Title: VSV-HIV VIRAL PARTICLES LACKING REVERSE TRANSCRIPTASE FUNCTIONALITY AND THERAPEUTIC APPLICATIONS THEREOF

Abstract: The present invention relates to the development of non-replicative virions comprising a human immunodeficiency virus (HIV) genome and vehiculized by the G protein of a vesicular stomatitis virus (VSV). Said virions are useful in medicine, specifically for use in generating vaccines and more specifically for treating AIDS. The present invention further relates to a method for obtaining a customized vaccine.
VSV-HIV Viral Particles Lacking Reverse Transcriptase Functionality and Therapeutic Applications Thereof

Field of the Invention

The present invention relates to HIV immunogens lacking a functional reverse transcriptase protein and vehiculized by the G protein of a vesicular stomatitis virus (VSV). The invention also relates to their preventive and therapeutic uses and to methods for obtaining activated immune cells using said immunogens.

Background of the Invention

It is estimated that more than 60 million people worldwide have been infected by the human immunodeficiency virus since 1982. Nearly half of these infected individuals have died of the resultant Acquired Immunodeficiency Syndrome (AIDS) during the same time frame. Although the virus spread seems to have reached a plateau lately, 2.7 million HIV new infections were reported in 2007. HIV still is a major public health problem. See UNAIDS, 2008 Report on the Global AIDS Epidemic. In the United States and Western Europe, the morbidity and mortality due to HIV infection have been reduced spectacularly after the introduction of Highly Active Antiretroviral Therapy (HAART).

Besides its high efficiency for controlling viral replication and for restoring immunity against opportunistic infections, HAART is incapable of eradicating the virus and recovering the specific responses against HIV in HIV-infected persons. In fact, after withdrawing HAART a rapid increase of viral load occurs in all cases. See Carcelain G, et al., J. Virol. 2001; 75(1):234-241. This implies that once HAART is started it must be continued throughout life, which is problematic due to the different types of serious side effects which it may involve, such as metabolic alterations and the cardiovascular risk that it entails.

Currently, the therapeutic arsenal against HIV infection is very complete and there are perspectives that it will continue to increase in a significant manner in the upcoming years. This situation is in contrast with the limited advances obtained in the
development of a preventive or therapeutic vaccine. This is due to, among other reasons, the genetic diversity, the fast replication rate and the high mutation frequency inherent to HIV, the difficulty for inducing neutralizing antibodies, the lack of a suitable animal model, the limited knowledge of the immune response capable of generating protection against HIV infection and the different transmission routes of the virus. See Autran B, et al., Nat. Rev. Immunol. 2003; 3(6):503-508 and Kaufmann S, et al., Nat. Med. 2005; 11(4 Suppl):S33-44).

A future objective for vaccine design consists of increasing their efficiency by enabling their accessibility to the greatest number of antigen presenting cells (APCs) possible and achieving high local concentrations required to induce strong immunological responses. By facilitating the proper penetration of vaccine compounds into the APC-rich immunization sites as well as the specific migration and activation of APCs, the efficiency of the new vaccines in inducting protective immune responses would be increased. The vaccines could be efficient for inducing strong and protective immune responses against pathogens if they penetrate properly and activate the dendritic cells (DCs) which play a unique role as inducers of antigen specific responses. In the case of HIV infection, the clearance of the virus depends on the generation of antibodies but also on the induction of effector and memory T cells, CD4+ and CD8+.

Different in depth studies on memory immune responses have been focused on the importance of forming a functional pool of memory CD8+ T cells for controlling the viral infection and requiring a suitable function of the APCs and of CD4+ T cell responses.

Currently, there is no preventive or therapeutic vaccine approved for use in HIV. None of the therapeutic vaccines used have shown long term efficacy with respect to the control of viral replication, the increase of CD4 lymphocytes or the progression to AIDS in randomized studies. Vaccines from attenuated live viruses have traditionally been used to prevent viral infections, such as, for example, measles, mumps or rubella. These vaccines are highly immunogenic because they imitate the natural infection both in the cell and humoral response, and they induce life-long immunity after one or two doses. In contrast, vaccines using dead organisms or subunits do not imitate the infection and the administration of these vaccines only induces a humoral response, periodic boosters are required to maintain the antibodies at suitable concentrations, and
furthermore adjuvants such as aluminum salts are used to increase these responses which are at times very poor. See McMichael A, et al., Nat. Med. 2003; 9:874-880.

Some of these traditional vaccine strategies cannot be used to prevent HIV infection or therapeutically due to safety problems or because they have had nil efficacy when used. See Mwau M, et al., J. Gene Med. 2003; 5:3-10 and Moss R, et al., Vaccine 2000; 18:1081-1087. For example, attenuated live vaccines are prohibited in humans since in experiments with animals the highly attenuated simian immunodeficiency virus (SIV) protecting adult macaques from HIV infection caused AIDS in infant macaques and some adult macaques subsequently developed AIDS. Moreover, the degree of attenuation was inversely correlated with immunogenicity. See Wyand M., et al., Nat. Med. 1997; 3:32-36. In addition, the vaccines of dead viruses have shown no positive results in clinical trials. See Moss R, et al., Vaccine 2000; 18:1081-1087.

Therefore, there exists a long-felt and continuing need in the art for HIV immunogens for the preparation of safer and more efficient vaccines, particularly autologous HIV therapeutic vaccines.

Summary of the Invention

The present invention refers to HIV immunogens lacking a functional reverse transcriptase protein and vehiculized by the G protein of a vesicular stomatitis virus (VSV) capable of generating an immune response.

In a first aspect, the invention relates to a non-replicative virion comprising a human immunodeficiency virus (HIV) genome containing a mutation in the pol gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus; and wherein said virion further comprises an envelope glycoprotein G from a vesicular stomatitis virus (VSV).

In another aspect, the invention relates to a method for obtaining an activated immune cell comprising the steps:

a) contacting an immune cell with a non-replicative virion of the invention,

b) maintaining the mixture obtained in step a) in conditions suitable for infecting said immune cells with the virion and for processing the antigens originating from the non-replicative virion, and
c) optionally, recovering the activated immune cells.

In another aspect, the invention relates to an *in vitro* method for obtaining a customized HIV vaccine for an HIV-infected subject, wherein said vaccine comprises activated immune cells, which comprises:

a) contacting immune cells *in vitro* with a non-replicative virion of the invention, wherein the HIV genome of the virion further comprises a mutation in at least a gene selected from the group consisting of the *gag*, *nef*, *tat*, *rev*, *vif*, *vpu* and *vpr* genes or in the sequence encoding the protease protein in the *pol* gene, wherein said mutation comprises the complete or partial deletion of said gene; and wherein the HIV genome of the virion further comprises a sequence homologous to that of the region deleted by means of the mutation in at least a gene selected from the group consisting of the *gag*, *nef*, *tat*, *rev*, *vif*, *vpu*, and *vpr* genes or in the sequence encoding the protease protein in the *pol* gene, originating from a second HIV genome, wherein the genome of said second HIV is different from the HIV genome containing the deleted region and in which the genome of said second HIV originates from an HIV of said HIV-infected subject;

b) maintaining the mixture obtained in step a) in conditions suitable for infecting said cells with the virion and for processing the antigens originating from the non-replicative virion;

c) detecting and quantifying the activation of the immune cells activated according to steps a) and b);

d) comparing the activation of said immune cells activated according to steps a) and b) with the activation of control immune cells;

e) selecting the virions which cause a greater activation of the immune cells activated according to steps a) and b) than the activation of the control immune cells;

f) infecting immune cells of said HIV-infected subject with the non-replicative virions selected in step e); and

g) recovering the immune cells activated according to step f).
In another aspect, the invention relates to an *in vitro* method for obtaining a customized HIV vaccine for an HIV-infected subject, wherein said vaccine comprises activated immune cells, which comprises:

a) contacting immune cells originating from said HIV-infected subject *in vitro* with a non-replicative virion of the invention, wherein the HIV genome of the virion further comprises a mutation in at least a gene selected from the group consisting of the *gag*, *nef*, *tat*, *rev*, *vif*, *vpu*, and *vpr* genes or in the sequence encoding the protease protein in the *pol* gene, wherein said mutation comprises the complete or partial deletion of said gene; and wherein the HIV genome of the virion further comprises a sequence homologous to that of the region deleted by means of the mutation in at least a gene selected from the group consisting of the *gag*, *nef*, *tat*, *rev*, *vif*, *vpu*, and *vpr* genes or in the sequence encoding the protease protein in the *pol* gene, originating from a second HIV genome, wherein the genome of said second HIV is different from the HIV genome containing the deleted region and wherein the genome of said second HIV originates from an HIV of said HIV-infected subject;

b) maintaining the mixture obtained in step a) in conditions suitable for infecting said immune cells with the virion and for processing the antigens originating from the non-replicative virion; and

c) recovering the immune cells activated according to step b).

In another aspect, the invention relates to a cell obtainable by a method according to the invention.

In another aspect, the invention relates to a HIV viral genome containing a first mutation in the *pol* gene, wherein said first mutation causes the virus produced solely from said viral genome to be a non-replicative virus; wherein said genome further comprises a second mutation in at least a gene selected from the group consisting of the *gag*, *nef*, *tat*, *rev*, *vif*, *vpu*, and *vpr* genes or in the sequence encoding the protease protein in the *pol* gene, wherein said second mutation comprises the complete or partial deletion of said gene.

In another aspect, the invention relates to a vector comprising a HIV viral genome of the invention.
In another aspect, the invention relates to an immunogenic composition or a vaccine comprising a non-replicative virion of the invention, a cell of the invention, a HIV viral genome of the invention or a vector of the invention together with a pharmaceutically acceptable carrier.

In further aspects, the invention relates to a non-replicative virion of the invention, a cell of the invention, a HIV viral genome of the invention, a vector of the invention or an immunogenic composition or vaccine of the invention for use in medicine as well as for use in the prevention or treatment of a HIV infection or a disease associated with a HIV infection.

In another aspect, the invention relates to a composition or kit comprising:

i) a polynucleotide comprising a human immunodeficiency virus (HIV) genome containing a mutation in the pol gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus; and

ii) a polynucleotide encoding the envelope glycoprotein G from a vesicular stomatitis virus (VSV).

In an additional embodiment, the composition or kit comprises at least one type of virion of the invention.

In a further aspect, the invention relates to a method for the generation of a recombinant HIV virion comprising:

i) co-transfecting a receptive cell with:

a) a polynucleotide encoding the envelope glycoprotein G from a vesicular stomatitis virus (VSV) and

b) a polynucleotide comprising a human immunodeficiency virus (HIV) genome containing a mutation in the pol gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus, wherein the HIV genome further comprises a deletion in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene and wherein the HIV genome further comprises a sequence homologous to that of the region deleted by means of the mutation in at least a gene selected
from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene, originating from a second HIV genome, wherein the genome of said second HIV is different from the HIV genome containing the deleted region,

ii) maintaining the cells obtained in i) under conditions adequate for formation of HIV, and

iii) recovering HIV virions from the cell.

Deposit of Microorganisms

The plasmids pNL4-3ΔRT, pNL4-3ΔRT/Δgag-[LacZ], pNL4-3ΔRT/Δenv-[LacZ], pNL4-3ΔRT/Δenv-nef-[LacZ], pNL4-3ΔRT/Δnef-[LacZ], pNL4-3ΔRT/Δ gag-pro-[LacZ], and pNL4-3ΔRT/Δ1136env were deposited on March 27th, 2012 at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), Inhoffenstraße 7 B, D-38124 Braunschweig, Federal Republic of Germany. The accession numbers assigned to said deposits were the following:

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Description of the Figures

Figure 1. A. Schematic depiction of the HIV genome pNL4-3. B. Schematic depiction of pNL4-3ΔRT. C. Schematic depiction of viral vectors pNL4-3ΔRT/Δ gag-[LacZ], pNL4-3ΔRT/Δ env-[LacZ], pNL4-3ΔRT/Δenv-nef-[LacZ], pNL4-3ΔRT/Δnef-[LacZ], and pNL4-3ΔRT/Δ gag-pro-[LacZ] (1 to 5, respectively), wherein the LacZ (β-
galactosidase) gene is substituting the gene fragments corresponding to the gag, env, env- nef, nef, or gag-pro genes, respectively. LTR, long terminal repeat; PR, protease; RT, reverse transcriptase; INT, integrase.

**Figure 2.** Measurement of luciferase activity to analyze the infection/replication capacity of the NL4-3 Ren WT, heat-inactivated NL4-3 Ren WT virions or NL4-3 ΔRT Ren (NL4-3 DRT Ren) virions after 24 or 96 hours (h) post-infection of MT-2 cells. Ren: renilla gene, WT: wild-type strain, RLUs: relative light units.

**Figure 3.** Measurement of luciferase activity to analyze the replicative capacity of the NL4-3 ΔRT (deltaRT) and NL4-3 (WT) virions after 72 hours post-infection of TZM-bl cells. WT, wild-type.

**Figure 4.** A. 293T cells co-transfected with pGAG-GFP (5 µg), pNL4-3 ΔRT (3 µg) + pGAG-GFP (2 µg), and pNL4-3WT (3 µg) + pGAG-GFP (2 µg). Upper panel, image overlay showing the cell nucleus, the cortical actin of the cell and the signal visualization of the expression of GAG-GFP. Lower panel, visualization of the expression of GAG-GFP by means of confocal microscopy. B. Infection of PBMC cells (peripheral mononuclear cells) with the virions obtained after co-transfecting 293T with pGAG-GFP (5 µg), pNL4-3WT (3 µg) + pGAG-GFP (2 µg), and pNL4-3 ΔRT (3 µg) + pGAG-GFP (2 µg). Upper panel, image overlay showing the cell nucleus, the cortical actin of the cell and the expression of GAG-GFP. Lower panel, visualization of the location of the GAG-GFP signal by means of confocal microscopy.

**Figure 5.** Visualization by means of transmission electron microscopy of the NL4-3 (A) and NL4-3 ΔRT (B) virion-producing 293T cells. Purified NL4-3 (C) and NL4-3 ΔRT (D) virions.

**Figure 6.** Western blot of protein extracts of the 293T cells transfected with pNL4-3 (NL4-3) and pNL4-3 ΔRT (ΔRT). An anti-p24 monoclonal antibody were used to study the processing profile of Gag in NL4-3/ΔRT and NL4-3 virions.

**Figure 7.** Sequential scheme of the *ex vivo* model of the MD-DC assay. MD-DC, monocyte-derived dendritic cells; CFSE, 5-6-carboxyfluorescein diacetate succinimidyl ester.

**Figure 8.** Assessment of the cellular response of the MD-DC cells against different immunogens: BaL30 (heat-inactivated R5 strain of HIV-1), heat-inactivated recombinant p24, NL4-3 WT, or NL4-3 ΔRT in an HIV patient. The proliferation index
(S.I.) (A), and the production of IL-2 (B) and interferon γ (IFN-γ) (C) are quantified.

**Figure 9.** Results of the cellular response obtained in the *ex vivo* model of MD-DC pulsed with the following immunogens: NL4-3 WT heat-inactivated (NL-4.3 heat) or NL4-3 ΔRT (NL-4.3-ΔRT). Test performed with 8 responding patients and against one and the same stock of immunogens with a degree of significance 0.01 **. WT, wild-type; MD-DC, monocyte-derived dendritic cells; IFN-γ, interferon γ; S.I., proliferation index.

**Figure 10.** Comparison of the results obtained using the MD-DC co-culture model and the *ex vivo* model established with PBMC. MD-DC, monocyte-derived dendritic cells; PBMC, peripheral mononuclear cells.

**Figure 11.** Visualization by means of electron microscopy of purified NL4-3 virions obtained by transfecting 293T cells in the absence (A) or in the presence (B) of Amprenavir (protease inhibitor).

**Figure 12.** A. Schematic depiction of the HIV genome pNL4-3/ΔRT/Δ1136env. B. Schematic depiction of pNL4-3/Δ3489pe. C. Schematic depiction of viral vectors pNL4-3 ΔRT/Δ1136env/Δgag-[LacZ], pNL4-3/ΔRT/Δ1136env/Δnef-[LacZ] and pNL4-3 ΔRT/Δ1136env/Δgag-pro-[LacZ], wherein the LacZ (β-galactosidase) gene is substituting the gene fragments corresponding to the gag, nef or gag-pro genes, respectively.

**Figure 13.** Visualization by means of transmission electron microscopy of the NL4-3 (A) and NL4-3 ΔRT (B) virion-producing 293T cells. Virions purified from said cells are also shown. NL4-3 ΔRT/Δ1136env/VSV virion-producing 293T cells as well as pseudovirions from 293-T cells transfected with pΔRT/Δ1136env + pVSV-G are also shown (C).

**Figure 14.** THP-1 cells infection with NL4-3, NL4-3/ΔRT and NL4-3/ΔRT-VSV virions. Lysosomes of THP-1 cells were labeled with a liso-tracker dye (red). Virions were labeled with Gag-GFP (green). Nuclei were labeled with DAPI (blue). Entrance in the cells were independent of lisosomal in NL4-3 and NL4-3/ΔRT virions meanwhile in the case of NL4-3/ΔRT-VSV is dependent of the lisosomal pathway shown by the colocalization of gag-GFP (green) and lisosomal dye (red).

**Figure 15.** Protein profile of NL4-3, NL4-3/ΔRT and NL4-3/ΔRT-VSV virions.
Purified virions by ultracentrifugation were used to extract proteins and separated them in a gradient 4-12% Tris-Glycine gel. An anti-p24 monoclonal antibody were used to study the processing profile of Gag in NL4-3/ΔRT, NL4-3/ΔRT-VSV and NL4-3 virions. There is a weaker processing of Gag in NL4-3/ΔRT and NL4-3/ΔRT-VSV virions represented by an increase in p55gag and MA-CA p41 forms in detriment of CA p24. At the same, an increment of intermediate forms is also shown in NL4-3/ΔRT virions.

**Figure 16. A.** Frequency of IFN-γ-ELISPOT positive responses and magnitude obtained against the immunogens indicated. White bars show the percentage of positive response (%) whereas each symbol represents the mean of SFC (spot forming cells/106 cells) ± SEM counted per well after basal background subtraction. **B.** Twenty-one cryopreserved PBMCs from HIV seropositive individuals were assessed by ELISPOT to detect IFN-γ production. PBMCs were pulsed with 200 ng/ml p24 equivalents in all cases. Magnitude response was significantly lower against WT inactivated virions (WT+AT-2) than against WT+APV+AT-2 (*p<0.05). Averaged values from duplicate wells normalized to SFC/10^6 PBMCs are shown for the stimulation conditions indicated. Plots represent mean ± standard error of the mean (SEM). Positivity threshold for each construct or antigen was defined as at least 50 SFC/10^6 PBMC and at least twice that of the control medium.

**Figure 17.** Assessment of the cellular response of the MD-DC cells against different immunogens: NL4-3 WT+AT-2, NL4-3 ΔRT/VSV, NL4-3 ΔRT and recombinant p24, and different concentration (0.5, 1, and 5 μg/mL of p24). Test performed with 9 responding patients and against one and the same stock of immunogens. The proliferation index in CD8^+ is quantified.

*Detailed Description of the Invention*

The present invention refers to non-replicative virions comprising a human immunodeficiency virus (HIV) genome lacking a functional reverse transcriptase protein and targeted by the envelope glycoprotein G of a vesicular stomatitis virus (VSV) for the purpose of obtaining autologous and heterologous vaccines, both
preventive and therapeutic, against HIV/AIDS infection.

By vectorizing these immunogens with VSV, the risk of HIV viral reversion is diminished and their immune response is increased, thus providing a solution more suitable for clinical application. The viral particles of the invention are capable of inducing an immune response higher than the immune response generated by other particles not vectorized by the glycoprotein G of VSV. See Figure 16.

The immunogens of the invention have additional advantages, since they are safer than other known HIV immunogens due to the fact that the virions of the invention are unable to replicate.

a) Definitions of general terms and expressions

The term “activated immune cell”, as used herein, refers to an immune cell which, after being treated with immunogenic factors, is capable of generating an immune response against said immunogens. Said immune cells can be, among others, lymphocytes or dendritic cells (DC).

The term “adjuvant”, as used herein, refers to an immunological agent that modifies the effect of an immunogen, while having few if any direct effects when administered by itself. It is often included in vaccines to enhance the recipient's immune response to a supplied antigen, while keeping the injected foreign material to a minimum. Adjuvants are added to vaccines to stimulate the immune system's response to the target antigen, but do not in themselves confer immunity. Non-limiting examples of useful adjuvants include mineral salts, polynucleotides, polyarginines, ISCOMs, saponins, monophosphoryl lipid A, imiquimod, CCR-5 inhibitors, toxins, polyphosphazenes, cytokines, immunoregulatory proteins, immunostimulatory fusion proteins, co-stimulatory molecules, and combinations thereof. Mineral salts include, but are not limited to, AlK(SO₄)₂, AlNa(SO₄)₂, AlNH(SO₄)₂, silica, alum, Al(OH)₃, Ca₃(PO₄)₂, kaolin, or carbon. Useful immunostimulatory polynucleotides include, but are not limited to, CpG oligonucleotides with or without immune stimulating complexes (ISCOMs), CpG oligonucleotides with or without polyarginine, poly IC or poly AU acids. Toxins include cholera toxin. Saponins include, but are not limited to, QS21, QS17 or QS7. An example of a useful immunostimulatory fusion protein is the fusion
protein of IL-2 with the Fc fragment of immunoglobulin. Useful immunoregulatory molecules include, but are not limited to, CD40L and CD1a ligand. Cytokines useful as adjuvants include, but are not limited to, IL-1, IL-2, IL-4, GMCSF, IL-12, IL-15, IGF-1, IFN-α, IFN-β, and interferon gamma. Also, examples of adjuvants are muramyl dipeptides, such as N-acetyl-muramyl-L-threonyl-D-isoglutamime (thr-DMP), N-acetylnornuramyl-L-alanyl-D-isoglutamime (CGP 11687, also referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1′2′-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, also referred to as MTP-PE), RIBI (MPL+TDM+CWS) in a 2% squalene/Tween® 80 emulsion, lipopolysaccharides, and its various derivatives, including lipid A, Freund's Complete Adjuvant (FCA), Freund's Incomplete Adjuvants, Merck Adjuvant 65, polynucleotides (e.g. poly IC and poly AU acids), wax D from Mycobacterium tuberculosis, substances found in Corynebacterium parvum, Bordetella pertussis, and members of the genus brucella, Titermax, Quil A, ALUN, Lipid A derivatives, cholera toxin derivatives, HSP derivatives, LPS derivatives, synthetic peptide matrixes or GMDP, Montanide ISA-51 and QS-21, CpG oligonucleotide, poly I:C, and GMCSF. See Osol A., Ed., Remington's Pharmaceutical Sciences (Mack Publishing Co., Easton, PA, US, 1980, pp. 1324-1341), Hunter R, US 5,554,372, and Jager E, et al., WO 1997/028816. Combinations of adjuvants can also be used.

The term “AIDS”, as used herein, refers to the symptomatic phase of HIV infection, and includes both Acquired Immune Deficiency Syndrome (commonly known as AIDS) and “ARC,” or AIDS-Related Complex. See Adler M, et al., Brit. Med. J. 1987; 294: 1145-1147. The immunological and clinical manifestations of AIDS are well known in the art and include, for example, opportunistic infections and cancers resulting from immune deficiency.

The term “complete deletion”, as used herein, refers to the loss of 100% of the nucleotides forming the nucleotide sequence of a gene.

The terms “comprising” or “comprises”, as used herein, denote also “consisting of” according to the generally accepted patent practice.

The term “control immune cells”, as used herein, refers to immune cells activated with viral preparations, such as viruses inactivated by means of heat or chemical treatment, or heterologous gene-carrying defective viral particles, immature
virions obtained by different mechanisms such as treatment with protease inhibitors or by mutations, recombinant proteins or peptide combinations.

The term “co-transfecting”, as used herein, refers to the simultaneous transfection with two separate, unrelated nucleic acid molecules.

The term “dendritic cell”, as used herein, refers to any member of a diverse population of morphologically similar cell types found in lymphoid or non-lymphoid tissues. Dendritic cells are a class of “professional” antigen presenting cells, and have a high capacity for sensitizing HLA-restricted T cells. Specifically, the dendritic cells include, for example, plasmacytoid dendritic cells, myeloid dendritic cells (generally used dendritic cells, including immature and mature dendritic cells), Langerhans cells (myeloid dendritic cells important as antigen-presenting cells in the skin), interdigitating cells (distributed in the lymph nodes and spleen T cell region, and believed to function in antigen presentation to T cells). All these DC populations are derived from bone marrow hematopoietic cells. Dendritic cells also include follicular dendritic cells, which are important as antigen-presenting cells for B cells, but who are not derived from bone marrow hematopoietic cells. Dendritic cells may be recognized by function, or by phenotype, particularly by cell surface phenotype. These cells are characterized by their distinctive morphology (having veil-like projections on the cell surface), intermediate to high levels of surface HLA-class II expression and ability to present antigen to T cells, particularly to naive T cells. See Steinman R., et al., Ann. Rev. Immunol. 1991; 9:271-196. The cell surface of dendritic cells is characterized by the expression of the cell surface markers CD1a+, CD4+, CD86+, or HLA-DR+.

The expression “disease associated with a HIV infection”, as used herein, includes a state in which the subject has developed AIDS, but also includes a state in which the subject infected with HIV has not shown any sign or symptom of the disease.

The term “env”, as used herein, refers to the gene encoding the gp160 protein of the HIV viral envelope.

The term “functional gene”, as used herein, refers to a gene capable of encoding a functional protein. Different methods of the state of the art can be used to analyze the function of the proteins encoded by gag, nef, tat, rev, vif, vpu, and vpr genes. Said methods will depend on the function of said protein.

The term “gag”, as used herein, refers to the gene encoding the p55 protein of
the capsid formed by 3 protein subunits (MA, CA, and NC).

The term “glycoprotein G”, as used herein, refers to a protein from the envelope of the VSV that enables viral entry, since mediates viral attachment to the host cell, where the virus is endocytosed, and then mediates fusion of the viral envelope with the endosomal membrane (e.g. Uniprot KB database accession no. Q6EH37). The term glycoprotein G is also used to refer to VSV glycoprotein G from other strains or isolates of the virus and also to functionally equivalent variants thereof. As it is used herein, “functionally equivalent variant of VSV glycoprotein G” is understood as a polypeptide capable of complementing the temperature-sensitive G mutant of VSV tsO45 at a nonpermissive temperature and having a minimal identity in the amino acid sequence with the sequence of the VSV glycoprotein G. See Lefkowitz E, et al., Virology 1990; 178(2):373-383. The functionally equivalent variants of VSV glycoprotein G include polypeptides showing at least 60%, 65%, 70%, 72%, 74%, 76%, 78%, 80%, 90%, 95%, 97%, 99% similarity or identity with the different natural variants of glycoprotein G mentioned above. The variants of VSV glycoprotein G can be both natural and artificial. The expression “natural variant” refers to all those variants of VSV glycoprotein G defined above which occur naturally in other strains. The expression “artificial variant” refers to a recombinant or synthetic polypeptide. The term “gp160 protein” refers to the product of the env gene and is a precursor of the gp120 and gp41 envelope proteins.

The term “HIV genome” or “HIV viral genome”, as used herein, refers to a RNA sequence approximately 9749 nucleotides long enclosed by the HIV capsid and encoding the genes gag, pol, env, tat, rev, vib, nef, vpr, vpu, vpx, and optionally, tev. The HIV genome sequence underlies high variability, for this reason, the HIV genome referred to in the invention is not limited to any specific sequence. Preferred sequences are those of the HIV types and subtypes recited herein.

The expression “HIV infected subject”, as used herein, refers to a human subject infected by HIV. HIV infected subjects are identified by clinical conditions associated with HIV infection and CD4+ T lymphocyte counts. From a practical standpoint, the clinician views HIV infection as a spectrum of disorders ranging from primary infection with or without the acute HIV syndrome to the symptomatic infection state of advanced disease. The Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, has established an authoritative definition for the diagnosis of AIDS, which informs a
skilled artisan whether the onset of AIDS has occurred: in a HIV-infected subject, the CD4+ T cell count must be below 200 cells per cubic mm of blood, or there must be the clinical appearance of an initial AIDS-defining opportunistic infection, such as PCP (i.e. *Pneumocystis carinii* pneumonia), oral candidiasis (i.e. thrush), pulmonary tuberculosis, or invasive cervical carcinoma. Methods of determining a subject’s CD4+ T cell count are known in the art.

The term “homologous sequence”, as used herein, refers to the sequence of a gene of a genome A equivalent to a sequence of that same gene in a genome B. By way of illustration, for the sequence of the *env* gene in a genome A (e.g. an HIV genome), its homologous sequence will be the sequence of the *env* gene of a genome B (e.g. a different HIV genome).

The term “human immunodeficiency virus” or “HIV”, as used herein, is meant to include HIV-1 and HIV-2. “HIV-1” means the human immunodeficiency virus type-1. HIV-1 includes, but is not limited to, extracellular virus particles and HIV-1 forms associated with HIV-1 infected cells. “HIV-2” means the human immunodeficiency virus type-2. HIV-2 includes, but is not limited to, extracellular virus particles and HIV-2 forms associated with HIV-2 infected cells. Preferably, HIV is HIV-1.

The term “immunogenic composition”, as used herein, refers to a composition that elicits an immune response that produces antibodies or cell-mediated immune responses against a specific immunogen.

The term “infectious”, as used herein, refers to a virion capable of infecting cells, either because the gp120 and gp41 envelope proteins of the virion interact with CD4 receptors and co-receptors (CCR5 or CXCR4) on the surface of the cell, therefore producing the fusion of the viral and cell membranes; or because the VSV-G mediates viral entry through endocytosis.

The term “kit”, as used herein, refers to a composition or combination of a set of components suitable to generate a virion of the invention in a receptive cell by co-transflecting a polynucleotide comprising a HIV genome of the invention and a polynucleotide encoding the VSV-G protein, together with one or more types of elements or components (for example, other types of biochemical reagents, containers, packaging suitable for its commercial sale, media, additives, cell lines, etc.). Both polynucleotides may be formulated as a single formulation or may be presented as
separate formulations of each of the components, which may be combined for joint use as a combined preparation. The composition may be a kit-of-parts wherein each of the components is individually formulated and packaged.

The expression “lack a functional gp160 protein”, as used herein, refers to the fact that the gp160 protein or their products gp120 or gp41 are unable to bind to the surface of the target cell. Particularly, a virion may lack a functional gp160 protein when: (i) the gp120 protein is unable to bind to the CD4 receptor of a target cell; (ii) the gp120 protein is capable of binding to the CD4 receptor of a target cell but unable to bind to CCR5 or CXCR4 co-receptors; (iii) the gp120 protein is unable to bind to the three receptors CD4, CCR5, and CXCR4; (iv) the gp41 protein is unable to assist in the fusion of the viral particle with the target cell, or (v) a combination of any of the above (i) to (iv). The expression “lack a functional gp160 protein” also refers to virions having a gp160 protein that is not processed into its products gp120 and gp41 and is, therefore, unable to produce functional gp120 or gp41 proteins.

The term “marker gene” or “reporter gene”, as used herein, refers to the gene encoding a product which gives rise to a signal that can be readily measured or detected. Marker genes include, but are not limited to, the GFP (green fluorescent protein) gene, the LacZ gene encoding the β-galactosidase protein, the renilla or firefly luciferase gene encoding the luciferase protein.

The term “mutation”, as used herein, refers to a change in a nucleic acid sequence. Said mutation include, but is not limited to, substitution (i.e. exchanging one or more nucleotides for others), inversion (i.e. a DNA segment inside a gene is inverted, to that end two 180° rotations are necessary, one for inverting the sequence and the other for maintaining the polarity of the DNA), translocation (i.e. a segment of a gene changes position to be in another different site of the same gene or in another site of the genome), and nucleotide insertions or deletions (i.e. the addition of one or more nucleotides (insertions or additions) or the loss of one or more nucleotides (deletions) having as a consequence changes in the reading frame, a reading error occurring during the translation ranging from the formation of non-functional proteins to the absence of said protein).

The term “ nef ”, as used herein, refers to the gene encoding the Nef protein which negatively controls CD4 and HLA molecules of the infected cell and plays a role
in the pathogenicity of the virus.

The term “non-functional RT protein”, as used herein, refers to the fact that the RT protein does not have reverse transcriptase function (i.e. it is not capable of synthesizing double-stranded DNA using single-stranded RNA as a template). Different methods of the state of the art can be used to analyze the function of a protein; said methods will depend on the function of said protein. Thus, for example, to determine whether the RT protein is functional, different assays can be performed, among others, reverse transcription reactions of an RNA sequence or analyzing the absence of replication of a virus as a consequence of having a non-functional RT protein by colorimetrically or fluorimetrically determining the presence of a marker gene of the viral genome in cells infected with a virion comprising the sequence encoding the RT protein to be analyzed.

The term “partial deletion”, as used herein, refers to the loss of at least 0.5%, 1%, 5%, 10%, 20%, 40%, 50%, 60%, 70%, 80%, 90% or 99% of the nucleotides forming the nucleotide sequence of a gene.

The expression “pharmaceutically acceptable carrier”, as used herein, refers to a vehicle or excipient that is involved in carrying or transporting a product of interest from a tissue, organ, or portion of the body to another tissue, organ, or portion of the body, and that is useful in preparing a pharmaceutical composition that is generally safe and non-toxic.

The term “pol”, as used herein, refers to the gene encoding the viral enzymes necessary for the viral replication process: protease (PRO), reverse transcriptase (RT), and integrase (INT).

The term “polynucleotide”, as used herein, relates to a polymeric form of nucleotides of any length and formed by ribonucleotides or deoxyribonucleotides. The term includes both single and double stranded polynucleotides, as well as modified polynucleotides (methylated, protected and similar). The terms “prevent,” “preventing,” and “prevention”, as used herein, refer to inhibiting the inception or decreasing the occurrence of a disease in a subject. Prevention may be complete (e.g. the total absence of pathological cells in a subject). The prevention may also be partial, such that for example the occurrence of pathological cells in a subject is less than that which would have occurred without the present invention. Prevention also refers to reduced
susceptibility to a clinical condition.

The term “protease”, as used herein, refers to the retroviral aspartyl protease encoded by the \textit{pol} gene that cleaves newly synthesized polyproteins at the appropriate places to create the mature protein components of an infectious HIV virion. Without effective protease, HIV virions remain uninfected. Thus, mutation of HIV protease’s active site or inhibition of its activity disrupts HIV’s ability to replicate and infect additional cells.

The term “pseudotyping”, as used herein, refers to providing the viral envelope carrying proteins codified by the genome, either partial or as a whole, from one virus species to another species.

The term “pseudovirions”, as used herein, refers to virions carrying a viral envelope with proteins codified by the genome, either partial or as a whole, from one virus species to another species.

The expression “recombinant HIV virion”, as used herein, refers to a virion obtained by recombinant techniques and having the HIV genome of the invention inside the capsid and the VSV G glycoprotein in the envelope.

The term “rev”, as used herein, refers to the gene encoding the Rev protein responsible for processing messenger RNA and transporting it to the cytoplasm.

The term “reverse transcriptase” or “RT”, as used herein, refers to the RNA-dependent DNA polymerase that transcribes the single-stranded RNA genome of a virus into single-stranded DNA, which is then integrated into the host genome and replicated along with it. The term “reverse transcriptase”, as used herein, is encoded by the \textit{pol} gene of a virus, particularly of the HIV-1 virus. The term HIV-1 reverse transcriptase is also used to refer to HIV-1 reverse transcriptase from other strains or isolates of the virus. The nucleic acid and amino acid sequence of a large number of HIV-1 reverse transcriptase are readily available to the public. See HIV Sequence Database, http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html, August 2012; Los Alamos HIV Databases and Compendia, http://www.hiv.lanl.gov/, August 2012.

The term “\textit{tat}”, as used herein, refers to the gene encoding the Tat protein, a viral messenger RNA transactivator and elongator.

The term “treat” or “treatment”, as used herein, refers to the administration of a pharmaceutical composition of the invention (i.e. an immunogenic composition or a
vaccine) or of a medicament containing it to control the progression of a disease after its clinical signs have appeared. Control of the disease progression is understood to mean the beneficial or desired clinical results that include, but are not limited to, reduction of the symptoms, reduction of the duration of the disease, stabilization of pathological states (specifically to avoid additional deterioration), delaying the progression of the disease, improving the pathological state and remission (both partial and total). The control of progression of the disease also involves an extension of survival, compared with the expected survival if treatment was not applied. Within the context of the present invention, the terms “treat” and “treatment” refer specifically to preventing or slowing the infection and destruction of healthy CD4+ T cells in a HIV infected subject. It also refers to the prevention and slowing the onset of symptoms of the acquired immunodeficiency disease such as extreme low CD4+ T cell count and repeated infections by opportunistic pathogens such as *Mycobacteria* spp., *Pneumocystis carinii*, and *Pneumocystis cryptococcus*. Beneficial or desired clinical results include, but are not limited to, an increase in absolute naïve CD4+ T cell count (range 10-3520), an increase in the percentage of CD4+ T cell over total circulating immune cells (range 1-50%), or an increase in CD4+ T cell count as a percentage of normal CD4+ T cell count in an uninfected subject (range 1-161%). “Treatment” can also mean prolonging survival of the infected subject as compared to expected survival if the subject did not receive any HIV targeted treatment.

The term “vaccine”, as used herein, refers to an immunogenic composition for *in vivo* administration to a host, which may be a primate, especially a human host, to confer protection against a disease, particularly a viral disease.

The term “vector”, as used herein, refers to a nucleic acid sequence comprising the necessary sequences so that after transcribing and translating said sequences in a cell a non-replicative viral particle is generated. Said sequence is operably linked to additional segments that provide for its autonomous replication in a host cell of interest. Preferably, the vector is an expression vector, which is defined as a vector, which in addition to the regions of the autonomous replication in a host cell, contains regions operably linked to the genome of the invention and which are capable of enhancing the expression of the products of the genome according to the invention. The vectors of the invention can be obtained by means of techniques widely known in the art. See Brown

The term “vif”, as used herein, refers to the gene encoding the Vif protein associated with the infectivity of the extracellular virions.

The term “virion”, as used herein, refers to a replication-deficient infectious viral particle comprising the HIV viral genome packed in a capsid and, optionally, in a lipid envelope surrounding the capsid, wherein the envelope of the viral particle comprises a glycoprotein G from a vesicular stomatitis virus (VSV). As it is used herein, “non-replicative virion” refers to a virion lacking capacity for multiplying its genetic material and therefore new copies of said virion cannot be formed. To determine whether a virion is non-replicative the same assays disclosed to determine if a RT protein is functional may be used. Specific assays are disclosed in sections 2.1 and 2.2 of the general procedures of the present description.

The term “vpr”, as used herein, refers to the gene encoding the Vpr protein which acts as an accelerator for the replication cycle at different levels.

The term “vpu”, as used herein, refers to the gene encoding the Vpu protein involved in the release of the virions.

The terms “VSV” or “vesicular stomatitis virus”, as used herein, refer to negative-stranded RNA virus of approximately 11 kb in size of the rhabdoviridae family. VSV is also known as “vesicular stomatitis indiana virus” or “VSIV”. At
present, eight VSV subtypes have been described in the art. See http://viralzone.expasy.org/complete_by_species/21.html#tab6, August 2012.

The term “VSV-G”, as used herein, refers to the viral vesicular stomatitis virus glycoprotein G belonging to the family rhabdoviridae.

b) Virions, viral genomes and vectors of the invention

In a first aspect, the invention relates to a non-replicative virion, hereinafter virion of the invention, comprising a human immunodeficiency virus (HIV) genome containing a mutation in the pol gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus; and wherein said virion further comprises an envelope glycoprotein G from a vesicular stomatitis virus (VSV).

In a particular embodiment, the HIV genome that has been used for obtaining the viral vectors of the invention corresponds to the HIV-1 genome pNL4-3 (GenBank accession no. M19921.1, (SEQ ID NO: 1)). See Figure 1.

In a particular embodiment, the HIV genome of the virion containing a mutation in the pol gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus, contains env, gag, nef, tat, rev, vif, vpu or vpr functional genes.

In a preferred embodiment, the HIV genome of the virion contains the following gene pairs in functional form: env and gag; env and nef; env and tat; env and rev; env andvif; env and vpu; env and vpr; gag and nef; gag and tat; gag and rev; gag and vif; gag and vpu; gag and vpr; nef and tat; nef and rev; nef and vif; nef and vpu; nef and vpr; tat and rev; tat and vif; tat and vpu; tat and vpr; tat and vpr; rev and vif; rev and vpu; rev and vpr; vif and vpu; vif and vpr; vpu and vpr.

In another preferred embodiment, the HIV genome of the virion contains the following groups of genes in functional form: env, gag and nef; env, gag and tat; env, gag and rev; env, gag and vif; env, gag and vpu; env, gag and vpr; env, nef and tat; env, nef and rev; env, nef and vif; env, nef and vpu; env, nef and vpr; env, tat and rev; env, tat and vif; env, tat and vpu; env, tat and vpr; env, rev and vif; env, rev and vpu; env, rev and vpr; env, vif and vpu; env, vif and vpr; env, vpu and vpr; gag, nef and tat; gag, nef and rev; gag, nef and vif; gag, nef and vpu; gag, nef and vpr; gag, tat and rev; gag,
tat and vif; gag, tat and vpu; gag, tat and vpr; gag, rev and vif; gag, rev and vpu; gag, rev and vpr; gag, vif and vpu; gag, vif and vpr; gag, vpu and vpr; nef, tat and rev; nef, tat and vif; nef, tat and vpu; nef, tat and vpr; nef, rev and vif; nef, rev and vpu; nef, rev and vpr; nef, vif and vpu; nef, vif and vpr; nef, vpu and vpr; tat, rev and vif; tat, rev and vpu; tat, rev and vpr; tat, vif and vpr; tat, vpu and vpr; rev, vif and vpu; rev, vif and vpr; rev, vpu and vpr; vif, vpu and vpr.

In another preferred embodiment, the HIV genome of the virion contains the following groups of genes in functional form: env, gag, nef and tat; env, gag, nef and rev; env, gag, nef and vif; env, gag, nef and vpu; env, gag, nef and vpr; env, gag, tat and rev; env, gag, tat and vif; env, gag, tat and vpu; env, gag, tat and vpr; env, gag, rev and vif; env gag, rev and vpu; env, gag, rev and vpr; env, gag, vif and vpu; env, gag, vif and vpr; env, gag, vpu and vpr; env, nef, tat and rev; env, nef, tat and vif; env, nef, tat and vpu; env, nef, tat and vpr; env, nef, rev and vif; env, nef, rev and vpu; env, nef, rev and vpr; env, nef, vif and vpu; env, nef, vif and vpr; env, nef, vpu and vpr; env, tat, rev and vif; env, tat, rev and vpu; env, tat, rev and vpr; env, tat, vif and vpu; env, tat, vif and vpr; env, tat, vpu and vpr; env, rev, vif and vpu; env, rev, vif and vpr; env, rev, vpu and vpr; env, vif, vpu and vpr; gag, nef, tat and rev; gag, nef, tat and vif; gag, nef, tat and vpu; gag, nef, tat and vpr; gag, nef, rev and vif; gag, nef, rev and vpu; gag, nef, rev and vpr; gag, nef, vif and vpu; gag, nef, vif and vpr; gag, nef, vpu and vpr; gag, tat, rev and vif; gag, tat, rev and vpu; gag, tat, rev and vpr; gag, tat, vif and vpu; gag, tat, vif and vpr; gag, tat, vpu and vpr; gag, rev, vif and vpu; gag, rev, vif and vpr; gag, rev, vpu and vpr; gag, vif, vpu and vpr; nef, tat, rev and vif; nef, tat, rev and vpu; nef, tat, rev and vpr; nef, tat, vif and vpu; nef, tat, vif and vpr; nef, tat, vpu and vpr; nef, rev, vif and vpu; nef, rev, vif and vpr; nef, rev, vpu and vpr; nef, vif, vpu and vpr; tat, rev, vif and vpu; tat, rev, vpu and vpr; rev, vif, vpu and vpr.

In a preferred embodiment, the non-replicative virion according to the invention comprises a HIV genome having the following elements in the 5’ to 3’ direction:

a) The 5’ LTR (long terminal repeats) or terminally redundant sequences containing various consensus sequences for transcription factors regulating viral expression;

b) gag, the gene encoding the p55 protein of the capsid formed by 3 protein subunits (MA, CA, and NC);
c) the mutated pol gene, encoding the viral enzymes necessary for the viral replication process: protease (PRO), reverse transcriptase (RT) and integrase (INT) containing a mutation that causes the virus produced solely from said genome to be a non-replicative virus. The pol gene overlaps at its 5’ end with the gag element and;

d) vif, the gene encoding the Vif protein associated with the infectivity of the extracellular virions, and it overlaps at its 5’ end with the pol gene and at its 3’ end with vpr;

e) vpr, the gene encoding the Vpr protein acting as an accelerator of the replication cycle at different levels, and it overlaps at its 5’ end with the vif gene;

f) tat, the gene encoding the Tat protein which is a transactivator, and its second exon is contained in the env sequence;

g) vpu, the gene encoding the Vpu protein involved in the release of the virions;

h) env, the gene encoding the gp160 protein of the viral envelope;

i) rev, the gene encoding the Rev protein, responsible for processing and transporting messenger RNA to the cytoplasm, and its second exon is contained in the env sequence;

j) nef, the gene encoding the Nef protein which negatively regulates CD4 and HLA molecules of the infected cell and plays a role in the pathogenicity of the virus and

k) the 3’ LTR.

In a particular embodiment, the mutation in the pol gene comprises the complete or partial deletion of said gene.

In a preferred embodiment, the mutation in the pol gene gives rise to a non-functional RT protein.

In a preferred embodiment, the mutation in the pol gene is a complete or partial deletion of the sequence encoding the RT protein and said mutation does not comprise a deletion of the sequence encoding the integrase protein or a deletion of the sequence encoding the protease protein.

In a preferred embodiment, the complete or partial deletion of the sequence encoding the RT protein is the deletion in said sequence encoding the RT protein in the construct pNL4-3 ΔRT deposited under accession number DSM 25917.
In another embodiment, the mutation in the *pol* gene is a complete or partial deletion of the sequence encoding the RT protein and the integrase protein.

In a preferred embodiment, the HIV genome of the virion further comprises a mutation in the *env* gene, wherein said mutation causes the virus produced from said genome to lack a functional gp160 protein.

In a particular embodiment, the HIV genome of the virion comprising a mutation in the *env* gene, wherein said mutation causes the virus produced from said genome to lack a functional gp160 protein, contains *gag, nef, tat, rev, vif, vpu* or *vpr* functional genes.

In a particular embodiment, the mutation in the *env* gene comprises the complete or partial deletion of said gene. In a particular embodiment, the sequence of the Env gp160 protein is SEQ ID NO: 3.

In a particular embodiment, the complete or partial deletion in the *env* gene corresponds to the deletion in the *env* gene in the construct pNL4-3 ΔRT/Δ1136env deposited under accession number DSM25923.

In another particular embodiment, the HIV genome of the virion further comprises a mutation in at least a gene selected from the group consisting of the *gag, nef, tat, rev, vif, vpu*, and *vpr* genes or in the sequence encoding the protease protein in the *pol* gene, wherein said mutation comprises the complete or partial deletion of said gene. In a particular embodiment, the HIV genome of the virion comprises a mutation in a gene selected from the group consisting of the *gag, nef, tat, rev, vif, vpu*, and *vpr* genes or in the sequence encoding the protease protein in the *pol* gene, wherein said mutation comprises the complete or partial deletion of said gene. In a particular embodiment, the HIV genome of the virion comprises a mutation in only one gene selected from the group consisting of the *gag, nef, tat, rev, vif, vpu*, and *vpr* genes or in the sequence encoding the protease protein in the *pol* gene, wherein said mutation comprises the complete or partial deletion of said gene. In a particular embodiment, the HIV genome of the virion further comprises a mutation in the *gag* gene. In a particular embodiment, the HIV genome of the virion further comprises a mutation in the *nef* gene. In a particular embodiment, the HIV genome of the virion further comprises a mutation in the *tat* gene. In a particular embodiment, the HIV genome of the virion further comprises a mutation in the *rev* gene. In a particular embodiment, the HIV
genome of the virion further comprises a mutation in the *vif* gene. In a particular embodiment, the HIV genome of the virion further comprises a mutation in the *vpu* gene. In a particular embodiment, the HIV genome of the virion further comprises a mutation in the *vpr* gene. In a particular embodiment, the HIV genome of the virion further comprises a mutation in the sequence encoding the protease protein in the *pol* gene. In a preferred embodiment, the mutation is in a gene selected from the group consisting of the *gag* and *nef* genes. In another preferred embodiment, the mutation is in the sequence encoding the protease protein in the *pol* gene. In another preferred embodiment, the mutation is in at least two genes, preferably in the *gag* gene and in the sequence encoding the protease protein in the *pol* gene. In another particular embodiment, the sequence of the Gag p55 protein is SEQ ID NO: 4. In another embodiment, the sequence of the Nef protein is SEQ ID NO: 5. In another particular embodiment, the sequence of the Tat protein is SEQ ID NO: 6. In another particular embodiment, the sequence of the Rev protein is SEQ ID NO: 7. In another particular embodiment, the sequence of the Vif protein is SEQ ID NO: 8. In another particular embodiment, the sequence of the Vpu protein is SEQ ID NO: 9. In another particular embodiment the sequence of the Vpr protein is SEQ ID NO: 10.

In another particular embodiment, the HIV genome of the virion further comprises a marker gene, wherein said marker gene is located at the site occupied by the region deleted by means of the mutation in at least a gene selected from the group consisting of the *gag*, *nef*, *tat*, *rev*, *vif*, *vpu*, and *vpr* genes or in the sequence encoding the protease protein in the *pol* gene.

In another particular embodiment, the HIV genome of the virion further comprises a sequence homologous to that of the region deleted by means of the mutation in at least a gene selected from the group consisting of the *gag*, *nef*, *tat*, *rev*, *vif*, *vpu*, and *vpr* genes or in the sequence encoding the protease protein in the *pol* gene, originating from a second HIV genome, wherein the genome of said second HIV is different from the HIV genome containing the deleted region.

In a preferred embodiment, the sequence homologous is different to the sequence of the region deleted, preferably has 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 80%, 70%, 60%, 50% identity or less with the sequence deleted.

The percentage of identity between two sequences indicates the proportion of
identical amino acids that share the two sequences that are compared. The percentage of identity between two sequences of amino acids is calculated by comparing two sequences aligned on a particular region, determining the number of positions wherein there are identical amino acids in both sequences to obtain the number of coincident positions by dividing the number of said positions by the number of total positions in the segment which is being compared and multiplying the result by 100. The degree of identity between two polypeptides is determined using algorithms implemented in a computer and methods which are widely known in the art. The identity between two amino acid sequences is preferably determined using the BLASTP algorithm. See Altschul S, et al., “BLAST Manual” (NCBI NLM NIH, Bethesda, MD, US, 1996) and Altschul S, et al., J. Mol. Biol. 1990; 215:403-410. In another preferred embodiment, said second HIV originates from a HIV-infected subject. In a particular embodiment, the HIV-infected subject is a subject for which a customized HIV vaccine is to be obtained.

The homologous sequences originating from the HIV genome of a subject can be obtained by means of different methods known in the state of the art, among others, from blood cells of the HIV-infected patient by means of the QIAmp DNA blood kit (Qiagen NV, Venlo, NL), and the viral RNA can be obtained from the plasma of an HIV-infected patient by means of the QIAmp kit (Qiagen NV, Venlo, NL). Said extracted HIV RNA is used as a template for obtaining cDNA, using techniques known in the art.

In another aspect, the invention relates to a HIV viral genome containing a first mutation in the pol gene, wherein said first mutation causes the virus produced solely from said viral genome to be a non-replicative virus; wherein said genome further comprises a second mutation in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene, wherein said second mutation comprises the complete or partial deletion of said gene.

All the embodiments disclosed for the non-replicative virion of the invention are also applicable to the HIV viral genome of the invention.

In a further aspect, the invention relates to a vector comprising a HIV viral genome of the invention.
c) Activated cells of the invention

The non-replicative virions of the invention may be used for activating immune cells. Thus, in another aspect, the invention relates to a method for obtaining an activated immune cell comprising the steps:

a) contacting an immune cell with a non-replicative virion of the invention,

b) maintaining the mixture obtained in step a) in conditions suitable for infecting said immune cells with the virion and for processing the antigens originating from the non-replicative virion, and

c) optionally, recovering the activated immune cells.

Suitable conditions for infecting the immune cells with the virion of the invention and the processing of antigens are known in the art. By way of illustration, it can be carried out, for example, by maintaining $5 \times 10^6$ immature monocyte-derived dendritic cells (MD-DC) in contact with $1 \times 10^9$ virions for 3 hours at 37°C.

The recovery of the activated immune cells of the invention can be carried out by means of several methods known in the art. For example, the recovery can be performed by detaching the cells adhered to the culture plate by using trypsin-EDTA (0.25% and 0.02%, respectively) and centrifuging subsequently at 650g for 4 minutes at room temperature.

In an embodiment, the immune cell is selected from a lymphocyte, a peripheral blood mononuclear cell and a monocyte-derived dendritic cell.

In another aspect, the invention relates to a cell obtainable according to the method of the invention for activating immune cells.

d) Immune and vaccine compositions of the invention

In another aspect, the invention relates to an in vitro method for obtaining a customized HIV vaccine for an HIV-infected subject, wherein said vaccine comprises activated immune cells, which comprises:
a) contacting immune cells originating from said HIV-infected subject *in vitro* with a non-replicative virion as in claims 12 to 15 as filed wherein the genome of said second HIV originates from an HIV of said HIV-infected subject;

b) maintaining the mixture obtained in step a) in conditions suitable for infecting said immune cells with the virion and for processing the antigens originating from the non-replicative virion; and

c) recovering the immune cells activated according to the previous step.

In a particular embodiment, the *in vitro* method further comprises, between steps b) and c), the following steps:

i) detecting and quantifying the activation of the immune cells activated according to steps a) and b);

ii) comparing the activation of said immune cells activated according to steps a) and b) with the activation of control immune cells;

iii) selecting the virions which cause a greater activation of the immune cells activated according to steps a) and b) than the activation of the control immune cells;

iv) infecting immune cells of said HIV-infected subject with the non-replicative virions selected in step iii).

In another aspect, the invention relates to an *in vitro* method for obtaining a customized HIV vaccine for an HIV-infected subject, wherein said vaccine comprises activated immune cells, which comprises:

a) contacting immune cells *in vitro* with a non-replicative virion of the invention wherein the HIV genome comprised in said virion, further comprises a mutation in at least a gene selected from the group consisting of the *gag, nef, tat, rev, vif, vpu,* and *vpr* genes or in the sequence encoding the protease protein in the *pol* gene, wherein said mutation comprises the complete or partial deletion of said gene; and wherein the HIV genome further comprises a sequence homologous to that of the region deleted by means of the mutation in at least a gene selected from the group consisting of the *gag, nef, tat, rev, vif, vpu,* and *vpr* genes or in the sequence encoding the
protease protein in the *pol* gene, originating from a second HIV genome, wherein the genome of said second HIV is different from the HIV genome containing the deleted region and in which the genome of said second HIV originates from an HIV of said HIV-infected subject;

b) maintaining the mixture obtained in step a) in conditions suitable for infecting said cells with the virion and for processing the antigens originating from the non-replicative virion;

c) detecting and quantifying the activation of the immune cells activated according to steps a) and b);

d) comparing the activation of said immune cells activated according to steps a) and b) with the activation of control immune cells;

e) selecting the virions which cause a greater activation of the immune cells activated according to steps a) and b) than the activation of the control immune cells;

f) infecting immune cells of said HIV-infected subject with the non-replicative virions selected in step e); and

g) recovering the immune cells activated according to step f).

The different steps comprised in the *in vitro* method for obtaining a customized HIV vaccine for an HIV-infected subject are known in the art. The method comprises first contacting immune cells *in vitro* with a non-replicative virion of the invention. In a particular embodiment, said immune cells are lymphocytes, peripheral blood mononuclear cells or monocyte-derived dendritic cells. The immune cells and the non-replicative virion of the invention must be maintained in suitable conditions so that said cells are infected by the virion and so that the antigens originating from the non-replicative virion of the invention are processed. In a particular embodiment, said conditions are 37°C for 7 days.

It is then necessary to compare the activation of said immune cells with the activation of control immune cells. Different techniques are known in the state of the art for analyzing the activation of immune cells. Non-limiting, illustrative examples are, among others, the detection of cytokine production, the detection of the number of IFNγ and IL-2 producing cells, or the frequency analysis of dividing antigen-specific CD4 and CD8 cells by means of flow cytometry analysis. The virions causing an activation
of the activated immune cells greater than the activation of the control immune cells are then selected and said non-replicative virions are used for infecting immune cells of said HIV-infected subject. Finally, the method comprises recovering said activated immune cells.

In another aspect, the invention relates to an in vitro method for obtaining a customized HIV vaccine for an HIV-infected subject, wherein said vaccine comprises activated immune cells, which comprises:

a) contacting immune cells originating from said HIV-infected subject in vitro with a non-replicative virion of the invention, wherein the HIV genome comprised in said virion, further comprises a mutation in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene, wherein said mutation comprises the complete or partial deletion of said gene; and wherein the HIV genome of the virion further comprises a sequence homologous to that of the region deleted by means of the mutation in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene, originating from a second HIV genome, wherein the genome of said second HIV is different from the HIV genome containing the deleted region and wherein the genome of said second HIV originates from an HIV of said HIV-infected subject;

b) maintaining the mixture obtained in step a) in conditions suitable for infecting said immune cells with the virion and for processing the antigens originating from the non-replicative virion; and

c) recovering the immune cells activated according to step b).

In a particular embodiment, the immune cell is selected from a lymphocyte, a peripheral blood mononuclear cell and a monocyte-derived dendritic cell.

In another particular embodiment, the methods for obtaining a customized HIV vaccine further comprise the step of subjecting the activated immune cells to a treatment to ensure the safety of said activated immune cells.

“Treatment to ensure the safety” refers to those treatments which prevent the virions of the invention used in generating a customized HIV vaccine, despite being
non-replicative, from being able to induce the development of AIDS in the vaccinated subject. To that end, different methods known in the art can be used, including treatment with HIV RT protein, protease or integrase inhibitors, or the inactivation of the virions with AT-2 or heat.

RT protein inhibitors suitable for use in the present invention are, among others, zidovudine (AZT), Retrovir®, didanosine (ddl), Videx®, zalcitabine (ddC), Hivid®, stavudine (d4T), Zerit®, lamivudine (3TC), Epivir®, abacavir (ABC), Ziajen®, emtricitabine (FTC), Emtriva®, tenofovir DF (TDF), Viread®, nevirapine (NVP), Viramune®, delavirdine (DLV), Rescriptor®, efavirenz (EFV), Sustiva®, etravirine (ETV) and Intelen®; and suitable HIV integrase inhibitors are, among others, Raltegravir (RTG) and Isentress®. The inactivation of the virions of the invention by heat can be carried out at 56°C for 60 minutes. The inactivation with AT-2 (aldriothiol-2) can be carried out by means of incubating the virions with AT-2 at a concentration of 1 mM overnight at 4°C. The inactivation with protease inhibitors is performed by treating the virus-producing 293T cells while changing medium after transfection with drugs like saquinavir (SQV), Invirase®, indinavir (IDV), Crixivan®, ritonavir (RTV), Norvir®, nelfinavir (NFV), Viracept®, fosamprenavir (FOSAPV), Telzir®, lopinavir/rtv, (LPV/r)-Kaletra®, atazanavir (ATV), Reyataz®, tipranavir (TPV), Aptivus®, darunavir (DRV), Prezista® at concentrations capable of inhibiting 90% of the viral maturation.

In another aspect, the invention relates to the activated immune cells obtained according to the methods of the invention.

In another aspect, the invention relates to an immunogenic composition or a vaccine comprising a non-replicative virion of the invention, a cell of the invention, a HIV viral genome of the invention or a vector of the invention together with a pharmaceutically acceptable carrier.

In another aspect, the invention relates to a non-replicative virion of the invention, a cell of the invention, a HIV viral genome of the invention, a vector of the invention or an immunogenic composition or vaccine of the invention for use in medicine. Alternatively, the invention relates to the use of a non-replicative virion of the invention, a cell of the invention, a HIV viral genome of the invention, a vector of the invention or an immunogenic composition or vaccine of the invention in medicine.
In a further aspect, the invention relates to a non-replicative virion of the invention, a cell of the invention, a HIV viral genome of the invention, a vector of the invention or an immunogenic composition or vaccine of the invention for use in the prevention or treatment of a HIV infection or a disease associated with a HIV infection. Alternatively, the invention relates to the use of a non-replicative virion of the invention, a cell of the invention, a HIV viral genome of the invention, a vector of the invention or an immunogenic composition or vaccine of the invention for the manufacture of a medicament for the prevention or treatment of a HIV infection or a disease associated with a HIV infection. Alternatively, the invention relates to a method for the prevention or treatment in a subject of a HIV infection or a disease associated with a HIV infection that comprises the administration to said subject of a non-replicative virion, a cell, a HIV viral genome of the invention, a vector of the invention or an immunogenic composition or vaccine of the invention.

The amount of virions of the invention and of activated immune cells of the invention, as well as the administration time, can be determined according to protocols known in the art. In fact, the administration of therapeutically effective amounts of virions of the invention and of activated immune cells of the invention can be achieved by means of a single administration, such as, for example, a single injection of a sufficient number of virions or cells to provide a therapeutic benefit to the patient who is subjected to such treatment. Alternatively, in some circumstances, it may be desirable to provide multiple or consecutive administrations, either for a relatively short or a relatively prolonged time period, which may be determined by the doctor supervising the administration of such compositions. In fact, in certain embodiments, it may be desirable to administer two or more compositions of different virions or cells of the invention either alone or in combination with one or more drugs to achieve the desired effects of the particular therapeutic regimen.

The vaccines of the invention will be administered in a pharmacologically effective amount producing an improvement of one or more symptoms of a viral disorder or preventing the progression of a viral disease or causing a regression of the disease or reducing the viral transmission. For example, a therapeutically effective amount preferably relates to the amount of a therapeutic agent which reduces the transmission index, reduces the HIV viral load or reduces the number of infected cells
by at least 5%, preferably by at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more. A therapeutically effective amount referring to an HIV also relates to the amount of a therapeutic agent which increases the CD4+ cell count, increases the time to progression to AIDS or increases the survival time by at least 5%, preferably by at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more.

The administration of the virions of the invention or of the immune cells of the invention can be performed together with a pharmaceutically acceptable carrier or support. Occasionally, said pharmaceutically acceptable carrier or support is an adjuvant; an aluminum salt or an oil in water emulsion; an emulsifying agent and a metabolizable oil; or an immunostimulating agent, and the composition is administered to the subject by means of injection.

Said vaccines may further comprise at least one adjuvant which increases or mediates an immune response chosen from an innate immune response or an adaptive immune response. The adjuvants which can be used can increase the immune response by activating the antigen presenting cells (APC), macrophages or by stimulating specific groups of lymphocytes.

An adjuvant that may be suitable for the present invention can be any ligand suitable for the activation of a pathogen recognition receptor (PRR) expressed in and on dendritic cells (DC), T cells, B cells or other antigen presenting cells. The ligands activating the nucleotide binding oligomerization domain (NOD) receptor pathway may be suitable for the purposes of the invention. The adjuvants suitable for these ligands may be derived from muramyl dipeptide. The ligands activating the toll-like receptors (TLR) may also be suitable for the purposes of the invention. These receptors are members of the PRR family and are widely expressed in a variety of innate immune cells including DC, macrophages, mast cells and neutrophils.

The following can be mentioned as examples of ligands which activate TLR: for TLR4, monophosphoryl lipid A, 3-O-deacetylated monophosphoryl lipid A, E. coli LPS, taxol, RSV fusion protein and host heat shock proteins 60 and 70; for TLR2, lipopeptides such as N-palmitoyl-S-2,3(bispalmitoioxy)-propyl-cysteinyll-seryl-(lysyl)3-lysine, S. aureus peptidoglycan, M. tuberculosis lipoproteins, S. cerevisiae
zymosan and highly purified *P. gingivalis* LPS; for TLR3, dsRNA, flagellin TLR5 and TLR7 of synthetic compounds such as imidazoquinoline; or for TLR9, certain types of CpG-rich DNA. Other adjuvants useful for the invention may be T helper epitopes.

A T helper epitope is a peptide normally derived from exogenous proteins which have undergone protein degradation and processing in the endocytic pathway of antigen presenting cells (APC). In those cells, the class II human leukocyte antigen (HLA II) is associated with those peptides in the endosomes. When transported to the surface of the APCs, this antigen can interact with a specific T cell receptor of CD4 T lymphocytes which causes the activation thereof. As is known in the art, the response of the T cell can be Th1 or Th2 type, depending on the helper epitope.

An example of a Th-oriented response epitope is the pan DR helper T cell epitope (PADRE). This epitope is manipulated so that it binds to the more common high affinity HLA-DRA molecules and so that it acts as a strong immunogen. It has been shown that the PADRE HTL epitope increases the strength of vaccines designed for stimulating a cellular immune response. See Alexander J, *et al.*, Immunol. Res. 1998; 18:79-92.

The formulation of vaccines according to the invention uses an effective amount of the antigen of interest. In other words, it will include an amount of antigen which, in combination with the adjuvant, causes the subject to produce a specific and sufficient immune response such that it confers protection to the subject against subsequent exposure to HIV. When it is used as an immunogenic composition, the formulation will contain an amount of antigen which, in combination with the adjuvant, causes the subject to produce specific antibodies that can be used for diagnostic or therapeutic purposes.

The vaccine compositions of the invention may be useful for preventing HIV infection or for HIV infection therapy. While all animals that may be affected with HIV or its equivalents can be treated in this manner, the vaccines of the invention are particularly aimed at their preventive and therapeutic uses in human beings. More than one administration may often be required to produce the desired prophylactic or therapeutic effect; the exact protocol (dose and frequency) can be established by means of standard clinical procedures.

The vaccine formulations will contain an effective amount of the selected
antigen(s) which, when combined with the adjuvant, causes the vaccinated subject to produce a sufficient and specific immune response to the antigen. The vaccine compositions can also be used therapeutically for treating subjects already infected with HIV.

In some embodiments, it may be desirable to administer combination vaccines having a component which causes an immune response mainly against HIV strains with tropism for CCR5 receptor and a second component which causes an immune response mainly against HIV strains with tropism for CXCR4 receptor. It may also be desirable for one or both components to be formed by a mixture of antigens, such as for example, a mixture of antigens each of which causes an immune response against a particular HIV strain or group of HIV strains.

e) Compositions, kits and methods for obtaining the virions of the invention

The invention also relates to compositions or kits containing the components necessary to generate the pseudotyped VSV-HIV virions of the invention and to methods for obtaining them by co-transfecting the polynucleotides encoding the HIV viral genome of the invention and the VSV G protein in a suitable cell.

Therefore, in another aspect, the invention relates to a composition or kit comprising:

i) a polynucleotide comprising a human immunodeficiency virus (HIV) genome containing a mutation in the pol gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus; and

ii) a polynucleotide encoding the envelope glycoprotein G from a vesicular stomatitis virus (VSV).

In a further aspect, the invention relates to a method for the generation of a recombinant HIV/VSV pseudovirion comprising:

i) co-transfecting a receptive cell with:

a) a polynucleotide encoding the envelope glycoprotein G from a vesicular stomatitis virus (VSV) and
b) a polynucleotide comprising a human immunodeficiency virus (HIV) genome containing a mutation in the pol gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus, wherein the HIV genome further comprises a deletion in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene and wherein the HIV genome further comprises a sequence homologous to that of the region deleted by means of the mutation in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene, originating from a second HIV genome, wherein the genome of said second HIV is different from the HIV genome containing the deleted region,

ii) maintaining the cells obtained in i) under conditions adequate for formation of HIV and

iii) recovering HIV/VSV pseudovirions from the cell.

For the production of the pseudovirions of the invention, it is necessary to first incorporate into a receptive cell both: i) a vector comprising a HIV genome containing a mutation in the pol gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus; and ii) an expression plasmid comprising the nucleotide sequence encoding the G protein of VSV in a cell by using any of the co-transfection methods known in the art. See Ausubel F, et al., Eds., “Current Protocols in Molecular Biology” (John Wiley and Sons, Inc., New York, NY, US, 2003). Non-limiting illustrative examples of transfection methods include co-precipitation with calcium phosphate, DEAE-dextran, polybrene, electroporation, microinjection, liposome-mediated fusion, lipofection, retrovirus infection and biolistic transfection. In a particular embodiment, the transfection is carried out by means of co-precipitation with calcium phosphate. The cell in which the vectors are incorporated must be capable of expressing the information encoded in said vectors and packing the proteins of the virus and the genetic material thereof to form the viral particles having the G protein of VSV in their envelope. Receptive cells suitable for carrying out the present invention
include, among others, cells from the 293T cell line, a cell line derived from the highly transfectable 293 cell line (derived from human embryonic kidney cells), to which cell the SV40 T antigen of SV40 has been inserted (e.g. ATCC). The pseudovirions produced are released into the culture medium of the cells transfected with the vectors. Therefore, to obtain the virions of the invention it is necessary to collect the supernatants after several hours post-transfection, preferably 24 and 48 hours post-transfection. The non-replicative virions of the invention can be stored at –80°C.

All the embodiments disclosed for the HIV viral genome of the invention are also applicable to the compositions or kits of the invention and to the methods of the invention.

All publications mentioned herein are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

General Procedures and Results

1. Production of virions (or pseudovirions)

1.1 Generation of viral vectors

To generate the pNL4-3ΔRT vector, HIV genome-1 pNL4-3 (GenBank accession no. M19921.1) was used. See SEQ ID NO: 1 and Figure 1A. To that end, restriction sites which allowed readily “extracting” particular DNA fragments from the provirus matrix (such as, for example, the gene encoding RT, the pol, gag, nef or env genes, etc.) were initially inserted by means of directed mutagenesis in order to substitute them with the same genes originating from the isolates of patients to be assessed. This system of “cloning” and generating a “chimera virus” allows studying the characteristics of the different viral proteins of the patients. Therefore, the following main modifications were performed:
a) first, the deletion of HIV-1 fragments, for example, the deletion of a fragment comprising the gene encoding reverse transcriptase (RT), specifically the fragment comprised between position 2592 and 3485 of the pNL4-3 proviral vector; and

b) then, the LacZ gene (encoding the beta-galactosidase enzyme) was inserted substituting different sequences of the viral genome, on one hand, to find out the generation frequency of recombinant viruses, and, on the other hand, to avoid the carry-over of sequences of wild-type viruses.

The HIV-based recombinant viral clones of the present invention are characterized in that they have the general structure depicted in Figure 1, comprising in direction 5’ to 3’ the following elements:

a) LTR (long terminal repeats) or terminally redundant sequences containing various consensus sequences for transcription factors regulating viral expression;

b) gag, the gene encoding the p55 protein of the capsid formed by 3 protein subunits (MA, CA, and NC);

c) pol, the gene encoding the viral enzymes necessary for the viral replication process: protease (PRO), reverse transcriptase (RT) and integrase (INT), and it overlaps at its 5’ end with the gag element;

d) vif, the gene encoding the Vif protein associated with the infectivity of the extracellular virions, and it overlaps at its 5’ end with the pol gene and at its 3’ end with vpr;

e) vpr, the gene encoding the Vpr protein acting as an accelerator of the replication cycle at different levels, and it overlaps at its 5’ end with the vif gene;

f) tat, the gene encoding the Tat protein which is a transactivator, and its second exon is contained in the env sequence;

g) vpu, the gene encoding the Vpu protein involved in the release of the virions;

h) env, the gene encoding the gp160 protein of the viral envelope;

i) rev, the gene encoding the Rev protein, responsible for processing and transporting messenger RNA to the cytoplasm, and its second exon is contained in the env sequence; and
j) *nef*, the gene encoding the Nef protein which negatively regulates CD4 and HLA molecules of the infected cell and plays a role in the pathogenicity of the virus.

The pNL4-3ΔRT[lacZ]/Ren viral vector described in EP 1752541 as IP HIV NL LacZ/RT Ren is characterized by having the substitution of the 896 bp fragment of the *pol* gene encoding the RT between NcoI/AgeI with the LacZ gene. pNL4-3ΔRT[lacZ] was generated from this construct by means of removing the marker gene encoding the *Renilla* (Ren) luciferase protein (SEQ ID NO: 13) and restoring the sequence of the *nef* gene (90 bp) which had been previously removed, located between the NotI/XhoI restriction sites (in positions 8797 and 8887, respectively). For this purpose, the area with the oligonucleotides specific for pNL4-3, 52EU: (SEQ ID NO: 11) and 104ED: (SEQ ID NO: 12), the NotI and XhoI restriction sites being included in these oligonucleotides, was previously amplified. Once the 90 bp region of the *nef* gene was amplified from pNL4-3, it was cloned into the NotI (8797)/XhoI (8887) position, where the *Ren* gene had been removed, the plasmid pNL4-3 ΔRT[lacZ] having a size of 14,483 bp being generated.

The following step was the removal of a NarI restriction site located in the plasmid pNL4-3 outside the coding sequence of the virus (i.e. pUC18). The previously generated plasmid (pNL4-3 ΔRT[lacZ]) was used and digested with the AatII and ApaI restriction enzymes, a 3613 bp fragment (insert) being obtained, where the NarI site had already been removed. This fragment was cloned with the same corresponding restriction enzymes into pNL4-3ΔRT[lacZ], converting it into plasmid pNL4-3 ΔRT[LacZ], NarI. The size of the generated plasmid is about 14,483 bp.

The LacZ gene encoding the β-galactosidase protein (SEQ ID NO: 14) cloned into NcoI/AgeI by means of digestion with the corresponding restriction enzymes and the subsequent filling of the sticky ends with the DNA polymerase I Klenow fragment (GE Healthcare) was then removed and re-ligated, giving rise to plasmid pNL4-3 ΔRT, NarI- hereinafter called pNL4-3 ΔRT. The size of this plasmid generated is about 13,983 bp. See Figure 1B.

Different constructs of the invention were generated from pNL4-3 ΔRT; to that end the LacZ gene was cloned into the pNL4-3ΔRT vector in the position of *gag* gene
(pNL4-3ΔRT/Δgag-[LacZ]), env gene (pNL4-3ΔRT/Δenv-[LacZ]), env-nef gene (pNL4-3ΔRT/Δenv-nef-[LacZ]), nef gene (pNL4-3ΔRT/Δnef-[LacZ]) and in gag-pro gene (pNL4-3ΔRT/Δgag-pro-[LacZ]) for the purpose of increasing the cloning efficacy and avoiding the carry-over of minority populations of the reference virus. See Figures 1C and 12, Table 1. Thus, the ligated clones without the insert from the patient gave blue colonies, whereas the plasmid which incorporated the gag gene, env gene, env-nef gene, nef gene, or gag-pro gene originating from the patient gave white colonies. The following viral vectors were generated from pNL4-3 ΔRT:

1) pNL4-3 ΔRT/Δgag [LacZ]: It is characterized by the removal of part of the gag gene, 1368 bp, between the NarI (in position 637) and ApaI (in position 2006) restriction sites of pNL4-3ΔRT; the 500 bp amino-terminal end of the LacZ gene was cloned in its place with the same enzyme. The LacZ gene was amplified by means of PCR of plasmid pUC19 (Invitrogen®, Life Technologies Corp., Carlsbad, CA, US) with the NarI-LacZ-UP (SEQ ID NO: 15) and ApaI-lacZ-D (SEQ ID NO: 16) oligonucleotides. The plasmid generated is referred to as pNL4-3 ΔRT/Δgag [LacZ] with a total size of 13,115 bp.

2) pNL4-3 ΔRT KspI+: A new KspI (SacII) restriction site was introduced in position 9433 of pNL4-3 by directed mutagenesis. In order to generate this plasmid, the BamHI-NaeI fragment of pNL4-3-NotI was first subcloned into pBluescript II SK(-) vector (Stratagene®, Agilent Technologies Inc., Santa Clara, CA, US) at the same sites, the pBluescript SK/NL BamHI-NaeI vector being generated, and on the latter directed mutagenesis was performed to generate the KspI site with the following oligonucleotides: M-KspI-UP (SEQ ID NO: 17) and M-KspI-Down (SEQ ID NO: 18), the plasmid pBluescript SK/NL BamHI-NaeI, KspI+, being generated; the BamHI-NaeI fragment was then extracted from the latter plasmid with KspI mutation and was cloned into pNL4-3 ΔRT at the same restriction sites, giving rise to pNL4-3 ΔRT KspI+. The presence of the new target KspI restriction was confirmed by means of sequencing in the corresponding position of pNL4-3. The size of the vector generated was
13,983 bp.

3) pNL4-3 ΔRT/Δ nef [LacZ]: The 640 bp nef gene between NotI (nt 8797 of pNL4-3) and KspI (SacII) (nt 9437) was removed from pNL4-3 ΔRT KspI+ and the 500 bp amino-terminal end of the LacZ gene was cloned in its place with the same enzymes. The LacZ gene was amplified by means of PCR of plasmid pUC19 (Invitrogen®, Life Technologies Corp., Carlsbad, CA, US) with the NotI-LacZ-UP (SEQ ID NO: 19) and KspI-LacZ-D (SEQ ID NO: 20) oligonucleotides. 13,843 bp plasmid pNL4-3 ΔRT/Δ nef [LacZ] was generated.

4) pNL4-3 ΔRT KspI+, NcoI+: A new NcoI restriction site was introduced in position 6113 in pNL4-3 by mutagenesis. The oligonucleotides used were: Mut-NcoI-Env-UP (SEQ ID NO: 21) and Mut-NcoI-Env-down (SEQ ID NO: 22). The presence of the new target NcoI restriction was confirmed by means of sequencing in the corresponding position of pNL4-3. The size is 13,838 bp.

5) pNL4-3 ΔRT/Δ env [LacZ]: The 2683 bp env gene between NcoI (nt 6113) and NotI (nt 8797) was removed from pNL4-3 ΔRT KspI+, NcoI+; the 500 bp amino-terminal end of the LacZ gene was cloned in its place with the same enzymes. The LacZ gene was amplified by means of PCR of the plasmid pUC19 (Invitrogen®, Life Technologies Corp., Carlsbad, CA, US) with the 127 (SEQ ID NO: 23) and Not-LacZ-down (SEQ ID NO: 24) oligonucleotides. 11,800 bp plasmid pNL4-3 ΔRT/Δ env [LacZ] was generated.

6) pNL4-3 ΔRT/Δ env-nef [LacZ]: The 3320 bp env and nef genes between the NcoI and KspI (SacII) restriction sites in pNL4-3 ΔRT, KspI+, NcoI+ were removed at the same time, the 500 bp amino-terminal end of the LacZ gene was cloned in their place with the same enzymes. The LacZ gene was amplified by means of PCR of the plasmid pUC19 (Invitrogen®, Life Technologies Corp., Carlsbad, CA, US) with the 127 (SEQ ID NO: 23), and KspI-LacZ-D (SEQ ID NO: 20) oligonucleotides. 11,160 bp plasmid pNL4-3 ΔRT/Δ env-Nef [LacZ] was generated.
7) pNL4-3 ΔRT, KspI\(^+\), NcoI\(^-\), AgeI\(^+\): A new AgeI restriction site was introduced in position 2572 according to the sequence of pNL4-3 by mutagenesis. The primers used were: Mut-AgeI-UP (SEQ ID NO: 25) and Mut-AgeI-Down (SEQ ID NO: 26). The presence of the new target AgeI restriction was confirmed by means of sequencing in the corresponding position of pNL4-3. The size is 13,838 bp.

8) pNL4-3 ΔRT/Δgag-pro[LacZ]: The 1935 bp fragment comprising the gag and pro genes comprised between the Nar I (in position 637) and AgeI (in position 2572) sites of the pNL4-3 ΔRT, KspI\(^+\), NcoI\(^-\), AgeI\(^+\) was removed; the 500 bp amino-terminal end of the LacZ gene was cloned in its place with the same enzymes. The LacZ gene was amplified by means of PCR of the plasmid pUC19 (Invitrogen\textsuperscript{®}, Life Technologies Corp., Carlsbad, CA, US) with the primers NarI-LacZ-UP (SEQ ID NO: 15) and AgeI-lacZ-D (SEQ ID NO: 27). 12,403 bp plasmid pNL4-3 ΔRT/Δgag-pro [LacZ] was generated.

9) pNL4-3 ΔRT/Δ1136env: In order to generate the vector NL4-3 ΔRT/Δ1136env the vector NL4-3 ΔRT was used. A double digestion at 37\(^\circ\)C using the enzymes NcoI and NheI, that cut in the position 6113 and 7249 of the plasmid, was performed during 2 hours. This double digestion generate 2 fragment of 1136 and 11972 pb each. The digestion was load in a 1% agarosa gel in order to separate and identify both fragments. Once the fragments were identify a purification of the 11972 fragment by its extraction from the gel was performed. Posteriorly, an extension of the plasmid for its subsequent ligation with the enzyme Klenow was done. The ligation of this fragment was performed by adding the enzyme ligase during 12 hours at 16\(^\circ\)C generating a new plasmid of 11972 pb with an envelope deletion of 1136 pb. Laterly, the plasmid was sequenced and transfected in 293T cells in order to generate and characterize the virions produced.

10) pNL4-3 ΔRT, AgeI\(^+\)/Δ1136env: The 1136 bp env gen between the NcoI (in position 6113) and NheI (in position 7249) restriction sites was subcloned
from pNL4-3 ΔRT/Δ1136env to pNL4-3 ΔRT, KspI⁺, NcoI⁺, AgeI⁺. 11,972 bp plasmid pNL4-3 ΔRT, AgeI⁺/Δ1136env was generated.

11) pNL4-3 ΔRT, AgeI⁺ Clal⁺/Δ1136env: A new Clai restriction site was introduced in position 4924 according to the sequence of pNL4-3 by mutagenesis in the pNL4-3 ΔRT. The oligonucleotides used were: Mut-Clai-Up (SEQ ID NO: 42) and Mut-Clai-Down (SEQ ID NO: 43). The size is 13,838 bp. AgeI⁺/Δ1136env plasmid. 12,847 bp plasmid pNL4-3 ΔRT, AgeI⁺ Clal⁺/Δ1136env was generated.

12) pNL4-3 ΔRT, AgeI⁺ NcoI⁺,Clal⁺/Δ1136env: A new NcoI⁺ restriction site was introduced in position 2590 according to the sequence of pNL4-3 by mutagenesis in the pNL4-3 ΔRT, AgeI⁺,Clai⁺/Δ1136env. The oligonucleotides used were: Mut-Ncoi-Up (3’CCGGTAAAAATTTAAGCCAGTCCTGGAGTGTATTATGACCCG5’)

and

Mut-Ncoi-Down (3’GGGTCATAATACACTCCATGGACTGGCTTTAATTTTACCGG5’).

. The size is 13,838 bp. 12,847 bp plasmid pNL4-3 ΔRT, AgeI⁺ NcoI⁺ Clai⁺/Δ1136env was generated.

13) pNL4-3 ΔRT, AgeI⁺ Clai⁺: A new Clai restriction site was introduced in position 4924 according to the sequence of pNL4-3 by mutagenesis in the pNL4-3 ΔRT, AgeI⁺ plasmid. The oligonucleotides used were: Mut-Clai-Up (SEQ ID NO: 42) and Mut-Clai-Down (SEQ ID NO: 43). 13,983 pb plasmid pNL4-3 ΔRT, AgeI⁺ Clai⁺ was generated.

14) pNL4-3/Δ1136env: The 1136 bp env gen between the NcoI (in position 6113) and Nhel (in position 7249) restriction sites was subcloned from pNL4-3 ΔRT/Δ1136env to pNL4-3. 13743 bp plasmid pNL4-3/Δ1136env was generated.

15) pNL4-3/Δ2353pol: The 2353 bp pol gen between the AgeI (in position 2573) and Clai (in position 4924) restriction sites of pNL4-3 ΔRT, AgeI⁺ Clai⁺ were removed. 12526 bp plasmid pNL4-3/Δ2353pol was generated.

16) pNL4-3/Δ3489pe: The 3489 bp pol-env gen between Clai (in position 4924) and AgeI (in position 2573) restriction sites of pNL4-3 ΔRT, Clai⁺ AgeI⁺ were removed. 11390 bp plasmid pNL4-3/Δ3689pe was generated.
17) pNL4-3/Δ3470pe: By directed mutagenesis two new restriction sites for Clal and NcoI were introduced in the position 4924 and 2590 respectively in the plasmid NL4-3 ΔRT/Δ1136env. Once the new restriction sites were generated a double digestion at 37°C using the enzymes NcoI and AgeI, that cut in the position 4924 and 2590 of the plasmid, was performed during 2 hours. This double digestion generate 2 fragment of 2334 and 11409 pb each. The digestion was load in a 1% agarosa gel in order to separate and identify both fragments. Once the fragments were identify a purification of the 11409 fragment by its extraction from the gel was performed. Posteriorly, an extension of the plasmid for its subsequent ligation with the enzyme Klenow was done. The ligation of this fragment was performed by adding the enzyme ligase during 12 hours at 16°C generating a new plasmid of 11409 pb. Laterly, the plasmid was sequenced and transfected in 293T cells in order to generate and characterize the virions produced.

<table>
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<th>Plasmid</th>
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<th>bp</th>
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Vectors/chimeras were then constructed by means of cloning the env, gag, gag-pro, env-nef or nef gene fragments of HIV-1-infected patients in the previously described corresponding vectors in order to subsequently generate virions and to analyze the induced immune response. See Figures 1C, 12C, and Table 1.

The total blood viral DNA of HIV-1 positive patients under study were obtained from the cell pellet by means of the QI Amp DNA blood kit (Qiagen NV, Venlo, NL).

For the extraction of viral RNA, 1 ml of plasma from the patients under study was used; the viral RNA was extracted by means of the QI Amp kit (Qiagen NV, Venlo, NL). The extracted HIV-1 RNA was used as a template to obtain cDNA. The RNA was initially transcribed and subsequently amplified using the specific primers for each of the areas to be characterized. The amplified fragments were:

1) gag fragment: 1793 bp were amplified by means of specific primers [(107 (SEQ ID NO: 28) / 122 (SEQ ID NO: 29)) and (163 (SEQ ID NO: 30) / 123 (SEQ ID NO: 31))] which include NarI and Apal restriction sites in primers 163 and 123, respectively, necessary for the subsequent cloning thereof into the pNL4-3 ΔRT/Δgag[LacZ], pNL4-3 ΔRT/Δ1136env/Δgag[LacZ], and pNL4-3/Δ3489pe/Δgag[LacZ] proviral vectors substituting the corresponding LacZ gene.

2) gag-pro fragment: 1935 bp were amplified by means of specific primers [(107 (SEQ ID NO: 28) / 125 (SEQ ID NO: 40)) and (163 (SEQ ID NO: 30) / 159(SEQ ID NO: 41))] which include NarI and AgeI restriction sites in primers 163 (SEQ ID NO: 30) and 159 (SEQ ID NO: 41), respectively, necessary for the subsequent cloning thereof into the pNL4-3 ΔRT/Δgag-pro[LacZ], pNL4-3 ΔRT/Δ1136env/Δgag-pro[LacZ], and pNL4-3 Δ3489pe/Δgag-pro[LacZ] proviral vectors, the LacZ gene being removed first.

3) nef fragment: 637 bp were amplified by means of specific primers [(117 (SEQ ID NO: 35) / 118 (SEQ ID NO: 34) and (119 (SEQ ID NO: 32) / 121 (SEQ ID NO: 33))] which include NotI and KspI sites in primers 119 (SEQ ID NO: 32) and 121 (SEQ ID NO: 33), respectively, necessary for the subsequent cloning thereof into the pNL4-3 ΔRT/Δnef[LacZ], pNL4-3 ΔRT/Δ1136env/Δnef[LacZ], and pNL4-3 Δ3489pe/Δnef[LacZ] proviral
vectors after removing the LacZ gene located in the position of the nef gene.

4) env fragment: 2683 bp were amplified by means of specific primers 101 (SEQ ID NO: 37) / 102 (SEQ ID NO: 36) and 126 (SEQ ID NO: 39) / 161 (SEQ ID NO: 38) which include NcoI and NotI restriction sites in primers 126 (SEQ ID NO: 39) and 161 (SEQ ID NO: 38), respectively, necessary for the subsequent cloning thereof into the pNL4-3 ΔRT/Δenv[LacZ] proviral vector, the LacZ gene being removed first.

5) env-nef fragment: 3320 bp were amplified by means of specific primers 101 (SEQ ID NO: 37) / 118 (SEQ ID NO: 34) and 126 (SEQ ID NO: 39) / 121 (SEQ ID NO: 33) which include NcoI and Kspl restriction sites in primers 126 (SEQ ID NO: 39) and 121 (SEQ ID NO: 33), respectively, necessary for the subsequent cloning thereof into the pNL4-3 ΔRT/Δenv-nef[LacZ] proviral vector, the LacZ gene being removed first.

After amplification, an enzymatic digestion of the amplified product and of the vector was performed with the corresponding enzymes in each case, according to the manufacturer’s instructions, and an in vitro ligation process was performed using T4 ligase according to the manufacturer’s instructions. See Table 1. All the plasmids were amplified in bacterial strain stbl2\textsuperscript{tm} (Invitrogen\textsuperscript{®}, Life Technologies Corp., Carlsbad, CA, US). They were subsequently purified by means of maxi-preparations with the NL-tip 500 kit (Qiagen NV, Venlo, NL) and stored at -20°C. All the plasmids were verified by means of restriction analysis and subsequent sequencing.

1.2 Production and concentration of virions

Virions were generated by means of transfecting 293T cells (ATCC) with the different viral vectors under study through the calcium phosphate method (Profection Kit; Promega Corp., Madison, WI, US). 5 µg of plasmid DNA from different viral vectors (Qiagen NV, Venlo, NL) were used. G-VSV pseudotyped HIV_env vectors were generated by co-transfection of 293T cells with NL 4-3 ΔRT/Δ1136env or pNL4-3/Δ3489pe, and an expression plasmid for the G protein of VSV. The day before transfection, 1.5x10\textsuperscript{6} cells were seeded in 10 mL of DMEM-10 (DMEM supplemented
with 10% (v/v) fetal bovine serum) in 75 cm² culture bottles. At 24 hours post-transfection, the medium was changed and at 48 hours the supernatants were collected, clarified and subsequently concentrated. Once the virions were obtained, they were stored at -80°C. In any case, a supernatant was obtained in cells treated in the same manner but transfected with the “empty vector” which was used as negative control. The supernatant collected after transfection was centrifuged in 30 ml threaded tubes for 32 minutes at 28,000 rpm in a Thermo scientific Sorvall® WX Ultra Centrifuge Series 80 centrifuge (Thermo Fisher Scientific Corp., Waltham, MA, US). The supernatant was discarded. The pellet obtained after centrifugation was resuspended in 500 μL of phosphate buffered saline (PBS) which was transferred to an Eppendorf tube. They were again centrifuged for 10 minutes at 40,000 rpm. The supernatant was discarded and the pellet was resuspended in 500 μL of PBS.

The detection of the p24 antigen by means of the ELISA/Ag HIV technique (Innogenetics NV, Ghent, BE) was used to quantify the viral immunogen. The quantification was always performed before and after its concentration.

2. Characterization of the virions

2.1 Assessment of the viral replication in MT-2 cells

The assessment of the viral replication was performed by means of using marker gene-carrying virions, specifically the Ren (renilla) gene. The assessment of the viral replication was analyzed by means of an assay in MT-2 cells (catalog number 237, NIHARRRP, US National Institutes of Health, Bethesda, MD, US). The virions were obtained by transfection with the corresponding pNL4-3 Ren WT or pNL4-3 ΔRT Ren viral vectors, described in EP1752541, in 293T cells. At 48 hours the supernatant was collected and the p24 antigen was quantified by capture ELISA (Innogenetics NV, Ghent, BE). Part of the NL4-3 Ren WT virions was heat-inactivated at 56°C for 60 minutes.

In order to assess the replication capacity, 5 x 10⁴ MT-2 cells were seeded per well in 96-well round bottom plates at a final volume of 200 μL. They were subsequently infected with different dilutions of the viral stocks (NL4-3 Ren WT, heat-
inactivated NL4-3 Ren WT or NL4-3 ΔRT Ren) which had previously been quantified by means of p24 antigen quantification. The assay was performed in triplicate. The infections were maintained at 37°C in 5% CO₂ atmosphere. At 24 and 96 hours of the infection, the luciferase activity was determined with the Renilla Luciferase Assay System (Promega Corp., Madison, WI, US) following the manufacturer’s recommendations. The cells were lysed in 100 µL of the lysis buffer for 5 minutes at 4°C. The luciferase activity of these cell extracts was evaluated by introducing 50 µL of the cell lysate in a plate illuminator (Orion L®, Titertek-Berthold Inc., Huntsville, AL, US) programmed for injecting 50 mL of substrate. This value provides the relative light units (RLUs) per mL of viral stock at 24 or 96 hours of infection in MT-2 cells. See Figure 2 and Table 2. As it can be appreciated, both the heat-inactivated NL4-3 Ren WT virions and the NL4-3 ΔRT Ren virions lack replicative capacity.

### Table 2

<table>
<thead>
<tr>
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<th>24 hours</th>
<th>96 hours</th>
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</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>143 RLUs</td>
<td>100 RLUs</td>
</tr>
<tr>
<td>NL4-3 Ren WT</td>
<td>261841 RLUs</td>
<td>5 x 10⁶ RLUs</td>
</tr>
<tr>
<td>Heat-inactivated NL4-3 Ren WT</td>
<td>129 RLUs</td>
<td>400 RLUs</td>
</tr>
<tr>
<td>NL4-3 ΔRT Ren</td>
<td>922 RLUs</td>
<td>200 RLUs</td>
</tr>
</tbody>
</table>

RLUs: Relative light units

2.2 Assessment of the replication of NL4-3 ΔRT virions in TZM-bl cells

The replication of the NL3.4 ΔRT (DRT) and NL4-3 (WT) virions was assessed by means of quantifying luciferase activity in TZM-bl cells.

The TZM-bl indicator cells express the CD4 receptor and the CCR5/CXCR4 co-receptors with an integrated LTR-Luc reporter system. After assessing the p24 antigen, 1:4 dilutions of the transfection supernatant where the virions are placed in 96-well plates were prepared in triplicate. 10,000 TZM-bl cells were added and the plate was maintained in an incubator at 37°C in 5% CO₂ atmosphere. After 72 hours, the luciferase activity present in the cell extracts was analyzed in a luminometer (Turner
Biosystems, Sunnyvale, CA, US). The NL4-3 ΔRT (DRT) virions lacked replicative capacity. See Figure 3.

2.3 Confocal microscopy assay

A) Transfections in 293T cells

293T cells were co-transfected with the plasmids: pGAG-GFP (5 µg), pNL4-3 WT (3µg) + pGAG-GFP (2 µg), pNL4-3 ΔRT (3µg) + pGAG-GFP (2 µg) and pNL4-3 ΔRT/Δ1136env (2µg) + pGAG-GFP (2 µg) + pVSV-G (1 µg). At 48 hours post-transfection, the supernatant was collected and used to subsequently infect new susceptible cells. The cells were fixed with FACS lysis solution for 20 minutes at room temperature. After washing with PBT (PBS, 0.1% Tween® 20) the cells were stained with 4’,6-diamidino-2-phenylindole (DAPI) (1/10000) and phalloidin (1/1000) for 30 minutes at room temperature. Subsequently, the cells were mounted with mounting medium (Vectashield®, Vector Labs., Inc., Burlingame, CA, US). See Figure 4 A.

B) Infection

The virion was quantified by means of assessing the p24 antigen by ELISA (Innogenetics NV, Ghent, BE). The PBMC cells (peripheral blood mononuclear cells) (25x10⁶) were infected with the amount of virions corresponding to 2 µg of p24 antigen of the following virions obtained by co-transfection in 293T cells: NL4-3 + GAG-GFP, NL4-3 ΔRT + GAG-GFP and 1 mL of GAG-GFP.

THP-1 cells were incubated with the liso-tracker dye for 1 hour. After the incubation, cells were infected with NL4-3 + GAG-GFP, NL4-3 ΔRT + GAG-GFP and ΔRT/Δ1136env + VSV + GAG-GFP virions.

Infected cells were incubated between 3-4 hours at 37°C. The cells were subsequently collected, fixed, stained and prepared for visualization by confocal microscopy following the same process as that for the transfected cells. The images were taken with a LEICA SP2 confocal microscope and processed with ImageJ.

Both the virions originating from the NL4-3WT + GAG-GFP co-transfection
and NL4-3 ΔRT + GAG-GFP were capable of infecting PBMCs. See Figure 4B.

Pseudovirions originated from ΔRT/Δ1136env + VSV + GAG-GFP were capable of infecting cells through lisosomal pathway. See Figure 14.

2.4 Visualization of the virion by means of electron microscopy

293T cells were transfected using the mammal transfection kit (Promega Corp., Madison, WI, US) with two different pNL4-3 (5 μg) and pNL4-3 ΔRT (5 μg) constructs. Pseudovirions were generated by cotransfection of pNL4-3 ΔRT/Δ1136env (3 μg) and pVSV-G (2 μg) constructs. 48 hours after transfection, the supernatant was removed and the cells were cleaned with 1X PBS. The PBS was removed and the cell monolayer was maintained in 10 mL of 2.5% glutaraldehyde in PB (phosphate solution) for 20 minutes at 4°C. After these 20 minutes, the cells were passed to a 15 mL falcon and were centrifuged for 5 minutes at 1500 rpm. See López-Iglesias C, et al., Ultrastruct. Mol. Struct. Res. 1988; 101(1):75-91. The supernatants were clarified and subsequently concentrated by centrifugation for 32 minutes at 28,000 rpm in a Thermo Scientific Sorvall® WX Ultra Centrifuge Series 80 centrifuge (Thermo Fisher Scientific Corp., Waltham, MA, US). The supernatant was discarded and the sediment obtained after centrifugation was resuspended in 500 μL of PBS. It was again centrifuged for 10 minutes at 40,000 rpm. The sediment was resuspended in 500 μL of 2.5% glutaraldehyde in PB. Once the sediment was properly resuspended, it was left for 30 minutes at 4°C with the glutaraldehyde and after that time the glutaraldehyde was changed for another 500 μL of new glutaraldehyde. It was left for 1 hour at 4°C, the glutaraldehyde was removed and washed two or three times with 1% PB and it was left in PB until it was used in electron microscopy. The samples were stained and processed in paraffin as previously described. See López-Iglesias, 1988, supra.

The NL4-3 viral particles were perfectly formed (arrows) and their core well defined, and gemmating viral particles were also observed. See Figure 5A. In contrast, the NL4-3 ΔRT virions were immature and neither the formed core (arrows) nor the typical compaction of a WT viral particle. See Figure 5B.

It can be observed that the NL4-3 viral particles were perfectly formed in 90% of the cases. See Figure 5C. Purified viral particles obtained from the 293T cells
transfected with the pNL4-3 ΔRT constructs can be observed. See Figure 5D. 93% of the population had immature viral particle morphology. Pseudovirions from 293-T cells transfected with pΔRT/Δ1136env + pVSV-G were also observed. See Figure 13C. After purification, the pseudovirions showed immature particles with a thicker envelope.

2.5 Analysis of the expression of the HIV-1 gag protein by means of Western blot (wb)

In order to analyze the expression of the HIV-1 gag protein, extracts from 293T cells and from purified virions obtained 48 hours post-transfection in 293T cells with the indicated plasmids (pNL4-3, pNL4-3 ΔRT or pΔRT/Δ1136env + pVSV-G) were used. Approximately 2 x 10^6 293T cells per experimental point collected at 48 hours of transfection were used for preparing the cell extracts. The cells were washed in PBS and in buffer A (10 mM Hepes pH 8, 10 mM KCl, 15 mM MgCl2 and 1 mM DTT), resuspended in 20 μL of buffer C (20 mM Hepes pH 8, 0.1% NP-40, 25% glycerol, 420 mM NaCl, 10 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA (ethylenediaminetetraacetic acid), 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin, 0.5 μg/mL aprotinin, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM DTT (dithiothreitol)), left to lyse for 15 minutes at 4°C and centrifuged at 4,062 g for 10 minutes at 4°C. The supernatant obtained was diluted in buffer D (20 mM Hepes pH 8, 20% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin, 0.5 μg/mL aprotinin, 1 mM PMSF and 1 mM DTT) depending on the desired final protein concentration. The assessment of protein concentration was determined by means of the Bradford reaction (Biorad Labs., Inc., Hercules, CA, US). See Bradford M, Anal. Biochem 1976; 72:248-254.

10 μg of the different extracts were migrated in 10% polyacrylamide-SDS (sodium dodecyl sulfate) gel to perform the Western blot (WB). The transfer of the gels to polyvinylidene fluoride (PVDF) membranes (Sigma-Aldrich Co., Saint Louis, MO, US) previously incubated for 5 minutes in methanol was performed in transfer buffer (20 mM glycine, 150 mM Tris-base and 20% methanol) using an electrotransfer apparatus (Pharmacia®, Pfizer, Inc., New York, NY, US). The membranes were blocked by incubation at 37°C for 1 hour with 5% milk powder in 0.1% PBS/Tween-20.
After blocking, the membranes were incubated with a monoclonal antibody directed against the p24 protein (clone 491, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, US) at a 1:1000 dilution. After three 5-minute washings with 0.1% PBS/Tween-20, they were incubated with peroxidase-bound mouse anti-immunoglobulin antibodies for one hour at 37°C. Three 5 minute-washings were subsequently performed with 0.1% PBS/Tween-20. The detection of the antigen-antibody interactions was performed by means of the SuperSignal WestPico Chemiluminescent Substrate (Pierce®, Thermo Fisher Scientific Corp., Waltham, MA, US) chemiluminescence system and exposure to photographic film (Hyperfilm ECL, GE Healthcare, General Electric Co., Fairfield, CT, US). Both the NL4-3 and NL4-3 ΔRT (ΔRT) virions produced the gag protein. See Figures 6. NL4-3 virions processed gag completely, while NL4-3 ΔRT (ΔRT) and ΔRT/Δ1136env + VSV (VSV) virions had a defect in gag processing. See Figure 15.

3. Assessment of the cellular immune response

Once the NL4-3 ΔRT and ΔRT/Δ1136env + VSV (VSV) virions were obtained and characterized, the cellular immune response was assessed in two ways. It was first assessed by means of an ex vivo model with monocyte-derived dendritic cells (MD-DC), the capacity for producing the specific anti-HIV cellular response in CD4+ and CD8+ cells was assessed. Assays were also performed with PBMC to detect the specific anti-HIV cellular response, among others, intracellular cytokine detection (ICS) and the assessment of the number of IFN-γ producing cells and by means of ELISPOT.

3.1 Ex vivo generation of monocyte-derived dendritic cells (MD-DC) pulsed with immunogen

The autologous MD-DCs were obtained from the monocytes present in the PBMC obtained from an anticoagulated sample. The PBMCs were washed three times in serum-free culture medium, X-VIVO15, and resuspended (5x10⁶/mL) in said culture medium supplemented with 1% inactivated autologous serum and they were deposited in culture vials at 37°C and 1% CO₂ for 2 hours, during which time the monocytes adhered to the surface of the vial. The non-adhered cells (lymphocytes) were removed
by suction. The adhered monocytes were cultured for 5 days in X-VIVO 15 supplemented with 5% inactivated autologous serum in the presence of human recombinant IL-4 and GM-CSF at the dose of 1000 IU/mL for each cytokine and with or without 1 mM of AZT to test whether or not it was better to suppress the possible viral replication in relation to patients without antiretroviral treatment. After 5 days in culture, the monocytes turned into immature dendritic cells (DC) and they were ready to be pulsed with the different immunogens. At least 5x10^6 immature MD-DC were used for pulsing, they were washed two times in medium and resuspended in 1 mL of medium containing the inactivated HIV-1 or immunogen aliquot (1x10^9 virions) as well as GM-CSF+ IL4 (1000 IU/ml of each), they were maintained at 37°C for 5 hours. A cocktail of human recombinant cytokines (TNF-alpha+IL-6+IL-1β) inducing the maturation of MD-DCs was then added into the culture and it was prolonged for about 40 additional hours. See Figure 7. All the MD-DCs in culture (at least 5x10^6) were collected and centrifuged, and the supernatant was aliquoted to perform the latest possible cytokine and remaining HIV-1 measurements. The MD-DCs were washed two times with X-VIVO10 culture medium.

As controls in all the experiments, the MD-DCs were also pulsed with HIV recombinant proteins (p24), as positive control, HIV-1 recombinant p24 (Protein Sciences Corp., Meridien, CT, US). They were also pulsed with the SEA superantigen as positive control. The same steps were repeated for the negative controls with the exception that the MD-DCs were not pulsed with the immunogen.

3.2 Measurement and analysis of the immune response

A) Labeling with CFSE and flow cytometry analysis

The PBMCs were labeled for 20 minutes at 37°C with a 1.5 mM CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester) solution. After 2 washings, the labeled PBMCs were stimulated with HIV-1 recombinant proteins and the relevant controls for 6 days in culture medium supplemented with 10% fetal calf serum (FCS) in an incubator at 37°C and 5% CO₂. Subsequently and after the corresponding surface labeling, the frequency of CD4 and CD8 antigen specific cells that had been divided for
each stimulus was evaluated by means of flow cytometry analysis. See Figure 7.

The dilution of the CSFE label was also evaluated after co-culturing for 2 days MD-DCs pulsed with the different types of immunogens and fresh autologous T cells in a DC:T cell ratio of 1:10. In vitro studies were performed by pulsing MD-DC cells with NL4-3 ΔRT virions and analyzing the response induced in the autologous T cells of HIV-1 patients. The data obtained indicated that after co-culturing MD-DCs pulsed with NL4-3 ΔRT and autologous T lymphocytes, an increase of cell proliferation and cytokine production (IL-2 and IFNα) with respect to that obtained with recombinant proteins or with the heat-inactivated autologous virus itself was generated in particular patients. See Figure 8.

B) Detection of the intracellular cytokine production of specific anti-HIV-1 CD4/CD8 lymphocytes

After culturing the PBMCs of the patients for 6 or 18 hours with groups of HIV-1 peptides, recombinant virions and co-stimulating antibodies (CD28 and CD49d) in the presence of Brefeldin A (10 μg/mL), the cells were fixed and permeated. The phenotypic markers of the CD4/CD8 lymphocytes were subsequently added and the HIV-1 specific T lymphocytes were identified and quantified by means of staining with anti-IFN-γ, anti-TNF-α and anti-IL-2, and activation labels (CD38, CD69) after the subsequent flow cytometry analysis.

Similarly, the measurement of CD69 expressing and IFN-γ producing CD4 and CD8 T cells was performed by means of intracellular staining and cytofluorometric analysis after re-stimulating the autologous T lymphocytes with CDDM for 1-2 days.

C) Analysis of the specific response of CD8+ T cells by means of ELISPOT

The analysis of the specific response of CD8+ T cells was performed by means of using a specific ELISPOT based on the determination of the production of IFN-γ by the CD8+ T cells against dominant epitopes of the HIV-1 proteins restricted for HLA-class I antigens. The cells were stimulated with pools of 15-mer overlapping synthetic peptides of the HIV-1 gag, pol, env, and nef proteins, chimeric recombinant virions.
Assays with depletion of cell subpopulations (CD4, B, NK) were also performed by means of immunomagnetic selection to analyze the degree of participation thereof in the response obtained.

3.3 High-resolution HLA typing

High-resolution HLA typing was evaluated by means of PCR-SBT of the genomic DNA of peripheral blood mononuclear or polynuclear cells or cells from B cell lines. The polymorphic exons of the genes encoding HLA (mainly exon 2 of the DRB1 gene) were amplified using generic primers. The PCR products were sequenced by using the automatic fluorescent sequencing method and the sequences obtained were analyzed by means of suitable software (PerkinElmer, Inc. Waltham, MA, US).

3.4 Optimization of the ex vivo assay for the detection and characterization of the immune responses against HIV-1 using defective recombinant and non-replicative virions

Samples of approximately 20-30 asymptomatic chronic HIV+ patients with a high CD4 number (>500/mm³) were used. An assay for intracellular cytokine detection (ICS) used with different pre-stimulations of the PBMC has fundamentally been used. The pre-stimulations tested were: low doses of recombinant IL-2 (20 U/mL) or a cocktail with CD28 and CD49d (1 µg/mL). No differences have been found between these 2 types of pre-stimulations although the use of the cocktail of antibodies produced greater reproducibility of IFNγ and IL-2 secretion by the CD4 and CD8 cells. The immunogenic antigens assayed were heat-inactivated NL4-3 WT, NL4-3 ΔRT, and BAL and MN (reference strains from laboratory isolates, occasionally known as HIV-BaL and HIV-MN, respectively) inactivated by heat or by means of treatment with 1 mM AT-2 (aldrithiol-2) at 4°C overnight (dose corresponding to 20 µg of p24/mL). After stimulating the cells for 18 to 24 hours with the mentioned stimuli, the results obtained were heterogeneous, a marked response being observed in 20-30% of the patients (n≥6), the response induced by the NL4-3 ΔRT being very similar to that obtained by the entirely heat-inactivated virions. See Figure 9.
3.5 Comparison of the results obtained using the ex vivo model established with PBMC with the MD-DC co-culture model

Majority of the ex vivo assays with PBMC (ICS) were performed in parallel using both MD-DC and T lymphocytes of the same HIV-infected patients. The preliminary results showed a practically complete parallelism between both assays, the patients responding to the NL4-3 ΔRT virions and other heat-inactivated reference virions can be perfectly discriminated from the non-responding patients. The production of cytokines (IFNγ, IL-2) and the induction of proliferation in CD4 and CD8 cells were assessed. See Figure 10. There is a parallel grouping of patients depending on their response to the different immunogens between both assays. These data suggest that the ex vivo assay with PBMC optimized for the detection of the anti-HIV immune response could allow a rapid in vitro assessment of the immunogenic activity of possible future candidates responding to therapeutic vaccines based on MD-DC since the results can be analyzed at 48-72 hours, whereas the results after co-culturing with MD-DC required the prior generation of MD-DC for 7 days and the patient has to be subjected to two blood donations.

3.6 Assays with PBMC to detect specific anti-HIV cellular response measured by the number of IFN-γ producing cells (ELISPOT)

Several assays were performed to detect specific anti-HIV cellular response, such as evaluating the immunogenicity of ΔRT against the WT-AT2 (pNL4-3) virion, evaluating the effect of the inactivation by AT2 (aldrithiol-2) (WT-AT2 vs. ΔRT-AT2), evaluating the efficiency of the tropism of the envelope X4 (ΔRT) vs. R5 (BaL and AC10) and evaluating the efficiency of an alternative envelope using VSV-G–pseudotyped virions (VSV-ΔRT).

After assaying the immunogenicity of the ΔRT against WT-AT2 (NL4-3) virion, it was observed that the ΔRT produced higher response, measured by the number of IFN-γ producing cells (ELISPOT), in a larger number of patients from the analyzed cohort of asymptomatic HIV-1 positive patients. After evaluating the effect of
inactivation by AT2 (WT-AT2 vs ΔRT-AT2), it was observed that this inactivation process did not influence the immunogenicity of the viral particle. After evaluating the efficacy with respect to the tropism of envelopes X4 (NL4-3) vs. R5 (BaL and AC10) within the ΔRT no significant differences was observed. Finally, VSV-G pseudotyped virions had significantly augmented immunogenicity (up to 90%), (Table 3). To clarify this point we have compared the percentages of positive response between WT-AT2 and all ΔRT variants. See Figure 16A. Significant differences have been observed between them, confirming the advantages reported for ΔRT constructions: more immunogenic with a safer profile. See Table 3. Moreover, the assessment of the cellular response of the MD-DC cells against different immunogens: NL4-3 WT+AT-2, NL4-3 ΔRT/VSV, NL4-3 ΔRT and recombinant p24 using different concentrations (0.5, 1, and 5 μg/mL of p24), has been performed. The test performed with 9 responding patients and against one and the same stock of immunogens shows that the proliferation index in CD8\(^+\) is substantially enhanced (see Fig. 17).

Table 3

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<tr>
<th>Patients /Virions</th>
<th>Response</th>
<th>Mean SFC</th>
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<tr>
<td>16 de 69 correspond to WT-AT2</td>
<td>23%</td>
<td>269</td>
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<tr>
<td>38 of 69 correspond to ΔRT (X4)</td>
<td>55%</td>
<td>418</td>
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<tr>
<td>33 of 52 correspond to ΔRT-AT2</td>
<td>63%</td>
<td>664</td>
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<tr>
<td>21 of 41 correspond to ΔRT/AC10 (R5)</td>
<td>51%</td>
<td>286</td>
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<tr>
<td>23 of 43 correspond to ΔRT/BaL (R5)</td>
<td>53%</td>
<td>462</td>
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<tr>
<td>18 of 20 correspond to VSV-ΔRT</td>
<td>90%</td>
<td>1181</td>
</tr>
<tr>
<td>14 of 21 correspond to WT/APV-AT2</td>
<td>67%</td>
<td>1006</td>
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Finally, the immunogenicity of ΔRT virions, which are immature, was evaluated compared to the WT-AT2 (NL4-3) virion which had been treated previously in their production with a protease inhibitor (Amprenavir, 50 μM APV), WT/APV-AT2. Consequently, treatment with the inhibitor caused the production of immature virions. The immature particles were analyzed by electron microscopy. See Figure 11. After evaluating the immunogenicity of ΔRT compared to the WT/APV-AT2 virion, it was observed that WT/APV-AT2 produced a response, measured by the number of IFN-γ producing cells (ELISPot), higher than its untreated homolog (67% responding patients being obtained in a population of 21 patients). See Table 3 and figure 16B.

The autologous chimeras were evaluated as a way of enhancing the response. After evaluating the immunogenicity of ΔRT/Δgag ("patient") virions in patients in whom there was material for amplifying, either DNA or RNA, compared to the different non-chimeric virions described previously, it was observed that the response intensified up to 49%. The heterologous responses observed in other individuals were very heterogeneous. In the responding patients, the use of autologous Gag (for example, patient 596) involved an increase of 33% in the number of SFC with respect to its ΔRT analog. In patients categorized as non-responding patients who have not responded to any of the viral constructs, they only responded to the autologous Gag at its maximum concentration, concentrations greater than 600 ng of p24 antigen. See Table 4.

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<tr>
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<th>Gag 70</th>
<th>Gag 596</th>
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<td>70.1CS</td>
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<td>255</td>
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4. Immunization

In each of the patients who are going to receive vaccinations (i.e. monocyte-derived dendritic cells (MD-DC) pulsed with the infectious non-replicative HIV-1), the first step is the isolation and large scale production of HIV-1 by means of transfecting the vector into cells under GMP conditions. Said non-replicative infectious virion
comprises a viral genome containing a mutation in the pol gene such that the RT protein is non-functional and, in addition, the sequence of another gene different from pol gene substituted with the sequence of the same gene originating from the HIV of the patient. The virus obtained is concentrated and kept at -80°C until it is needed to pulse the MD-DCs of the same patient.

The MD-DCs are freshly prepared from the PBMC monocytes of a 150 mL venous blood sample which is drawn one week prior to each immunization and is immediately processed. After 5 days of culturing the monocytes to generate immature MD-DCs, they are pulsed with the non-replicative infective immunogen for 2 additional culture days, adding thereto a cocktail of MD-DC maturation-inducing cytokines. These will then be washed and administered immediately to the patient through sub-dermal injection close to the axillae. See Garcia F, et al., J. Infect. Dis. 2011, 203:473-478.
Example 1

The mutant NL4-3 delta RT + VSV as described in point 3 (Assessment of the cellular immune response, under General Procedures) was used to assess cellular response and immunogenicity. After evaluating the efficacy with respect to the tropism of envelopes X4 (NL4-3) vs. R5 (BaL and AC10) within the ΔRT no significant differences was observed: the envelopes R5 appeared to be slightly less immunogenic. Finally, VSV-G pseudotyped virions had significantly augmented immunogenicity (up to 90%), (Table 3). To clarify this point the percentages of positive response between WT-AT2 and all ΔRT variants have been compared. See Figure 16. Significant differences have been observed between them, confirming the advantages reported for ΔRT constructions: more immunogenic with a safer profile. See Table 3. Moreover, the assessment of the cellular response of the MD-DC cells against different immunogens: NL4-3 WT+AT-2, NL4-3 ΔRT/VSV, NL4-3 ΔRT and recombinant p24 using different concentrations (0.5, 1, and 5 μg/mL of p24), has been performed. The test performed with 9 responding patients and against one and the same stock of immunogens shows that the proliferation index in CD8⁺ is substantially enhanced (see Fig. 17).

Example 2

In order to generate the vector NL4-3 ΔRT/Δ1136env the vector NL4-3 ΔRT was used. A double digestion at 37°C using the enzymes NcoI and NheI, that cut in the position 6113 and 7249 of the plasmid, was performed during 2 hours. This double digestion generate 2 fragment of 1136 and 11972 pb each. The digestion was load in a 1% agarosa gel in order to separate and identify both fragments. Once the fragments were identify a purification of the 11972 fragment by its extraction from the gel was performed. Posteriorly, an extension of the plasmid for its subsequent ligation with the enzyme Klenow was done. The ligation of this fragment was performed by adding the enzyme ligase during 12 hours at 16°C generating a new plasmid of 11972 pb with an envelope deletion of 1136 pb. Laterly, the plasmid was sequenced and transfected in 293T cells in order to generate and characterize the virions produced.
Example 3

pNL4-3/Δ3470pe: By directed mutagenesis two new restriction sites for ClaI and NcoI were introduced in the position 4924 and 2590 respectively in the plasmid NL4-3 ΔRT/Δ1136env. Once the new restriction sites were generated a double digestion at 37°C using the enzymes NcoI and AgeI, that cut in the position 4924 and 2590 of the plasmid, was performed during 2 hours. This double digestion generate 2 fragment of 2334 and 11409 pb each. The digestion was load in a 1% agarosa gel in order to separate and identify both fragments. Once the fragments were identify a purification of the 11409 fragment by its extraction from the gel was performed. Posteriorly, an extension of the plasmid for its subsequent ligation with the enzyme Klenow was done. The ligation of this fragment was performed by adding the enzyme ligase during 12 hours at 16°C generating a new plasmid of 11409 pb. Laterly, the plasmid was sequenced and transfected in 293T cells in order to generate and characterize the virions produced.
0-1 Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)  

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10-19  

1-3 Identification of deposit  

1-3-1 Name of depository institution  

DSMZ DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH  

1-3-2 Address of depository institution  

Inhoffenstr. 7B, D-38124 Braunschweig, Germany  

1-3-3 Date of deposit  

27 March 2012 (27.03.2012)  

1-3-4 Accession Number  

DSMZ DSM 25917  

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Claims

1. A non-replicative virion comprising a human immunodeficiency virus (HIV) genome containing a mutation in the pol gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus; and wherein said virion further comprises an envelope glycoprotein G from a vesicular stomatitis virus (VSV).

2. A virion as in claim 1, wherein said mutation in the pol gene comprises the complete or partial deletion of said gene.

3. A virion as in claims 1 or 2, wherein said mutation in the pol gene gives rise to a non-functional RT protein.

4. A virion as in claim 3, wherein said mutation in the pol gene is a complete or partial deletion of the sequence encoding the RT protein.

5. A virion as in claim 4, wherein said complete or partial deletion of the sequence encoding the RT protein corresponds to the deletion in said sequence encoding the RT protein in the construct pNL4-3 ΔRT deposited under accession number DSM 25917.

6. A virion as in claims 1 to 5, wherein the HIV genome of the virion further comprises a mutation in the env gene, wherein said mutation causes the virus produced from said genome to lack a functional gp160 protein.

7. A virion as in claim 6, wherein said mutation in the env gene comprises the complete or partial deletion of said gene.

8. A virion as in claim 7, wherein the complete or partial deletion in the env gene corresponds to the deletion in the env gene in the construct pNL4-3 ΔRT/Δ1136env deposited under accession number DSM25923.
9. A virion as in claims 1 to 8, wherein the HIV genome of the virion further comprises a mutation in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene, wherein said mutation comprises the complete or partial deletion of said gene.

10. A virion as in claim 9, wherein the mutation is in a gene selected from the group consisting of the gag and nef genes.

11. A virion as in claim 9, wherein the mutation is in the sequence encoding the protease protein in the pol gene.

12. A virion as in claims 9 to 11, wherein the HIV genome of the virion further comprises a sequence homologous to that of the region deleted by means of the mutation in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene, originating from a second HIV genome, wherein the genome of said second HIV is different from the HIV genome containing the deleted region and wherein the sequence homologous is different to the sequence of the region deleted.

13. A virion as in claim 12, wherein said second HIV originates from a HIV-infected subject.

14. A virion as in claims 9 to 13, wherein the HIV genome of the virion further comprises a marker gene, wherein said marker gene is located at the site occupied by the region deleted by means of the mutation in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene.
15. A virion as in claims 1 to 14, wherein the mutations in the sequence encoding the RT protein and in the env gene correspond to the all delta construct pNL4-3/Δ3489pe.

16. A method for obtaining an activated immune cell comprising the steps:
   a) contacting an immune cell with a non-replicative virion as in claims 1 to 15,
   b) maintaining the mixture obtained in step a) in conditions suitable for infecting said immune cells with the virion and for processing the antigens originating from the non-replicative virion, and
   c) optionally, recovering the activated immune cells.

17. An in vitro method for obtaining a customized HIV vaccine for an HIV-infected subject, wherein said vaccine comprises activated immune cells, which comprises:
   a) contacting immune cells in vitro with a non-replicative virion as in claims 12 to 15 in which the genome of said second HIV originates from an HIV of said HIV-infected subject;
   b) maintaining the mixture obtained in step a) in conditions suitable for infecting said cells with the virion and for processing the antigens originating from the non-replicative virion;
   c) detecting and quantifying the activation of the immune cells activated according to steps a) and b);
   d) comparing the activation of said immune cells activated according to steps a) and b) with the activation of control immune cells;
   e) selecting the virions which cause a greater activation of the immune cells activated according to steps a) and b) than the activation of the control immune cells;
   f) infecting immune cells of said HIV-infected subject with the non-replicative virions selected in step e); and
   g) recovering the immune cells activated according to step f).

18. An in vitro method for obtaining a customized HIV vaccine for an HIV-infected subject, wherein said vaccine comprises activated immune cells, which comprises:
a) contacting immune cells originating from said HIV-infected subject in vitro with a non-replicative virion as in claims 12 to 15 wherein the genome of said second HIV originates from an HIV of said HIV-infected subject;
b) maintaining the mixture obtained in step a) in conditions suitable for infecting said immune cells with the virion and for processing the antigens originating from the non-replicative virion; and
c) recovering the immune cells activated according to step b).

19. The method as in claims 16 to 18, wherein said immune cell is selected from a lymphocyte, a peripheral blood mononuclear cell and a monocyte-derived dendritic cell.

20. The method as in claims 17 or 18, further comprising the step of subjecting the activated immune cells to a treatment to ensure the safety of said activated immune cells.

21. A cell obtainable according to the method as in claims 16 to 20.

22. A HIV viral genome containing a first mutation in the pol gene, wherein said first mutation causes the virus produced solely from said viral genome to be a non-replicative virus; wherein said genome further comprises a second mutation in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene, wherein said second mutation comprises the complete or partial deletion of said gene.

23. The HIV viral genome as in claim 22, wherein said first mutation in the pol gene comprises the complete or partial deletion of said gene.

24. The HIV viral genome as in claims 22 or 23, wherein said first mutation in the pol gene gives rise to a non-functional RT protein.
25. The HIV viral genome as in claims 22 to 24, wherein said genome further comprises a third mutation in the env gene, wherein said mutation causes the virus produced from said genome to lack a functional gp160 protein.

26. The HIV viral genome as in claim 25, wherein said third mutation in the env gene comprises the complete or partial deletion of said gene.

27. The HIV viral genome as in claims 22 to 26, wherein said second mutation is in a gene selected from the group consisting of the gag and nef genes.

28. The HIV viral genome as in claims 22 to 26, wherein the second mutation is in the sequence encoding the protease protein in the pol gene.

29. The HIV viral genome as in claims 22 to 28, further comprising a sequence homologous to that of the region deleted by means of said second mutation, originating from a second HIV genome, wherein the genome of said second HIV is different from the HIV genome containing the deleted region.

30. The HIV viral genome as in claim 29, wherein the sequence homologous is different to the sequence of the region deleted.

31. The HIV viral genome as in claims 29 or 30, wherein said second HIV originates from an HIV infected subject.

32. The HIV viral genome as in claims 22 to 28, further comprising a marker gene, wherein said marker gene is located at the site occupied by the region deleted by means of said second mutation.

33. A vector comprising a genome as in claims 22 to 32.
34. An immunogenic composition or a vaccine comprising a non-replicative virion as in claims 1 to 15, a cell as in claim 21, a HIV viral genome as in claims 22 to 31, or a vector as in claim 33 together with a pharmaceutically acceptable carrier.

35. A non-replicative virion as in claims 1 to 15, a cell as in claim 21, a HIV viral genome as in claims 22 to 31, or a vector as in claim 33 or an immunogenic composition or vaccine according to claim 34 for use in medicine.

36. A non-replicative virion as in claims 1 to 15, a cell as in claim 21, a HIV viral genome as in claims 22 to 32, or a vector as in claim 33 or an immunogenic composition or vaccine according to claim 34 for use in the prevention or treatment of a HIV infection or a disease associated with a HIV infection.

37. A composition or kit comprising:

(i) a polynucleotide comprising a human immunodeficiency virus (HIV) genome containing a mutation in the pol gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus; and

(ii) a polynucleotide encoding the envelope glycoprotein G from a vesicular stomatitis virus (VSV).

38. The composition or kit as in claim 37, wherein said mutation in the pol gene comprises the complete or partial deletion of said gene.

39. The composition or kit as in claims 37 or 38, wherein said mutation in the pol gene gives rise to a non-functional RT protein.

40. The composition or kit as in claims 37 to 39, wherein the HIV genome further comprises a mutation in the env gene, wherein said mutation causes the virus produced from said genome to lack a functional gp160 protein.
41. The composition or kit as in claim 40, wherein said mutation in the env gene comprises the complete or partial deletion of said gene.

42. The composition or kit as in claims 37 to 41, wherein the HIV genome further comprises a mutation in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene, wherein said mutation comprises the complete or partial deletion of said gene.

43. The composition or kit as in claim 42, wherein the mutation is in a gene selected from the group consisting of the gag and nef genes.

44. The composition or kit as in claim 42, wherein the mutation is in the sequence encoding the protease protein in the pol gene.

45. The composition or kit as in claims 42 to 44, wherein the HIV genome further comprises a marker gene, wherein said marker gene is located at the site occupied by the region deleted by means of the mutation in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene.

46. The composition or kit as in claims 42 to 45, wherein the HIV genome further comprises a sequence homologous to that of the region deleted by means of the mutation in at least a gene selected from the group consisting of the gag, nef, tat, rev, vif, vpu, and vpr genes or in the sequence encoding the protease protein in the pol gene, originating from a second HIV genome, wherein the genome of said second HIV is different from the HIV genome containing the deleted region and wherein the sequence homologous is different to the sequence of the region deleted.

47. The composition or kit as in claim 46, wherein said second HIV originates from a HIV-infected subject.
48. Method for the generation of a recombinant HIV virion comprising:
   (i) co-transfected a receptive cell with:
      a) a polynucleotide encoding the envelope glycoprotein G from a
         vesicular stomatitis virus (VSV) and
      b) a polynucleotide comprising a human immunodeficiency virus (HIV)
         genome containing a mutation in the pol gene, wherein said mutation
         causes the virus produced solely from said genome to be a non-
         replicative virus, wherein the HIV genome further comprises a
         deletion in at least a gene selected from the group consisting of the
         gag, nef, tat, rev, vif, vpu and vpr genes or in the sequence encoding
         the protease protein in the pol gene and wherein the HIV genome
         further comprises a sequence homologous to that of the region
         deleted by means of the mutation in at least a gene selected from the
         group consisting of the gag, nef, tat, rev, vif, vpu and vpr genes or in
         the sequence encoding the protease protein in the pol gene,
         originating from a second HIV genome, wherein the genome of said
         second HIV is different from the HIV genome containing the deleted
         region, and wherein the sequence homologous is different to the
         sequence of the region deleted,
   (ii) maintaining the cells obtained in (i) under conditions adequate for formation
        of HIV, and
   (iii) recovering HIV virions from the cell.

49. The method as in claim 48, wherein said mutation in the pol gene comprises the
    complete or partial deletion of said gene.

50. The method as in claims 48 or 49, wherein said mutation in the pol gene gives rise
to a non-functional RT protein.
51. The method as in claims 48 to 50, wherein the HIV genome further comprises a mutation in the env gene, wherein said mutation causes the virus produced from said genome to lack a functional gp160 protein.

52. The method as in claim 51, wherein said mutation in the env gene comprises the complete or partial deletion of said gene.

53. The method as in claims 48 to 52, wherein the mutation is in a gene selected from the group consisting of the gag and nef genes.

54. The method as in claims 48 to 52, wherein the mutation is in the sequence encoding the protease protein in the pol gene.

55. The method as in claims 48 to 55, wherein said second HIV originates from a HIV-infected subject.
FIG. 2

[Graph showing RLU values for different samples over time: Negative control, NL4.3-Ren (wt), Inactivated NL4.3-Ren (wt), NL4.3DRT-Ren. The graph compares 24 h and 96 h time points.]
A

Overlay
GAG-GFP
ART+GAG-GFP
WT+GAG-GFP

B

Overlay
GAG-GFP
ART+GAG-GFP
WT+GAG-GFP

FIG. 4
A

NL4-3 virion-producing 293T cells

B

NL4-3 ΔRT virion-producing 293T cells

FIG. 5
C

Purified NL4-3 virions

D

Purified NL4-3 ΔRT virions

FIG. 5 (cont.)
FIG. 6
FIG. 7
**A**  
![S.I. 5GS](image)

**B**  
![IL-2 5GS 48 hours](image)

**C**  
![IFN-g 5GS 6 days](image)

*FIG. 8*
NL-4.3-ΔRT induced HIV specific immunoresponse

% CD8+ T cell proliferation

% CD4+ T cell proliferation

SI

**

FIG. 9 (cont.)
**Ex vivo model of MDDC**

**Assays in PBMC**

**20MC**

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<td>P24</td>
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**Stimuli**

- PMA + iono
- Gag mix
- NL4-3, IRT

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**3AM**

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**Stimuli**

- PMA + iono
- Gag mix
- NL4-3, IRT

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**13LN**

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<tr>
<td>Be lat, Be lat-2</td>
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**Stimuli**

- PMA + iono
- Gag mix
- NL4-3, IRT

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**Non-responder**

**Responder**

**Good responder**

FIG. 10
A

Purified NL4-3 virions without treatment with Amprenavir

B

Purified NL4-3 virions treated with Amprenavir

FIG. 11
A
NL4-3 virion-producing 293T cells

B
NL4-3 ΔRT virion-producing 293T cells
FIG. 13

NL4-3 ΔRT/Δ1136env/VSV virion-producing 293T cells

FIG. 14

NL4-3 + Gag-GFP
NL4-3 ΔRT + Gag-GFP
NL4-3 ΔRT/Δ1136env/VSV + Gag-GFP
**FIG. 16**

A

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<tr>
<td>VSV-ΔRT</td>
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Bar graph showing % of positive responses for various conditions.

B

Scatter plot showing SFC in 10^6 PBMC for WT-AT2 and WT+ADV+AT2 conditions.

Legend:
- * indicates statistical significance
- ** indicates higher significance
- *** indicates highest significance
FIG. 17
### A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N7/00  C12N7/04  C12N5/10  A61K39/21

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

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N  A61K

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

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

EPO-Internal, BIOSIS, WPI Data

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>1-21, 37-55</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

**Special categories of cited documents:**

- **A** document defining the general state of the art which is not considered to be of particular relevance
- **E** earlier application or patent but published on or after the international filing date
- **L** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- **O** document referring to an oral disclosure, use, exhibition or other means
- **P** document published prior to the international filing date but later than the priority date claimed

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

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

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

**A** document member of the same patent family

Date of the actual completion of the international search: 9 December 2013

Date of mailing of the international search report: 20/12/2013

Authorized officer: Galli, Ivo

Form PCT/ISA/210 (second sheet) (April 2005)
<table>
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This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

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

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

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

see additional sheet

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

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.
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This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-21, 37-55
   (i) A non-replicative virion comprising a human HIV genome containing a mutation in the pos gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus; and therein said virion further comprises an envelope glycoprotein G from a vesicular stomatitis virus.
   (ii) A method of obtaining an activated immune cell using said virion, and a cell so obtainable.
   (iii) In vitro methods of obtaining a customized HIV vaccine using said virion.
   (iv) A kit comprising a polynucleotide comprising a human HIV genome containing a mutation in the pos gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus, and a polynucleotide encoding the G protein of VSV.
   (v) A method of producing a recombinant HIV virion using the content of the kit.

2. claims: 22-36

A non-replicative virion comprising a human HIV genome containing a mutation in the pos gene, wherein said mutation causes the virus produced solely from said genome to be a non-replicative virus. Immunogenic compositions thereof and corresponding medical uses.