (ICS assay).
-Multifunctional anti-HIV CD8 T cell response compared to pre-vaccination and post-vaccination time points.
-Frequency of CD4 T cells that produce double-spliced HIV mRNA after in vitro stimulation.

1.4 Efficacy evaluation-Phase II-Number and frequency of genetically modified CD4 T cells.
-Maintaining viral suppression by maraviroc monotherapy (<2,000 vRNA copies per ml but 2 consecutive weekly blood draws not exceeding 5×10^4 vRNA copies per ml are allowed). ..
Viral suppression during and after the Maraviroc washout.
-Stable CD4 T cell count.

(Example 21)
Generating a population of CD4+ T cells through depletion of CD8+ T cells prior to peptide stimulation CD8+ T cell overgrowth significantly affected the proliferation of target CD4+ T cells, so that CD8+ T cell It was depleted at the beginning of proliferation to determine if it improved CD4+ T cell proliferation. Current CD8+ T cell depletion

methods require cells to pass through a magnetic column. To avoid possible effects of the procedure on antigen presenting cells and CD4+ T cells, cell depletion was performed after peptide stimulation and before lentiviral transduction, where cells were better able to withstand mechanical stress. .

More specifically, HIV-positive human peripheral blood was obtained. PBMCs were separated using Ficoll-Paque PLUS (GE Healthcare, Catalog No. 17-1440-02). Freshly isolated PBMCs (1×10^{7}) were added to PepMix™ HIV(GAG) Ultra (catalog number PM-HIV-GAG, JPT Peptide Technologies, Berlin, Germany) in 1 mL medium in 24-well plates. I was stimulated for 18 hours. CD8+ T cells were depleted with PE anti-human CD8 antibody and anti-PE microbeads. Negatively selected cells were selected from IL-7 (170-076-111, Miltenyi Biotech, Bergisch Gladbach, Germany), IL-15 (170-076-114, Miltenyi Biotech, Bergisch Gladbach, Germany catalog number and 46). , NIH AIDS Reagent Program, German, MD) in TexMACS GMP medium (catalog number 170-076-309, Miltenyi Biotech, Bergisch Gladbach, Germany) at 2×10^{6} /mL. Lentivirus AGT103 was added at MOI 5 after 24 hours. Fresh medium containing IL-7, IL-15 and saquinavir was added every 2-3 days during growth. The final concentration of IL-7/IL-15 was 10 ng/mL. The final concentration of saquinavir was 100 nM. On days 12-16, $2\text{-}3 \times 10^{6}$ cells were collected for peptide restimulation and intracellular cytokine staining (ICS) analysis. A schematic diagram of this depletion protocol is shown in FIG.

HIV-specific CD4 T cell proliferation was significantly improved when CD8+ T cells were depleted (FIGS. 25A-C). However, overgrowth with Vδ1 T cells (PTID 01-006) (FIG. 25A) and NK cells (PTID 01-008) (FIG. 25C) was observed.

Referring to FIG. 25A, on day 0, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 44.5%, 55.5%, 0. It had a fluorescence intensity of 032%, and 0%. On day 0, GagPepMix's lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 44.2%, 55.3%, 0.48%, and 0. It had a fluorescence intensity of 053%. On day 12, without CD8 depletion, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 79.8%, 20.1%, 0. It had a fluorescence intensity of 12% and 0.018%. On day 12, without CD8 depletion, GagPepMix left lower quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 58.9%, 19.2%, and 21. It had a fluorescence intensity of 2% and 0.69%. On day 12, with CD8 depletion, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 64.4%, 35.0%, 0. It had a fluorescence intensity of 44% and 0.14%. On day 12, with CD8 depletion, GAGPepMix's lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 61.9%, 32.9%, and 3. It had a fluorescence intensity of 47% and 1.70%.

On day 12, gating data with CD8 depletion was also generated using CD4 and CD8 as variables. Lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant are 45.5%/45.3%, 44.9%, 9.26%, and 0.35%, respectively. It had a fluorescence intensity of. In addition, gating data was generated using Vδ1 and Vδ2 as variables. The lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant have fluorescence intensities of 16.9%, 82.8%, 0.14%, and 0.12%, respectively. did.

Referring to FIG. 25B, on day 0, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 33.6%, 66.4%, and 5. It had a fluorescence intensity of 9E-4% and 1.78E-3. On day 0, GagPepMix's lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 33.7%, 66.3%, 0.011%, and 0. It had a fluorescence intensity of 016%. On day 16, without CD8 depletion, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 78.4%, 21.2%, 0. It had a fluorescence intensity of 30% and 0.018%. On day 16, in the absence of CD8 depletion, GagPepMix's lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 76.3%, 20.2%, and 2. It had a fluorescence intensity of 95% and 0.61%. On day 16, with CD8 depletion, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 50.9%, 48.7%, 0. It had a fluorescence intensity of 36%, and 0.10%. On day 16, with CD8 depletion, GagPepMix's lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 51.6%, 44.4%, 0. It had a fluorescence intensity of 43% and 3.60%.

Referring to FIG. 25C, on day 0, the control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 65.4%, 34.5%, 0. It had a fluorescence intensity of 096%, and 7.71E-4. On day 0, the lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant of GagPepMix were 65.4%, 34.3%, 0.20%, and 0. It had a fluorescence intensity of 10%. On day 16, without CD8 depletion, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 87.9%, 12.1%, 0. It had a fluorescence intensity of 028%, and 6.24E-3%. On day 16, without CD8 depletion, lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant of GagPepMix were 82.3%, 12.1%, and 5. It had a fluorescence intensity of 38% and 0.23%. On day 16, with CD8 depletion, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 87.8%, 12.0%, 0. It had a fluorescence intensity of 22% and 0.013%. On day 16, with CD8 depletion, GagPepMix left lower quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 87.8%, 11.1%, 0. It had a fluorescence intensity of 30% and 0.78%.

On day 16, gating data with CD8 depletion was also generated using the variables CD3 and CD4, showing a fluorescence intensity of 83.1% in the indicated area. In addition, gating data was generated using the variables CD56 and CD4 and showed a fluorescence intensity of 65.7% in the indicated area.

(Example 22)
Generating a population of CD4+ T cells via depletion of CD8+, γδ, NK, and B cells prior to peptide stimulation When deficient in CD8+ T cells, γδ or NK cell overgrowth is observed in multiple patients Was done. Therefore, we depleted CD8, γδ, NK or B cells and tested whether it improved CD4+ T cell proliferation. Cell depletion was performed after peptide stimulation and before lentiviral transduction.

HIV positive human peripheral blood was obtained. PBMCs were separated using Ficoll-Paque PLUS (GE Healthcare, Catalog No. 17-1440-02). Freshly isolated PBMCs (1×10^{7}) were added to PepMix™ HIV(GAG) Ultra (catalog number PM-HIV-GAG, JPT Peptide Technologies, Berlin, Germany) in 1 mL medium in 24-well plates. I was stimulated for 18 hours. CD8+ T, γδ, NK, or B cells were depleted with PE-labeled specific antibody and anti-PE microbeads. Negatively selected cells were selected from IL-7 (170-076-111, Miltenyi Biotech, Bergisch Gladbach, Germany), IL-15 (170-076-114, Miltenyi Biotech, Bergisch Gladbach, Germany catalog number and 46). , NIH AIDS Reagent Program, German, MD) in TexMACS GMP medium (catalog number 170-076-309, Miltenyi Biotech, Bergisch Gladbach, Germany) at 2×10^{6} /mL. Lentivirus AGT103 was added at MOI 5 after 24 hours. Fresh medium containing IL-7, IL-15 and saquinavir was added every 2-3 days during growth. The final concentration of IL-7/IL-15 was 10 ng/mL. On days 12-16, $2\text{-}3\times10^{6}$ cells were collected for peptide restimulation and intracellular cytokine staining (ICS) analysis. A schematic of this depletion protocol is shown in FIG.

When depleted of additional cell subsets, HIV Gag-specific CD4 T cells expanded to higher levels (Fig. 27A-B). Overgrowth of CD8, γδ, or NK cells appears to inhibit CD4 T cell proliferation or kill lentivirus transducing antigen-specific CD4 T cells. This optimized protocol is suitable for scale-up and cell production.

Referring to FIG. 27A, on day 0, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 56.4%, 43.5%, 0. It had a fluorescence intensity of 034% and 7.44E-4%. On day 0, the GagPepMix left lower quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 54.8%, 44.8%, 0.30%, and 0. It had a fluorescence intensity of 055%. Without depletion after 18 hours, the control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 83.9%, 16.0%, and 0.061%, respectively. , And a fluorescence intensity of 0.027%. Without depletion after 18 hours, the lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant of GagPepMix were 77.6%, 15.4%, and 6.39%, respectively. , And 0.54% fluorescence intensity. In the case of depletion after 18 hours, the control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 41.9%, 57.9%, and 0.094%, respectively. , And 0.099% of fluorescence intensity. In the case of depletion after 18 hours, the lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant of GagPepMix are 43.3%, 50.7%, and 3.00%, respectively. , And 2.98% fluorescence intensity. With CD8 and γδ depletion after 18 hours, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 40.4%, 59.3%, 0, respectively. It had fluorescence intensities of 0.12% and 0.13%. With CD8 and γδ depletion 18 hours later, the lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant of GagPepMix were 38.3%, 54.7%, 3 respectively. It had fluorescence intensities of 0.14% and 3.86%. With 18 hours of CD8, γδ, and B depletion, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 46.2% and 53.6, respectively. %, 0.13%, and 0.080%. With CD8, γδ, and B depletion after 18 hours, the lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant of GagPepMix were 42.1% and 48.5, respectively. %, 4.28%, and 5.06%.

Referring to FIG. 27B, on day 0, control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 42.6%, 57.4%, and 2. 71E-3%, and 0.0% fluorescence intensity. On day 0, GagPepMix's lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 42.5%, 57.4%, 0.031%, and 0. It had a fluorescence intensity of 048%. Without depletion after 18 hours, the control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 79.5%, 20.5%, and 0.017%, respectively. , And a fluorescence intensity of 9.73E-3%. Without depletion after 18 hours, the lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant of GagPepMix were 78.9%, 19.5%, and 0.93%, respectively. , And a fluorescence intensity of 0.65%. In the case of depletion after 18 hours, the control lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant were 51.4%, 48.4%, and 0.11%, respectively. , And a fluorescence intensity of 0.063%. In the case of depletion after 18 hours, the lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant of GagPepMix were 51.7%, 43.0%, and 0.22%, respectively. , And a fluorescence intensity of 5.03%. With CD8, CD56, γδ, and B depletion after 18 hours, the lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant of unstimulated cells were 12.8 respectively. %, 87.0%, 0.14%, and 0.10%. With CD8, CD56, γδ, and B depletion after 18 hours, GagPepMix had lower left quadrant, lower right quadrant, upper left quadrant, and upper right quadrant respectively 13.2%, 79%. It had fluorescence intensities of 0.4%, 0.27%, and 7.17%.

(Example 23)
Method for measuring the transduction efficiency of AGT103 lentivirus In order to improve the proliferation of CD4+ T cells, the target cells are lentivirus AGT103 transducing antigen-specific CD4+ T cells. Transduction efficiency was measured using a lentivirus with GFP. Since intracellular staining causes a marked loss of GFP signal, CCS was used to identify antigen-specific CD4+ T cells and GFP positive cells to identify transduced cell subsets.

1×10^{7} PBMCs from HIV positive patients were placed in PepMix™ HIV(GAG) Ultra (catalog number PM-HIV-GAG, JPT Peptide Technologies, Berlin, Germany) in 1 mL medium in 24-well plates. I was stimulated for 18 hours. CD8, γδ, NK or B cells were depleted with PE-labeled specific antibody and anti-PE microbeads. Negatively selected cells were selected from IL-7 (170-076-111, Miltenyi Biotech, Bergisch Gladbach, Germany), IL-15 (170-076-114, Miltenyi Biotech, Bergisch Gladbach, Germany catalog number and 46). , NIH AIDS Reagent Program, German, MD) in TexMACS GMP medium (catalog number 170-076-309, Miltenyi Biotech, Bergisch Gladbach, Germany) at 2×10^{6} /mL. Lentivirus with GFP was added at MOI 5 after 24 hours. Fresh medium containing IL-7, IL-15 and saquinavir was added every 3 days during growth. The final concentration of IL-7/IL-15 was 10 ng/mL. On day 12-16, $2\text{-}3 \times 10^{6}$ cells were collected. Peptide restimulation and CCS assays were performed to assess IFN-γ positive antigen-specific CD4+ T cells and GFP signaling was used to assess transduction efficiency. All experiments were performed according to the manufacturer's instructions.

IFN-γ positive antigen-specific CD4+ T cells showed significantly better transduction efficiency compared to other cell subsets in culture (Figure 28). This is reasonable given that antigen-specific CD4+ T cells were TCR stimulated, proliferated faster and were more easily infected by the lentivirus. As shown in FIG. 28, the lower right quadrant (68.6% fluorescence) and the upper right quadrant (12.6% fluorescence) had 41.5% and 67.8% GFP, respectively. It had transduction efficiency. This is in contrast to the lower left quadrant (9.75% fluorescence) and the upper left quadrant (2.46% fluorescence), which had GFP transduction efficiencies of 35.6% and 43.3%, respectively. is there.

(Example 24)
Method for Determining the Relationship between Percentage of Transduced Cells and Vector Copy Number Since the target cells are AGT103 lentivirus transduced HIV-specific CD4 T cells, how many target cells are included in the final cell product It is important to know if it will be done. However, there are no detectable markers in the clinical grade AGT103 lentivirus. As a result, transduction efficiency was measured by detecting the vector copy number (VCN) by qPCR. Lentiviruses with GFP can be used to establish the percentage of transduced cells based on VCN in the final cell product by establishing the relationship between the percentage of transduced cells and VCN. it can.

1×10^{7} PBMCs from HIV positive patients were placed in PepMix™ HIV(GAG) Ultra (catalog number PM-HIV-GAG, JPT Peptide Technologies, Berlin, Germany) in 1 mL medium in 24-well plates. I was stimulated for 18 hours. CD8, γδ, NK or B cells were depleted with PE-labeled specific antibody and anti-PE microbeads. Negatively selected cells were selected from IL-7 (170-076-111, Miltenyi Biotech, Bergisch Gladbach, Germany), IL-15 (170-076-114, Miltenyi Biotech, Bergisch Gladbach, Germany catalog number and 46). , NIH AIDS Reagent Program, German, MD) in TexMACS GMP medium (catalog number 170-076-309, Miltenyi Biotech, Bergisch Gladbach, Germany) at 2×10^{6} /mL. Lentivirus with GFP was added at MOI 5 after 24 hours. Fresh medium containing IL-7, IL-15 and saquinavir was added every 3 days during growth. The final concentration of IL-7/IL-15 was 10 ng/mL. The final concentration of saquinavir was 100 nM. On day 12-16, $2\text{-}3 \times 10^{6}$ cells were collected. Peptide restimulation and CCS assays were performed to assess antigen-specific CD4+ T cells and GFP signaling was used to assess transduction efficiency. QPCR was performed to detect vector copy number. All experiments were performed according to the manufacturer's instructions.

After testing four samples, a positive correlation was observed between the percentage of transduced cells and vector copy number (Figure 29).

Sequences The following sequences are cited herein:

Although some preferred embodiments of the present invention have been described and specifically exemplified above, the present invention is not intended to be limited to such embodiments. Various modifications may be made therein without departing from the scope and spirit of the invention.

Patent Citations (11)

Publication number	Priority date	Publication date	Assignee	Title

WO2010127166A2 *	2009-04-30	2010-11-04	The Regents Of The University Of California	Combination anti-hiv vectors, targeting vectors, and methods of use
JP2012533299A *	2009-07-15	2012-12-27	カリミューン, インコーポレーティッド	Dual vector for human immunodeficiency virus inhibition
WO2016061232A2 *	2014-10-14	2016-04-21	Texas Tech University System	Multiplexed shrnas and uses thereof
Family To Family Citations				
WO2005028634A2 *	2003-09-18	2005-03-31	Emory University	Improved mva vaccines
CN101160055A *	2005-02-16	2008-04-09	莱蒂恩公司	Lentiviral vectors and their use
WO2010051521A1 *	2008-10-31	2010-05-06	Lentigen Corporation	Cell therapy product for the treatment of hiv infection
EP2191834A1 *	2008-11-26	2010-06-02	Centre National De La Recherche Scientifique (Cnrs)	Compositions and methods for treating retrovirus infections
CN102782136A *	2010-02-18	2012-11-14	爱默蕾大学	Vectors expressing HIV antigens and GM-CSF and related methods for generating an immune response
WO2017007994A1 *	2015-07-08	2017-01-12	American Gene Technologies International Inc.	Hiv pre-immunization and immunotherapy
EP3413926A4 *	2016-02-08	2019-10-09	American Gene Technologies International, Inc.	Hiv vaccination and immunotherapy
AU2017292582C1 *	2016-07-08	2021-11-11	American Gene Technologies International Inc.	HIV pre-immunization and immunotherapy

* Cited by examiner, † Cited by third party

Non-Patent Citations (4)

Title
AIDS RES HUM RETROVIRUSES, vol. 16, no. 3, JPN7021000067, 2000, pages 259 - 271, ISSN: 0004970585 *
CLINICAL AND VACCINE IMMUNOLOGY, vol. 14, no. 9, JPN6021000472, 2007, pages 1196 - 1202, ISSN: 0004970586 *
MOLECULAR THERAPY, vol. 23, JPN6021046908, 2015, pages 310 - 320, ISSN: 0004970584 *
THE JOURNAL OF INFECTIOUS DISEASES, vol. 210, JPN6021000471, 2014, pages 99 - 110, ISSN: 0004970587 *

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Publication number	Priority date	Publication date	Assignee	Title
Family To Family Citations				
WO2010045659A1	2008-10-17	2010-04-22	American Gene Technologies International Inc.	Safe lentiviral vectors for targeted delivery of multiple therapeutic molecules
US10137144B2	2016-01-15	2018-11-27	American Gene Technologies International Inc.	Methods and compositions for the activation of gamma-delta T-cells
EP4310500A3	2016-01-15	2024-04-03	American Gene Technologies International Inc.	Methods and compositons for the activation of gamma-delta t-cells
EP3413926A4	2016-02-08	2019-10-09	American Gene Technologies International, Inc.	Hiv vaccination and immunotherapy
WO2017156311A2	2016-03-09	2017-09-14	American Gene Technologies International Inc.	Combination vectors and methods for treating cancer
AU2017292582C1	2016-07-08	2021-11-11	American Gene Technologies International Inc.	HIV pre-immunization and immunotherapy
EP3487507A4	2016-07-21	2020-04-08	American Gene Technologies International, Inc.	Viral vectors for treating parkinson's disease
US11820999B2	2017-04-03	2023-11-21	American Gene Technologies International Inc.	Compositions and methods for treating phenylketonuria

* Cited by examiner, † Cited by third party, ‡ Family to family citation

Similar Documents

Publication	Publication Date	Title
JP7162895B2	2022-10-31	HIV pre-immunization and immunotherapy
JP7153332B2	2022-10-14	HIV vaccination and immunotherapy
JP7260170B2	2023-04-18	HIV immunotherapy without prior immunization step
JP2021138721A	2021-09-16	Hiv pre-immunization and immunotherapy
JP2021519069A	2021-08-10	Method for producing genetically modified lymphocytes

Priority And Related Applications

Child Applications (1)

Application	Priority date	Filing date	Relation	Title
JP2022189475A	2017-01-09	2022-11-28	Division	Hiv immunotherapy without advance immunization step

Priority Applications (1)

Application	Priority date	Filing date	Title
JP2022189475A	2017-01-09	2022-11-28	Hiv immunotherapy without advance immunization step

Applications Claiming Priority (3)

Application	Filing date	Title
US201762444147P	2017-01-09	
US62/444,147	2017-01-09	
PCT/US2018/012998	2018-01-09	Hiv immunotherapy with no pre-immunization step

Legal Events

Date	Code	Title	Description
2020-05-20	A521	Request for written amendment filed	**Free format text**: JAPANESE INTERMEDIATE CODE: A523 **Effective date**: 20200520
2020-12-17	A621	Written request for application examination	**Free format text**: JAPANESE INTERMEDIATE CODE: A621 **Effective date**: 20201217
2021-08-30	A521	Request for written amendment filed	**Free format text**: JAPANESE INTERMEDIATE CODE: A523 **Effective date**: 20210830
2021-11-26	A131	Notification of reasons for refusal	**Free format text**: JAPANESE INTERMEDIATE CODE: A131 **Effective date**: 20211126
2022-02-28	A601	Written request for extension of time	**Free format text**: JAPANESE INTERMEDIATE CODE: A601 **Effective date**: 20220228

2022-04-26	A521	Request for written amendment filed	**Free format text**: JAPANESE INTERMEDIATE CODE: A523 **Effective date**: 20220426
2022-07-28	A02	Decision of refusal	**Free format text**: JAPANESE INTERMEDIATE CODE: A02 **Effective date**: 20220728
2022-11-28	A521	Request for written amendment filed	**Free format text**: JAPANESE INTERMEDIATE CODE: A523 **Effective date**: 20221128
2022-11-28	C60	Trial request (containing other claim documents, opposition documents)	**Free format text**: JAPANESE INTERMEDIATE CODE: C60 **Effective date**: 20221128
2022-12-06	C11	Written invitation by the commissioner to file amendments	**Free format text**: JAPANESE INTERMEDIATE CODE: C11 **Effective date**: 20221206
2022-12-27	A521	Request for written amendment filed	**Free format text**: JAPANESE INTERMEDIATE CODE: A821 **Effective date**: 20221209
2023-01-04	A911	Transfer to examiner for re-examination before appeal (zenchi)	**Free format text**: JAPANESE INTERMEDIATE CODE: A911 **Effective date**: 20221228
2023-01-04	C21	Notice of transfer of a case for reconsideration by examiners before appeal proceedings	**Free format text**: JAPANESE INTERMEDIATE CODE: C21 **Effective date**: 20230104
2023-01-24	A131	Notification of reasons for refusal	**Free format text**: JAPANESE INTERMEDIATE CODE: A131 **Effective date**: 20230124
2023-02-16	A521	Request for written amendment filed	**Free format text**: JAPANESE INTERMEDIATE CODE: A523 **Effective date**: 20230216
2023-03-01	TRDD	Decision of grant or rejection written	
2023-03-03	A01	Written decision to grant a patent or to grant a registration (utility model)	**Free format text**: JAPANESE INTERMEDIATE CODE: A01 **Effective date**: 20230303
2023-04-03	A61	First payment of annual fees (during grant procedure)	**Free format text**: JAPANESE INTERMEDIATE CODE: A61

			Effective date: 20230330
2023-04-10	R150	Certificate of patent or registration of utility model	**Ref document number**: 7260170 **Country of ref document**: JP **Free format text**: JAPANESE INTERMEDIATE CODE: R150

Concepts

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Name	Image	Sections	Count	Query match
immunization		title,abstract,description	31	0.000
immunization		title,abstract,description	30	0.000
immunotherapy		title,abstract,description	14	0.000
vector		claims,abstract,description	267	0.000
method		claims,abstract,description	169	0.000
peripheral blood mononuclear cell		claims,abstract,description	167	0.000
genetic effect		claims,abstract,description	92	0.000
virological effect		claims,abstract,description	61	0.000
stimulating effect		claims,abstract,description	29	0.000
HIV Infections		claims,abstract,description	27	0.000
chemical substances by application		claims,abstract,description	27	0.000
HIV infectious disease		claims,abstract,description	25	0.000
human immunodeficiency virus infectious disease		claims,abstract,description	25	0.000
leukocyte		claims,abstract,description	25	0.000
treatment		claims,abstract,description	25	0.000
transducing effect		claims,abstract,description	14	0.000
culturing		claims,abstract,description	13	0.000
cell		claims,description	472	0.000

T-lymphocyte	claims,description	287	0.000
processed proteins & peptides	claims,description	115	0.000
plasmid	claims,description	112	0.000
MicroRNAs	claims,description	91	0.000
microRNA	claims,description	82	0.000
small Interfering RNA	claims,description	74	0.000
particle	claims,description	66	0.000
infectious disease	claims,description	64	0.000
(ribonucleotides)n+m	claims,description	61	0.000
Small hairpin RNA	claims,description	61	0.000
Bacterial small RNA	claims,description	56	0.000
therapeutic effect	claims,description	54	0.000
natural killer cell	claims,description	53	0.000
b-lymphocyte	claims,description	49	0.000
vaccine	claims,description	49	0.000
manufacturing process	claims,description	46	0.000
Nucleic acid sequence	claims,description	45	0.000
antigen	claims,description	42	0.000
antigens	claims,description	42	0.000
antigens	claims,description	42	0.000
HIV vaccine	claims,description	40	0.000
Virion infectivity factor	claims,description	40	0.000
packaging method and process	claims,description	37	0.000
inhibitory effect	claims,description	35	0.000
regulatory T cell	claims,description	35	0.000

CD8-positive T-lymphocyte	claims,description	34	0.000
natural killer T-cell	claims,description	31	0.000
depleting effect	claims,description	27	0.000
CCR5	claims,description	23	0.000
Homo sapiens	claims,description	23	0.000
erythrocyte	claims,description	23	0.000
T cell proliferation	claims,description	21	0.000
targeting	claims,description	21	0.000
Envelope protein	claims,description	19	0.000
Protein X	claims,description	19	0.000
reductive effect	claims,description	17	0.000
Gag polyprotein	claims,description	12	0.000
measurement	claims,description	12	0.000
pol Genes	claims,description	12	0.000
rev Genes	claims,description	12	0.000
basophil	claims,description	10	0.000
eosinophil	claims,description	10	0.000
gag Genes	claims,description	10	0.000
neutrophil	claims,description	10	0.000
treatment regimen	claims,description	7	0.000
rev gene	claims,description	5	0.000
Syncytin-1	claims	4	0.000
C-C chemokine receptor type 5	claims	1	0.000
C-C chemokine receptor type 5	claims	1	0.000
transduction	abstract,description	75	0.000

transduction	abstract,description	73	0.000
prevention	abstract,description	2	0.000

Show all concepts from the description section

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