(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 October 2006 (12.10.2006)

PCT

(10) International Publication Number
WO 2006/106435 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/IB2006/001058

(22) International Filing Date: 5 April 2006 (05.04.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0507003.2 6 April 2005 (06.04.2005) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2006/106435 A2

(54) Title: THERAPEUTIC

(57) Abstract: A fusion protein comprising a Nef double mutant (V 153 L, E 177 G) polypeptide (hereinafter Nef7) as shown in Seq ID No: 1 associated with a heterologous polypeptide.

Therapeutic

Background of the Invention

The present invention relates to a fusion protein comprising HIV-1 Nef mutant and its use in therapy and diagnosis, particularly, but not exclusively in the field of cancer, and infectious diseases.

Protein delivery into eukaryotic cells is a field of investigation having a great deal of potential applications. The general aim is delivering selected molecules, like drugs or prodrug modifying enzymes, to appropriate cell targets. The technical procedures currently available rely on the ability of liposomes (1) and nanoparticles (2) to be internalized by cells. Other approaches exploit the unusual properties of some cellular or viral proteins to actively cross the cell membrane, as for *Drosophila antennapedia* homeotic transcription factor (3), basic Fibroblast Growth Factor (4), Human Immunodeficiency Virus (HIV)-1 Tat (5-6), Herpes Simplex Virus (HSV) VP-22 (7), and Hepatitis B Virus PreS2 (8). However, their therapeutic potentialities are limited by the difficulties in the cell targeting. On the other hand, precise cell targeting could be ensured by the insertion of desired receptors in virus-like particle (VLP) envelopes.

VLPs have been already generated by engineering the genomes of a wide number of virus species, including Human Papilloma virus (9, 10), Human Hepatitis B and C virus (11, 12) retroviruses (13) and lentiviruses (for a review, see 14).

With regard to HSV-TK delivery, in one instance, HSV-1 TK was delivered to cells upon fusion with 11 amino acids from the basic domain of HIV-1 Tat, which impart cell membrane translocating ability (15). Another approach attempted to deliver HSV-1 TK upon the formation of complexes with protein-lipid artificial structures (16). However, neither approach guarantees the cell targeting selectivity, which may be achieved by expressing the appropriate receptor in packaging cells.

The most significant result in terms of protein delivery by means of retroviral particles has been recently published by Galla et al. (17). In this study, the viral mRNA-dependent delivery of a derivative of the bacteriophage endonuclease Cre into target

cells is described. Since the cargo activity strictly depended on the presence of viral mRNA, the genetic inactivation of critical duplication functions of the viral genome was required, with the undesired residual possibility of viral cDNA integration, virus expression and replication. In contrast, we have now demonstrated the efficiency of Nef7 as a cargo molecule even in the absence of the viral genome inside the viral particles, which represents a great improvement in terms of safety.

The progression of AIDS deeply depends on alterations in immunological functions. On the basis of molecular epidemiology data in humans, and results obtained in animal models, a key role in AIDS pathogenesis has been proposed for the HIV regulatory protein Nef. Following the studies on animals, the conclusions drawn by former observations demonstrating the lack of disease in macaques inoculated with *nef* deleted SIV strain, have been strongly enforced by results obtained in trangenic mice. In particular, it has been reported that the Nef expression in cells of the lympho-monocytic lineage induced severe AIDS-like pathologies, mainly correlating with the presence of the Nef Src Homology-3 (SH3) motif binding domain. Consistently, a huge mass of data consistently indicates that Nef interferes with the signaling pathways in lymphocytic cells.

Despite its original denomination (i.e., NEgative Factor), Nef acts as an enhancer of the viral infectivity. While previous observations suggested that the expression of Nef in the producer cells increases the retrotranscription activity in the target cells, more recent papers suggested that Nef acts by increasing the viral infectivity through the stimulation of the intravirion retrotranscription activity, or by increasing the cytoplasmic delivery of virions through a mechanism acting at the level of viral entry. Finally, the possibility that Nef influences the functions of the viral envelope has been also proposed.

We have now found that high levels of Nef7 may be incorporated into virion. Despite the fact that all wild type (wt) Nef isoforms are expected to be incorporated in virions, there is no data in the literature describing Nef alleles with increased virion incorporation activity. Hence, the high levels of Nef7 virion incorporation described herein is surprising.

In lentiviruses, it has been widely demonstrated that non-structural proteins like Vif, Vpr, and Nef are incorporated in viral particles (for reviews, see 18, 19 and 20). In general, the respective amounts appear too limited to allow efficient protein delivery strategies. However, in the case of Vpr, a number of papers reporting the efficient packaging of Vpr with either a bacterial nuclease (21), GFP or CAT proteins (21-24), with the C-terminal part of HIV-1 Vpu protein (24), or with single-chain antibodies (24), have been published. Of note, large bodies of evidence demonstrated that the incorporation of Vpr-fusion proteins can lead to a severe reduction of the virus infectivity by targeting multiple steps of viral morphogenesis (22, 23). In contrast, we have found that the presence of Nef7-related fusion products in packaging cells did not appear to hinder either the production or the infectivity of the lentiviral virions. In addition, while it is well established that Vpr has apoptotic/cell-cycle arresting activities (for a review, see 25), we detected no major anti-cellular effects were by Nef7.

We have also found that Nef7 based VLP targeted to HIV infected cells may be used in the treatment of persistently HIV-1 infected cells, that in AIDS patients represent virus reservoirs resistant to HAART therapies.

Statements of the Invention

According to one aspect of the present invention there is provided a fusion protein comprising a Nef double mutant (V 153 L, E 177 G) polypeptide (hereinafter Nef7) as shown in Seq ID No: 1 associated with a heterologous polypeptide.

In one embodiment the heterologous polypeptide is associated with the C terminus of Nef7.

In one embodiment the heterologous polypeptide is a therapeutic or diagnostic polypeptide.

In one embodiment the therapeutic polypeptide is a tumor antigen selected from the group consisting of BAGE-1, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GnTV^{f}, HERV-K-MEL, KM-HN-1, LAGE-1, MAGE-A1,

MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A9, MAGE-A10, MAGE-A12, MAGE-C2, MART-1, mucin^{k}, NA-88, NY-ESO-1/LAGE-2, SAGE, Sp17, SSX-2, SSX-4TRP2-INT2^{g} tumor antigens.

In a preferential embodiment the tumor antigen is MAGE-A3

In another embodiment the therapeutic polypeptide has antitumor properties

In another embodiment the therapeutic polypeptide is an enzyme capable of converting a non-toxic prodrug into an active cytotoxic drug selected from the group consisting of :

a) Herpes Simplex Virus derived Thimidine Kinase (TK)
b) E.coli derived cytosine deaminase
c) Mammalian cytocrome P450

In a preferred embodiment the therapeutic polypeptide is TK.

In another embodiment the therapeutic polypeptide is a viral antigen.

In a preferred embodiment viral antigen is selected from the group consisting of:

a) Human Papilloma Virus derived oncoproteins E6 and E7 and derivatives thereof
b) Human Hepatitis C derived NS3, NS5, E1, E2, Core Antigen, and derivatives thereof
c) HIV derived Tat and Env and derivative thereof

In another embodiment the diagnostic polypeptide is a detectable moiety.

According to another aspect of the present invention there is provided a polynucleotide encoding the protein of the present invention.

According to another aspect of the present invention there is provided an expression vector comprising the polynucleotide of the present invention.

According to another aspect of the present invention there is provided a viral particle (or VLP) comprising the protein or expression vector of the present invention. Also referred to herein as Nef 7-based VLP.

In one embodiment the viral particle is derivable from a retrovirus or lentivirus, such as HIV or MLV.

In one embodiment the viral particle is pseudotyped.

In one embodiment the viral particle is pseudotyped with the viral envelope protein VSV-G, RD114 glycoprotein, MLV glycoprotein or a chimeric protein comprising the extracellular and transmembrane domains of RD114 and cytoplasmic domain of MLV glycoproteins.

In another embodiment the viral particle is pseudotyped with HIV cell receptors.

In a preferred embodiment the viral particle is pseudotyped with CD4 and CXCR4 or with CD4 and CCR5 HIV cell receptors.

In another embodiment the viral particle is pseudotyped with a tumor cell surface antigens.

In another embodiment the viral particle is pseudotyped with a ligand of a tissue marker.

According to another aspect of the present invention there is provided a cell comprising the protein, the polynucleotide, the expression vector or the viral particle of the present invention.

In one embodiment the cell is a host cell.

In another embodiment the cell is a tumor cell.

In another embodiment the cell is a dendritic cell, a lymphocyte or a monocyte.

According to another aspect of the present invention there is provided a method comprising exposing a cell obtainable from an individual to the protein or the viral particle of the present invention.

According to another aspect of the present invention there is provided a cell obtainable from the method of the present invention.

According to another aspect of the present invention there is provided a pharmaceutical composition comprising the protein, the cell or the viral particle of the present invention, together with a pharmaceutically acceptable carrier, excipient or diluent.

According to another aspect of the present invention there is provided the protein, the polynucleotide, the expression vector, the cell or the viral particle of the present invention for use in medicine.

According to yet another aspect of the present invention there is provided use of the protein, the polynucleotide, the expression vector, the cell or the viral particle of the present invention for the preparation of a medicament for the treatment of cancer.

According to a further aspect of the present invention there is provided use of the protein, the polynucleotide, the expression vector, the cell or the viral particle of the present invention for the treatment of infectious diseases.

Some Key Advantages of the Invention

The surprisingly high efficiency of Nef7 virion incorporation enables us to provide a novel strategy of protein delivery based on the use of HIV-1-based VLPs. Nef7-based VLPs may be a useful tool for example: i) as part of alternative protocols for gene therapy-based cell suicide strategies targeted to malignancies; ii) for the therapeutic targeting of infected cells;iii) in cancer vaccines and for the preparation of vaccines against infectious diseases and iv) for the targeting to specific cells and tissues of therapeutic or diagnostic polypeptide.

In more detail, our data shows that the efficient delivery of adequate amounts of TK would render the transduction of HSV-TK expressing vectors unnecessary for cell suicide therapeutic designs, with obvious advantages in terms of the overcoming of both technical and safety limitations due to the transfer, insertion, and expression of foreign DNA in target cells.

In addition, VLPs can selectively recognize a cell target in which pathologic alterations lead to the expression on the cell membrane of specific markers. This can occur in tumors (e.g. melanoma-associated antigens) (26) as well as in infected cells. In the latter case, a therapeutic intervention could rely on the delivery of Nef7/fusion molecules by means of the "inverse" fusion between VLPs carrying the viral cell receptors, and the infected cells expressing the virus receptor.

One of the most attracting new anti-cancer strategy relies on the development of cancer vaccines (for a review, see 27). This essentially consists of loading dendritic cells (DC) with tumor associated antigens in an attempt to induce an immune response effective in selectively destroying tumor cells (for a review, see 28). In this respect, different strategies have been considered, including the use of recombinant tumor antigens, *ex vivo* treatment with lysates from tumor cells, and electroporation of mRNA expressing tumor-associated antigens. In any case, the strongest anti-tumor effect is expected when the tumor-associated antigen related products are presented in both Class I and II MHC. In this regard, the use as immunogen of tumor antigens delivered by Nef7-based VLPs may have an advantage from the fact that, as previously demonstrated for HIV virions (21), they can recognize the so-called "cross- presentation" process, leading to the exposition in both Class I and Class II MHC.

With a similar approach and with the same advantages Nef7-based VLPs can also be used for the preparation of vaccine against infectious diseases.

In conclusion, the Nef7-based VLPs of the present invention can be regarded as a both reliable and versatile protein delivery system exploitable for a variety of experimental/therapeutic purposes for example, both human and murine systems.

The present invention also relates to a novel anti-HIV therapeutic approach based on the recovery of anti-HIV-1 Env targeted VLPs carrying a fusion protein of the present invention and expressing T- or M-specific HIV cell receptor complexes. Such VLPs deliver the fusion protein upon the "inverse fusion" process. In a therapeutic context this may ultimately lead to cell death. For example, use may be made of a Nef/TK product in the presence GCV. In particular (CD4-CXCR4) and (CD4-CCR5) Nef7-based VLPs efficiently enter cells infected by T- or M-tropic HIV-1 strains, respectively. Importantly, the delivery of the VLP-associated Nef7/TK correlated with the induction of cell death upon 5 to 7 days re-culture with GCV. Of interest, VLPs were effective also against *ex vivo*, non-replicating human monocyte-derived macrophages infected by HIV-1 *in vitro*. Thus HIV-targeted VLPs may be used for the treatment of persistently HIV-1 infected cells, that in AIDS patients represent virus reservoirs resistant to HAART therapies.

Depending on the envelope protein incorporated in the VLP, Nef7 based VLPs can be targeted to specific cells and tissue with great efficiency. This system allows to deliver therapeutic or diagnostic polypeptide to a target cells or tissues.

Detailed Description of the Invention

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example. Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc (as well as the complete version Current Protocols in Molecular Biology).

Fusion Protein

The present invention relates to a fusion protein, or conjugate, which is a molecule comprising at least one Nef7 protein linked to at least one heterologous polypeptide (or peptide or protein) such as may be formed through genetic fusion or chemical coupling. By linked we mean that the first and second sequences are associated such that the second sequence is able to be transported by the first sequence in a viral

vector. Thus, the fusion protein of the present invention includes proteins in which the Nef7 protein is linked to a heterologous polypeptide via their polypeptide backbones through genetic expression of a DNA molecule encoding these proteins, directly synthesised proteins and coupled proteins in which pre-formed sequences are associated by a cross-linking agent. The term is also used herein to include associations, such as aggregates, of the Nef7 with the heterologous polypeptide. According to one embodiment the second sequence may comprise a polynucleotide sequence. This embodiment may be seen as a protein/nucleic acid complex.

The second sequence is heterologous in the sense that it is different to the Nef7 protein. However, the second sequence may be from the same species as the first sequence.

The Nef7 may be coupled directly to the heterologous polypeptide or indirectly through a spacer, which can be a single amino acid, an amino acid sequence or an organic residue.

Nef

Nef is a myristoylated regulatory protein expressed by HIV-1/2 and SIV having molecular weights of 27 and 34 kDa, respectively, and lacking any enzymatic activity (for reviews, see 30-32).

The Nef N-terminal myristoylation ensures a preferential cell membrane localization, correlating with its virion incorporation occurring upon the viral protease-mediated cleavage at the amino acid positions 57-61 (depending on the allele considered) (33-36). The biological significance of the Nef virion incorporation remains to be fully elucidated, even if recently it was suggested that virionic Nef is involved in the HIV penetration through the cortical actin network (37). As for the mechanism of Nef virion incorporation, whilst not wishing to be bound by any theory it is believed that the Nef virion incorporation occurs mainly as the consequence of the disposition of Nef at the cell membrane, and more in particular at the "detergent resistant microdomains" (rafts) (38), from which HIV preferentially buds (39). This is

consistent with the observation that Nef incorporates also in heterologous retroviruses, such as the Moloney Leukemia Virus (34).

By back-mutating the F12-Nef allele (32), we isolated a Nef mutant (Nef7) showing an efficiency of incorporation so high that it allows its use as a carrier molecule.

In more detail, we cloned a Nef double mutant (the $^{V}153^{L}$, $^{E}177^{G}$, here defined Nef7 and having the sequence shown in SEQ ID NO:1) having the unique property to undergo virion incorporation at quite high levels. This can occur whether Nef7 is expressed *in cis* or *in trans* with respect to the viral genome. Through densitometric analysis, we estimated a 20-50 fold increase of the Nef7 virion incorporation compared with wild-type (wt) Nef. (wt HIV-1 Nef incorporates in virions at low levels, i.e. about 10 molecules per viral particle.) We have also found that Nef7 may be used with viral particles other than HIV since HIV-products are not required for the high incorporation of Nef7 into the virions. Thus, the Nef7 mutant is useful for both lenti- and retroviral particles, and indeed other types of viral particles.

We have also found that advantageously the inclusion of the Nef7-based fusion products of the present invention has no major negative effects on viral particle assembling.

We have further found that the Nef7-based fusion products are incorporated at high levels in empty virions. This is advantageous from a safety point of view.

We have further found that the Nef7-based fusion products of the present invention may be used to efficiently kill both replicating and resting cells.

Viral Particles or Viral Like Particles (VLPs)

We have found that the Nef-7 mutant is able to transfer proteins using viral particles. The viral particles may be empty in the sense that they do not contain genetic material usually associated with viral particles, and are hence often referred to as viral like particles or VLPs.

Thus, the VLPs of the present invention, together with the use of the Nef7 mutant represent a new drug delivery system. This drug delivery system may be applicable in general for the delivery of a wide range of polypeptides, peptides and proteins. Compared to the use of fusion proteins with viral sequences per se, encapsidation into VLPs has the advantage that the protein of interest is protected inside a stable protein shell from external proteases. It is also possible to modify the surface of the VLP so as to allow targeting of specific cell types. During in vitro assembly, different proteins of interest could be encapsidated and administered simultaneously with, for example, a defined ratio.

During the past decade, gene therapy has been applied to the treatment of disease in hundreds of clinical trials. Various tools have been developed to deliver genes into human cells. Whilst the present invention relates to the delivery of proteins, use may be made of viral particle systems initially designed for gene therapy application.

Heterologous Polypeptide

The heterologous polypeptide of the present invention may be any polypeptide of interest, but is generally one which has a therapeutic effect or a potential therapeutic effect. By "therapeutic" we include diagnostic and preventative uses. The heterologous polypeptide may be obtainable from or produced by any suitable source, whether natural or not. The heterologous polypeptide may be designed or obtained from a library of peptides. It may be a peptidomimetic, a peptide cleaved from a whole protein, a peptide synthesised synthetically (such as, by way of example, either using a peptide synthesiser) or by recombinant techniques or combinations thereof. It may also be a peptide under test.

The term "polypeptide" as used herein includes peptides and proteins. The term "polypeptide" includes single-chain polypeptide molecules as well as multiple-polypeptide complexes where individual constituent polypeptides are linked by covalent or non-covalent means.

It will be understood that the polypeptide sequences for use in the invention are not limited to the particular sequences or fragments thereof but also include homologous

sequences obtained from any source, for example related viral/bacterial proteins, cellular homologues and synthetic peptides, as well as variants or derivatives thereof. Polypeptide sequences of the present invention also include polypeptides encoded by the polynucleotides of the present invention.

Peptides of the present invention may be administered therapeutically to patients. It is preferred to use peptides that do not consisting solely of naturally-occurring amino acids but which have been modified, for example to reduce immunogenicity, to increase circulatory half-life in the body of the patient, to enhance bioavailability and/or to enhance efficacy and/or specificity.

A number of approaches have been used to modify peptides for therapeutic application. One approach is to link the peptides or proteins to a variety of polymers, such as polyethylene glycol (PEG) and polypropylene glycol (PPG) – see for example U.S. Patent Nos. 5,091,176, 5,214,131 and US 5,264,209.

Replacement of naturally-occurring amino acids with a variety of uncoded or modified amino acids such as D-amino acids and N-methyl amino acids may also be used to modify peptides

Another approach is to use bifunctional crosslinkers, such as N-succinimidyl 3-(2 pyridyldithio) propionate, succinimidyl 6-[3-(2 pyridyldithio) propionamido] hexanoate, and sulfosuccinimidyl 6-[3-(2 pyridyldithio) propionamido]hexanoate (see US Patent 5,580,853).

It may be desirable to use derivatives of the peptides of the invention which are conformationally constrained. Conformational constraint refers to the stability and preferred conformation of the three-dimensional shape assumed by a peptide. Conformational constraints include local constraints, involving restricting the conformational mobility of a single residue in a peptide; regional constraints, involving restricting the conformational mobility of a group of residues, which residues may form some secondary structural unit; and global constraints, involving the entire peptide structure.

The active conformation of the peptide may be stabilised by a covalent modification, such as cyclization or by incorporation of gamma-lactam or other types of bridges. For example, side chains can be cyclized to the backbone so as create a L-gamma-lactam moiety on each side of the interaction site. See, generally, Hruby et al., "Applications of Synthetic Peptides," in Synthetic Peptides: A User's Guide: 259-345 (W. H. Freeman & Co. 1992). Cyclization also can be achieved, for example, by formation of cysteine bridges, coupling of amino and carboxy terminal groups of respective terminal amino acids, or coupling of the amino group of a Lys residue or a related homolog with a carboxy group of Asp, Glu or a related homolog. Coupling of the .alpha-amino group of a polypeptide with the epsilon-amino group of a lysine residue, using iodoacetic anhydride, can be also undertaken. See Wood and Wetzel, 1992, Int'l J. Peptide Protein Res. 39: 533-39.

Another approach described in US 5,891,418 is to include a metal-ion complexing backbone in the peptide structure. Typically, the preferred metal-peptide backbone is based on the requisite number of particular coordinating groups required by the coordination sphere of a given complexing metal ion. In general, most of the metal ions that may prove useful have a coordination number of four to six. The nature of the coordinating groups in the peptide chain includes nitrogen atoms with amine, amide, imidazole, or guanidino functionalities; sulfur atoms of thiols or disulfides; and oxygen atoms of hydroxy, phenolic, carbonyl, or carboxyl functionalities. In addition, the peptide chain or individual amino acids can be chemically altered to include a coordinating group, such as for example oxime, hydrazino, sulfhydryl, phosphate, cyano, pyridino, piperidino, or morpholino. The peptide construct can be either linear or cyclic, however a linear construct is typically preferred. One example of a small linear peptide is Gly-Gly-Gly-Gly which has four nitrogens (an N_4 complexation system) in the back bone that can complex to a metal ion with a coordination number of four.

A further technique for improving the properties of therapeutic peptides is to use non-peptide peptidomimetics. A wide variety of useful techniques may be used to elucidating the precise structure of a peptide. These techniques include amino acid sequencing, x-ray crystallography, mass spectroscopy, nuclear magnetic resonance spectroscopy, computer-assisted molecular modelling, peptide mapping, and

combinations thereof. Structural analysis of a peptide generally provides a large body of data which comprise the amino acid sequence of the peptide as well as the three-dimensional positioning of its atomic components. From this information, non-peptide peptidomimetics may be designed that have the required chemical functionalities for therapeutic activity but are more stable, for example less susceptible to biological degradation. An example of this approach is provided in US 5,811,512.

Retroviruses

Genetically engineered retroviruses are currently the most popular tool for gene delivery, and use may be made of such viruses in the present invention. Most of the systems contain vectors that are capable of accommodating proteins of interest and helper cells that can provide the viral structural proteins. Retroviridae is a family of retroviruses that differs in nucleotide and amino acid sequence, genome structure, pathogenicity, and host range. This diversity provides opportunities to use viruses with different biological characteristics to develop different therapeutic applications. As with any delivery tool, the efficiency, the ability to target certain tissue or cell type, and the safety of retroviral-based systems are important. Significant efforts have been dedicated to these areas of research in recent years in relation to their use in gene therapy. Various modifications have been made to retroviral-based vectors and helper cells to target delivery, improve viral titers, and increase safety. The present invention represents an improvement in this design process in that it acts to efficiently deliver proteins of interest into such viral vectors. In addition, since the VLPs of the present invention may be designed so that they do not contain viral genomic material, they are incapable of replication and hence represent a major advantage over the use of viral particles in a gene therapy context.

Viruses from several different families have been modified to generate viral vectors for gene delivery, and may also be used in the present invention. These viruses include retroviruses, adenoviruses, adeno-associated viruses, herpes simplex viruses, picornaviruses, and alphaviruses. The present invention preferably employs retroviruses, including lentiviruses.

An ideal retroviral vector for gene delivery must be efficient, cell-specific, regulated, and safe. The efficiency of delivery is important because it can determine the efficacy of the therapy. Safety is a major issue for viral gene delivery because most viruses are either pathogens or have a pathogenic potential. It is important that during gene delivery, the patient does not also inadvertently receive a pathogenic virus that has full replication potential. As indicated above, the present invention avoids such problems in that the VLPs of the present invention lack the genomic material of the wild-type virus.

Retroviruses are RNA viruses that replicate through an integrated DNA intermediate. Retroviral particles encapsidate two copies of the full-length viral RNA, each copy containing the complete genetic information needed for virus replication. Retroviruses possess a lipid envelope and use interactions between the virally encoded envelope protein that is embedded in the membrane and a cellular receptor to enter the host cells. Using the virally encoded enzyme reverse transcriptase, which is present in the virion, viral RNA is reverse transcribed into a DNA copy. This DNA copy is integrated into the host genome by integrase, another virally encoded enzyme. The integrated viral DNA is referred to as a provirus and becomes a permanent part of the host genome. The cellular transcriptional and translational machinery carries out expression of the viral genes. The host RNA polymerase II transcribes the provirus to generate RNA, and other cellular processes modify and transport the RNA out of the nucleus. A fraction of viral RNAs are spliced to allow expression of some genes whereas other viral RNAs remain full-length. The host translational machinery synthesizes and modifies the viral proteins. The newly synthesized viral proteins and the newly synthesized full-length viral RNAs are assembled together to form new viruses that bud out of the host cells. In the present invention, this viral RNA which has the ability to be reverse transcribed and subsequently integrated in the host genome is omitted from the VLP.

Based on their genome structures, retroviruses can be classified into simple and complex retroviruses. Simple and complex retroviruses encode gag (group-specific antigen), pro (protease), pol (polymerase), and env (envelope) genes. In addition to these genes, complex retroviruses also encode several accessory genes. These may be removed, if appropriate, in the present VLPs.

Retroviruses can also be classified into oncoviruses, lentiviruses, and spumaviruses. Most oncoviruses are simple retroviruses. Lentiviruses, spumaviruses, and some oncoviruses are complex retroviruses. Currently, all three types of viruses are being exploited as gene therapy tools and may be used in the present VLPs. Examples of each type will be discussed below.

Murine leukemia virus (MLV) is example of an oncovirus, human immunodeficiency virus 1 (HIV-1) is an example of a lentivirus, and human foamy virus is an example of a spumavirus.

When a replication-competent retrovirus infects a natural host cell, it can form a provirus in the host genome, express viral genes, and release new infectious particles to infect other hosts. In most gene therapy applications, it is not desirable to deliver a replication-competent virus into a patient because the virus may spread beyond the targeted tissue and cause adverse pathogenic effects. Therefore, in most retroviral systems designed for gene delivery, the viral components are divided into a vector and a helper construct to limit the ability of the virus to replicate freely.

The term vector generally refers to a modified virus that contains the gene(s) of interest and cis-acting elements needed for gene expression and replication. In the present invention this is replaced by the Nef7 fusion protein of the present invention.

Helper cells are engineered culture cells expressing viral proteins needed to propagate retroviral vectors; this is generally achieved by transfecting plasmids expressing viral proteins into culture cells. Most helper cell lines are derived from cell clones to ensure uniformity in supporting retroviral vector replication. Helper viruses are not used often because of the likelihood that a replication-competent virus could be generated through high frequency recombination. Helper functions can also be provided by transient transfection of helper constructs to achieve rapid propagation of the retroviral vectors.

Most retroviral vectors are maintained as bacterial plasmids to facilitate the manipulation and propagation of the vector DNA. These double-stranded DNA

vectors can be introduced into helper cells by conventional methods such as DNA transfection, lipofection, or electroporation. The helper cell expresses all of the viral proteins (Gag, Gag-Pol, and Env) but lacks RNA containing the packaging signal. As previously discussed, viral RNA is necessary for the formation and release of infectious viral particles, but it is not necessary for the formation of "empty" noninfectious viral particles. We have found that the Nef7 fusion protein of the present invention is efficiently packaged into viral particles. The viral particles contain viral proteins expressed from helper constructs and the Nef7 fusion protein of the present invention. In one embodiment the Nef7 fusion protein of the present invention is expressed from an expression vector which has been incorporated into the helper cell. These viral particles can infect target cells, and deliver the protein of interest to the target cell. However, because the vector does not express any viral proteins, it cannot generate infectious viral particles that can spread to other target cells.

Helper cells are designed to support the propagation of retroviral vectors. The viral proteins in the helper cells are expressed from helper constructs that are transfected into mammalian cells. Helper constructs vary in their mode of expression and in the genes they encode.

Split-Genome Helper Constructs

The safety concern associated with the generation of replication-competent viruses has provoked the design of many helper cell lines using "split genomes", including CRIP, GP+envAm12, and DSN. In these helper cells, the viral Gag/Gag-Pol polyproteins are expressed from one plasmid and the Env proteins are expressed from another plasmid. Use may be made of such constructs in the present invention. These are particularly useful when a pseudotyped VLP is being prepared.

Inducible Helper Constructs

In contrast to the helper cell lines described above that express viral proteins constitutively, some helper cell lines have been designed to express the viral proteins in an inducible manner. One rationale for the generation of an inducible helper cell

line is that some viral proteins are cytotoxic and cannot be easily expressed at high levels. By using an inducible system, expression of the cytotoxic proteins can be limited to the stage in which virus is propagated. By controlling the expression of the cytotoxic proteins, high viral titers can be achieved. Examples of the inducible helper cells include the 293GPG cells and HIV-1 helper cell lines.

Transient Transfection Systems

With the development of efficient transfection methods, transient transfection systems have also been developed for propagation of retroviral vectors. In these systems, helper functions are generally expressed from two different constructs, one expressing gag-pol and another expressing env. These two constructs generally share little sequence homology. The retroviral vector and the helper constructs are transfected into cells, and viruses are harvested a few days after transfection.

Systems That Generate Pseudotyped Viruses

Pseudotyping refers to viral particles containing a viral genome from one virus and part (or all) of the viral proteins from a different virus. The most common form of pseudotyping involves one virus using the envelope protein of another virus. Some of the helper cell lines contain helper constructs that express gag-pol from one virus and env from another virus. Since the Gag polyproteins select the viral RNA, the viral vector to be propagated contains an RNA that is recognized by the Gag polyprotein expressed in these cells. However, the viral particles produced contain the Env protein derived from another virus. Therefore, these viral particles can only infect cells that express a receptor that can interact with the heterologous envelope protein. For example, the helper cell line PG13 expresses gag-pol from MLV and env from gibbon ape leukemia virus (GaLV). Because the PG13 cell line expresses MLV Gag polyprotein, it can efficiently package MLV-based retroviral vectors. It has also been shown that some envelopes derived from viruses of a different family can also pseudotype retroviruses and generate infectious viral particles. For example, the G protein of vesicular stomatitis virus (VSV), a rhabdovirus, can be used to generate pseudotyped retroviral vectors. These VSV G pseudotyped viruses exhibit a very

broad host range and can infect a variety of cells that cannot normally be infected with retroviruses.

Pseudotyping may involve for example a retroviral genome based on a lentivirus such as an HIV or equine infectious anaemia virus (EIAV) and the envelope protein may for example be the amphotropic envelope protein designated 4070A. Alternatively, envelope protein may be a protein from another virus such as an Influenza haemagglutinin. In another alternative, the envelope protein may be a modified envelope protein such as a mutant or engineered envelope protein. Modifications may be made or selected to introduce targeting ability or to reduce toxicity or for another purpose.

For example to obtain the specific targeting of hematopoietic cells, the viral particle can be pseudotyped with a chimeric glycoprotein comprising the extracellular and the transmenbrane domains derived from the glycoprotein of the feline endogenous virus RD114 and the cytoplasmic domain of the glycoprotein derived from MLV-A (WO03091442)

In another embodiment, and particularly for use in anti-HIV therapy, the viral particle may express T- or M-specific HIV cell receptor complexes. In one embodiment the viral particle may incorporate CD4 and CXCR4 or CCR5 in their envelope.

Systems Containing Genetically Modified env for Cell or Tissue Targeting

Interactions between the viral envelope proteins and the cellular receptors determine the host range of the virus. Strategies have been developed to target virus delivery into certain cell types by modifying the viral Env. After translation and modification, the SU portion of Env interacts with a cellular receptor. The modification of the SU portion of Env is often achieved by deletion of a part of the coding region for SU and replacing it with regions of other proteins. Proteins that have been used to modify the SU portion of Env include erythropoietin, heregulin, insulin-like growth factor I, and single-chain variable fragment antibodies against various proteins.

Vectors Derived from Oncoviruses

Vectors derived from three different oncoviruses will be described here to represent some of the most widely used retroviral vectors. Oncoviruses can only infect dividing cells; therefore, vectors that are derived from oncoviruses can only be used to efficiently deliver genes into dividing cells. The requirement for cell proliferation can sometimes be used as an advantage to selectively target rapidly dividing cells (for example, cancer cells). However, protein delivery through the use of VLPs is independent of the cell replication even if the VLP derives from an oncoretrovirus. This is an additional advantage of the present invention.

1. Murine Leukemia Virus-Based Vectors.

Currently, MLV-based retroviral vectors and helper cells are the most frequently used system for gene delivery. The development and availability of engineered vectors and helper cell lines has promoted the popularity of MLV-based vectors and they may be effectively used in the present invention.

MLV-based vectors can be propagated in all of the MLV helper cell lines efficiently. There are several MLV envelope proteins that dictate the host range of MLV vectors. Viruses that use the ecotropic envelope can infect mouse cells but not cells derived from other species. Viruses that use the amphotropic envelope can infect both mouse cells and cells derived from other species, including human cells. Viruses that use the xenotropic envelope cannot infect mouse cells but can infect cells derived from other species. In addition, MLV vectors can also be propagated in spleen necrosis virus (SNV)-based helper cell lines. SNV is an avian virus that is distantly related to MLV.

2. Spleen Necrosis Virus-Based Vectors.

The required viral sequences in these vectors are very similar to those of the MLV vectors. SNV-based vectors can be propagated in SNV-based helper cell lines such as C3A2, DSDH, DSH134G, and DSN.

3. Rous Sarcoma Virus- and Avian Leukosis Virus-Based Vectors.

RSV is the onlyknown acute oncogenic retrovirus that is replication-competent. ALV has also been modified to generate vectors that require helper cells for their propagation.

Vectors Derived from Lentiviruses

In contrast to the oncoviruses, some lentiviruses have been shown to infect nondividing, quiescent cells. Lentiviruses are complex retroviruses. As examples of lentivirus-based vectors, HIV-1- and HIV-2-based vectors are commonly used.

Vectors Derived from Spumaviruses

Foamy viruses are unconventional retroviruses in that many features in their replication cycle are different from those of oncoviruses and lentiviruses. Although these viruses can be toxic to cultured cells, none of the foamy viruses are known to cause any disease in hosts.

Vectors Targeted to Specific Cells

An important goal for gene therapists is to develop a means to target gene delivery to specific cell types or tissues. At least two strategies have been used in an effort to target gene delivery using retroviral vectors. One strategy is designed to control gene delivery at the point of virus entry into the host cell by using natural or genetically engineered envelope proteins that interact with cell-type-specific receptors. Another strategy is designed to control expression of the therapeutic gene in specific cell types by using tissue-specific promoters.

Virus Host Range

The nature of the viral envelope protein determines whether a certain virus can enter a target cell. Therefore, it is important to consider whether the target cells have the correct cell surface receptor before the selection of an envelope protein that will be used for virus production (as discussed above).

Therapeutic Applications

Therapy approaches involving the viral delivery system of the present invention may be used to treat different types of diseases where use of a protein is appropriate. Traditionally, the development of drugs has focused on small molecule therapeutics. However, with recent advances in recombinant protein technology the potential of proteins as therapeutics is starting to be realized. Already there are protein drugs on the market, including naturally occurring proteins and engineered proteins.

For example, diseases that are amenable to protein therapy include a variety of cancers, heart attacks, strokes, cystic fibrosis, Gaucher's disease, diabetes, anaemia and haemophilia and graft-versus-host disease after bone marrow transplantation. The direct introduction of proteins into cells may be useful to a variety of fields including cell cycle regulation, control of apoptosis, oncogenesis and transcription regustion. Several different therapy strategies may be used to treat a variety of cancers. These strategies include elimination of cancer cells by suicide therapy, reversion of cancer cells to normal cells by delivery of a functional tumor suppressor protein, and modification of cancer cells to elicit stronger immune responses.

Another potential application of protein therapy is to prevent severe graft-versus-host disease that often results from allogeneic bone marrow transplantation. For example, a bone marrow donors' lymphocytes may be first transduced with a retroviral vector comprising herpes simplex virus thymidine kinase enzyme (HSV-tk). HSV-tk is not toxic by itself; however, HSV-tk can phosphorylate a nontoxic prodrug named ganciclovir (GCV) to activate the toxicity of the drug. The HSV-tk-containing cells may then used for bone marrow transplantation.

The present invention allow to deliver therapeutic proteins specifically to HIV-1 infected cells. Generally, these treatments have involved modification of the syngeneic lymphocytes *ex vivo* using retroviral vectors and are designed to suppress the expression of viral genes.

Cytotoxic proteins

The Nef7 mutant may be associated with such proteins, and used as a delivery tool for introducing the protein into a VLP. In a preferred embodiment the protein is an anti-cancer agent.

Drug-sensitivity proteins

One approach to increase the differential response of anti-cancer treatments between tumor and normal tissue is suicide therapy. This involves the transfer of enzymes that convert non-toxic pro-drugs into toxic antimetabolites. Examples of such enzyme are E.coli cytosine deaminase, TK and the mammalian cytochrome P450 (CYP2B1), which confer sensitivity to 5-fluorocytosine, GCV and cyclophosphamide (CPA), respectively. The present invention may employ any such pro-drug; although in a preferred embodiment of the present invention use is made of TK.

Cancer Vaccines

Dendritic cell (DC)-based vaccines are being developed for treatment of patients with cancer, in part because DCs are potent inducers of CD8+ CTL. DC MHC class I:antigenic peptide complexes that are required for CTL elicitation are most often generated by incubating DCs with peptides or by transfecting (or transducing) DCs with cDNAs or viral vectors that encode protein antigens. The use of viral vectors may be hampered by the preparation of such viral vectors. The present invention allows for the efficient delivery of the tumor antigen by the VLP directly to the target cell. Any useful tumor antigen, e.g. the SV40 large T antigen, may be employed in the present invention. Table A shows a list of known tumor antigens which could be used in the present invention.

Table A

Gene	HLA[a]	HLA Frequency[b] (%)	Peptide	Position	Lymphocyte Stimulation Method
BAGE-1	Cw16	7	AARAVFLAL	2-10	autologous tumor cells
GAGE-1,2,8	Cw6	18	YRPRPRRY	9-16	autologous tumor cells
GAGE-3,4,5,6,7	A29	6	YYWPRPRRY	10-18	autologous tumor cells
GnTV[f]	A2	44	VLPDVFIRC(V)	intron	autologous tumor cells
HERV-K-MEL	A2	44	MLAVISCAV	1-9	autologous tumor cells
KM-HN-1	A24	20	NYNNFYRFL	196-204	peptide
	A24	20	EYSKECLKEF	499-508	peptide
	A24	20	EYLSLSDKI	770-778	peptide
LAGE-1	A2	44	MLMAQEALAFL	ORF2 (1-11)	autologous tumor cells
	A2	44	SLLMWITQC	157-165	peptide
	A31	5	LAAQERRVPR	ORF2 (18-27)	autologous tumor cells
	A68	8	ELVRRILSR	103-111	adenovirus-dendritic cells
	DP4	75	SLLMWITQCFLPVF	157-170	peptide
	DR3	21	QGAMLAAQERRVPRAAEVPR	ORF2 (14-33)	protein
	DR4	24	AADHRQLQLSISSCLQQL	139-156	protein
	DR11	25	CLSRRPWKRSWSAGSCPGMPHL	ORF2 (81-102)	peptide
	DR12	5	CLSRRPWKRSWSAGSCPGMPHL	ORF2 (81-102)	peptide
	DR13	19	ILSRDAAPLPRPG	108-120	autologous tumor cells
MAGE-A1	A1	26	EADPTGHSY	161-169	autologous tumor cells
	A2	44	KVLEYVIKV	278-286	peptide
	A3	22	SLFRAVITK	96-104	poxvirus-dendritic cells[c]
	A68	8	EVYDGREHSA	222-231	poxvirus-dendritic cells

	B7	17	RVRFFFPSL	289-298	poxvirus-dendritic cells
	B35	20	EADPTGHSY	161-169	poxvirus-dendritic cells
	B37	3	REPVTKAEML	127-136	autologous tumor cells
	B53	2	DPARYEFLW	258-266	poxvirus-dendritic cells
	B57	8	ITKKVADLVGF	102-112	ALVAC-dendritic cells
	Cw2	10	SAFPTTINF	62-70	poxvirus-dendritic cells
	Cw3	17	SAYGEPRKL	230-238	poxvirus-dendritic cells
	Cw16	7	SAYGEPRKL	230-238	autologous tumor cells
	DR13	19	LLKYRAREPVTKAE	114-127	protein
	DR15	20	EYVIKVSARVRF	281-292	protein
MAGE-A2	A2	44	YLQLVFGIEV	157-166	peptide
	A24	20	EYLQLVFGI	156-164	peptide
	B37	3	REPVTKAEML	127-136	autologous tumor cells
	Cw7	41	EGDCAPEEK	212-220	lentivirus-dendritic cells
	DR13	19	LLKYRAREPVTKAE	121-134	protein
MAGE-A3	A1	26	EVDPIGHLY	168-176	autologous tumor cells
	A2	44	FLWGPRALV[d]	271-279	peptide
	A2	44	KVAELVHFL	112-120	peptide
	A24	20	TFPDLESEF	97-105	peptide
	B18	6	MEVDPIGHLY	167-176	adeno-dendritic cells
	B35	20	EVDPIGHLY	168-176	poxvirus-dendritic cells
	B37	3	REPVTKAEML	127-136	autologous tumor cells
	B40	6	AELVHFLLL[i]	114-122	adeno-dendritic cells
	B44	21	MEVDPIGHLY	167-176	peptide
	B52	5	WQYFFPVIF	143-151	retrovirus-dendritic cells[h]
	Cw7	41	EGDCAPEEK	212-220	lentivirus-dendritic cells
	DP4	75	KKLLTQHFVQENYLEY	243-258	protein
	DQ6	63	KKLLTQHFVQENYLEY	243-258	peptide
	DR1	18	ACYEFLWGPRALVETS	267-282	protein
	DR4	24	VIFSKASSSLQL	149-160	peptide

	DR7	25	VIFSKASSSLQL	149-160	peptide
	DR11	25	GDNQIMPKAGLLIIV	191-205	peptide
	DR11	25	TSYVKVLHHMVKISG	281-295	protein
	DR13	19	AELVHFLLLKYRAR	114-127	protein
	DR13	19	LLKYRAREPVTKAE	121-134	protein
MAGE-A4	A1	26	EVDPASNTY[j]	169-177	peptide after tetramer sorting
	A2	44	GVYDGREHTV	230-239	adeno-dendritic cells
	A24	20	NYKRCFPVI	143-151	peptide
	B37	3	SESLKMIF	156-163	poxvirus-dendritic cells
MAGE-A6	A34	1	MVKISGGPR	290-298	autologous tumor cells
	B35	20	EVDPIGHVY	168-176	autologous tumor cells
	B37	3	REPVTKAEML	127-136	autologous tumor cells
	Cw7	41	EGDCAPEEK	212-220	lentivirus-dendritic cells
	Cw16	7	ISGGPRISY	293-301	autologous tumor cells
	DR13	19	LLKYRAREPVTKAE	121-134	protein
MAGE-A9	A2	44	ALSVMGVYV	223-231	peptide
MAGE-A10	A2	44	GLYDGMEHL	254-262	autologous tumor cells
	B53	2	DPARYEFLW	290-298	poxvirus-dendritic cells
MAGE-A12	A2[g]	44	FLWGPRALV[e]	271-279	peptide
	Cw7	41	VRIGHLYIL	170-178	autologous tumor cells
	Cw7	41	EGDCAPEEK	212-220	lentivirus-dendritic cells
	DR13	19	AELVHFLLLKYRAR	114-127	protein
MAGE-C2	A2	44	LLFGLALIEV	191-200	autologous tumor cells
	A2	44	ALKDVEERV	336-344	autologous tumor cells
mucin [k]			PDTRPAPGSTAPPAHGVTSA		transfected B cells
NA-88	B13	6	QGQHFLQKV		autologous tumor cells
NY-ESO-1 / LAGE-2	A2	44	SLLMWITQC	157-165	autologous tumor cells
	A2	44	MLMAQEALAFL	ORF2 (1-11)	autologous tumor cells
	A31	5	ASGPGGGAPR	53-62	autologous tumor cells

	A31	5	LAAQERRVPR	ORF2 (18-27)	autologous tumor cells
	B7	17	APRGVRMAV	ORF2 (46-54)	adenovirus-APC
	B35	20	MPFATPMEA	94-102	autologous tumor cells
	B51	12	MPFATPMEA	94-102	adenovirus-APC
	Cw3	17	LAMPFATPM	92-100	adenovirus-PBMC
	Cw6	18	ARGPESRLL	80-88	adenovirus-PBMC[d]
	DP4	75	SLLMWITQCFLPVF	157-170	peptide
	DP4	75	LLEFYLAMPFATPMEAELARRSLAQ	87-111	peptide
	DR1	18	LLEFYLAMPFATPMEAELARRSLAQ	87-111	peptide
	DR1	18	EFYLAMPFATPM	89-100	protein
	DR2	25	RLLEFYLAMPFA	86-97	protein
	DR3	21	QGAMLAAQERRVPRAAEVPR	ORF2 (14-33)	protein
	DR4	24	PGVLLKEFTVSGNILTIRLT	119-138	peptide and protein
	DR4	24	VLLKEFTVSG	121-130	peptide
	DR4	24	AADHRQLQLSISSCLQQL	139-156	protein
	DR4	24	LLEFYLAMPFATPMEAELARRSLAQ	87-111	peptide
	DR7	25	PGVLLKEFTVSGNILTIRLTAADHR	119-143	peptide
	DR7	25	LLEFYLAMPFATPMEAELARRSLAQ	87-111	peptide
SAGE	A24	20	LYATVIHDI	715-723	peptide
Sp17	A1	26	ILDSSEEDK	103-111	protein
SSX-2	A2	44	KASEKIFYV	41-49	autologous tumor cells
	DP1	14	EKIQKAFDDIAKYFSK	19-34	peptide
	DR3	21	WEKMKASEKIFYVYMKRK	37-54	peptide
	DR11	25	KIFYVYMKRKYEAM	45-58	protein
SSX-4	DP10	2	INKTSGPKRGKHAWTHRLRE	151-170	peptide
	DR3	21	YFSKKEWEKMKSSEKIVYVY	31-50	peptide
	DR11	25	LGFKVTLPPFMRSKRAADFH	61-80	peptide
	DR15	20	KSSEKIVYVYMKLNYEVMTK	41-60	peptide
TRP2-INT2[g]	A68	8	EVISCKLIKR	intron 2	autologous tumor cells

Anti-HIV therapy

HIV enters cells expressing the specific receptors and co-receptors (typically CD4 and CXCR4 or CCR5), thereby establishing a type of virus/host interaction that depends on both the cell type and the cell activation state (for a review, see (40)). In particular, HIV can either lead to cell death, mostly in activated CD4 lymphocytes, or establish a persistent infection in cells controlling the extent of HIV expression and resisting its cytopathic effect, such as macrophages and follicular dendritic cells. In addition, HIV can generate a latent infection in the case the viral genome remains silent upon integration in the host DNA. Latently infected cells could be induced to express HIV by the treatment with cytokines such as IL-2, anti-CD3, γIFN, or TNFα (for a review, see (41)), or with drugs, like prostratin (42, 43, 44) or 12-deoxyphorbol-13 acetate (45), leading to cell activation in the absence of cell replication. In these cases, HIV Env molecules undergo cell membrane exposition, thus rendering the infected cells readily and specifically recognizable by viral particles carrying the respective cell receptor complex. In this respect, we sought to recover VLPs incorporating CD4 and CXCR4 or CCR5 in their envelope for the delivery of the fusion protein of the present invention in HIV infected cells. Whilst not wishing to be bound by theory, this was expected to occur by means of the "inverse fusion" process, mimicking the fusion events naturally occurring between HIV and target cells, however in a swapped virus to cell receptor configuration.

We have found that Nef7-based VLPs carrying the viral cell receptor complexes enter HIV-1 infected cells thereby, for example in an embodiment using Nef7/TK VLPs, inducing cell killing in both replicating and resting HIV-1 infected cells cultured with GCV. Thus the VLPs of the present invention comprising a Nef7 fusion product of the present invention provide new therapeutic tools against HIV infected cells, particularly those with longer half-life. In particular, the present invention has a therapeutic relevance even during the HAART treatment.

Thus, the present invention allows for new VLP-based therapeutic strategies, and in particular for targeting persistently HIV infected cells such as monocyte/macrophages, follicular dendritic cells, and resting T lymphocytes. In

addition, the treatment may impact also against latently infected T lymphocytes in the case the HIV expression is pharmacologically induced.

In one embodiment use may be made of HSV/TK-derived enzymes with stronger activity (46, 47, 48), and/or nucleosides analogues with shorter time of action. Furthermore, due that ligands of both CXCR4 and CCR5 co-receptors may restrict the VLP infectivity *in vivo*, in another embodiment use may be made of VLPs comprising the Nef7 fusion based product of the present invention, and in particular Nef7/TK-based VLPs, incorporating a VSV-G deletion mutant ("G stem") that has been demonstrated to relieve the CXCR4 or CCR5 requirement for CD4-dependent HIV-1 Env mediated fusion (49). G stem incorporating VLPs may be equally effective against cells infected by T- or M-tropic HIV isolates.

Furthermore, as discussed above, Nef7 is incorporated at high levels in VLPs such as Simian Immunodeficiency Virus and Moloney Leukemia Virus viral particles, therefore, the VLPs of the present invention may be employed for both experimental and pre-clinical tests of new peptidic drugs in primates as well as in rodents. In addition, as with the already described Vpr-GFP (50), Nef7 associated with a reporter gene, for example Nef7/GFP, may be used as a reporter/fluorescent HIV-1 particle for the study of the mechanisms underlying inverse fusion.

As well as direct delivery into patients, the treatment protocol may involve an ex vivo approach involving removing the target cells from the patients, delivering the protein product/polypeptide of interest *ex vivo*, and putting the cells back into the patients.

Nef7-based VLPs as vaccines for infectious diseases

The ideal HPV/HCV therapeutic vaccine should efficiently stimulate the immune system to raise an effective and long-lasting humoral and cellular response.

Dendritic cells (DCs) are the most potent professional antigen presenting cells for naïve T cell priming of the immune system; they have the unique ability to take up and process antigens in the peripheral blood and tissues. Then they migrate to draining lymph nodes where present antigens to the resting lymphocytes. DCs loaded with

antigens, such as tumor associated antigen, are used to study the response of the different antigens and also as a vaccine in vivo. To date, most DC vaccines have been used particularly to stimulate immune response against cancer. A promising vaccine delivery vehicle is represented by the virus-like particles. In this respect, it has been recently reported that both DCs and Langerhans cells are able to cross-present HPV-like particles-associated antigen to CD8+. In addition, recent studies have shown that the adoptive transfer of autologous DCs loaded in vitro with inactivated HIV-1 induced protective antiviral immunity in hu-PBL-SCID mice.

We have developed a strategy to produce retro- and lentiviral VLPs able to incorporate fusion products based on a mutated HIV-1 Nef protein. We already showed that this mutated Nef can be used as a potential carrier of heterologous proteins. VLPs containing high levels of HPV/HCV products fused to Nef7 MAY act as a vaccine by means of the internalization into DCs leading to the induction of an effective immune response selectively destroying infected cells. Of a major importance, such a response is expected to be driven by both Class I and II MHC-mediated mechanisms, as, besides the exposition in Class II typical of the exogenous products, the lentivirus-based VLPs have been demonstrated to undergo the so called "cross-presentation", leading to the exposition of viral antigens in Class I MHC. Thus, both humoral and cellular responses may be induced upon DC internalization of Nef7-based VLPs.

The expected advantages of the use of Nef7-based VLPs as vaccines can be summarized as follows:

- large amounts of the desired antigens either alone or in combination can be internalized by DCs;
- the viral antigen may be presented in both Class I and Class II MHC, thus eliciting a potent immunologic response;
- the viral antigens are protected from the host antibody neutralization;
- the antigens may be presented to the DC proteasome machinery in a rather natural conformation, thus guaranteeing a relevant immunologic response.

Diagnostic Use

Nef7-based VLP can be used for diagnostic purposes by pseudotyping the virion with an envelope protein able to interact with a specific cell or tissue and by associating Nef7 to a detectable moiety. As used herein the term detectable moiety refers to, a polypeptide having the property of rendering its presence detectable. For example, a polypeptide labelled with a radioactive isotope which permits cells or the tissue in which VLP is present to be detected in immunohistochemical assays. A detectable moiety is a polypeptide detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, the polypeptide can be labelled with radioisotopes (e. g.,3H,35S,32p,51Cr,or 125I) or can be linked to fluorescent dyes, electron-dense reagents, biotin, digoxigenin, or haptens. Other possible example of detectable polypeptide are enzymes such as alkaline phosphatase, horseradish peroxidase, or others commonly used in an ELISA.

Nucleotide vectors

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells.

Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" means that the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The control sequences may be modified, for example by the addition of further

transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators.

Vectors of the invention may be transformed or transfected into a suitable host cell as described below to provide for expression of a protein of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the protein, and optionally recovering the expressed protein.

The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used, for example, to transfect or transform a host cell.

Control sequences operably linked to sequences encoding the protein of the invention include promoters/enhancers and other expression regulation signals. These control sequences may be selected to be compatible with the host cell for which the expression vector is designed to be used in. The term "promoter" is well-known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal promoters to promoters including upstream elements and enhancers.

Vectors/polynucleotides of the invention may introduced into suitable host cells using a variety of techniques known in the art, such as transfection, transformation and electroporation. Where vectors/polynucleotides of the invention are to be administered to animals, several techniques are known in the art. In the present invention use is preferably made of VLPs as discussed above.

Administration

The present invention may involve an *ex vivo* approach in which modified cells are (re-)introduced into a patient or an *in vivo* or direct transfer approach in which the VLPs are used to deliver the heterologous polypeptides directly into the body of a

patient. In an *in vivo* approach, the composition of the invention may be introduced by direct injection. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration.

Typically, each protein of interest may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

In one embodiment the substance of the invention is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration.

Generally in an *ex vivo* approach the cells which are infected with the VLP of the present invention will be returned to the same patient. Examples of cells include HIV-permissible cells, tumor cells, lymphocytes, monocytes, stem cells, dendritic cells and bone marrow cells. It will be appreciated that a cell type appropriate for the treatment may be selected.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

Both human and veterinary treatments are within the scope of the present invention.

Various further preferred features and embodiments of the present invention will now be described by reference to the following accompanying Figures and Examples.

Description of the Figures

FIG. 1. Nef7 is strongly and specifically incorporated in both lenti- and retroviral particles. (A). Western blot analysis for the expression and virion incorporation of

Nef7 expressed either *in cis* (top) or *in trans* (bottom). 293T cells were transfected with HIV-1 NL4-3 molecular clones expressing either wt Nef or the Nef7 mutant, or, as control, with the isoform defective for the *nef* expression (Δ*nef*). Alternatively, cells were transfected with the Δ*nef* NL4-3 alone or with vectors expressing either wt Nef or Nef7. In both cases, non-transfected cells (Ctrl) were used as control. Forty-eight hours thereafter, both cells and supernatants were harvested for the Western blot analyses. Thirty μg of cell lysates were analyzed for the expression of Nef and actin using 1:1000 dilutions of the ARP444 anti-Nef polyclonal Abs and of an anti-human actin mAb, respectively. At the same time, supernatants were concentrated, viral contents measured by anti Gag p24 ELISA, and 20 ng of HIV-1 particles were run and analyzed for the relative amounts of virionic Nef by incubating the filters with 1:1000 dilutions of ARP 444 anti-Nef polyclonal Abs or the anti-p24 Gag HIV-1 AG3.0 mAb. (B). Western blot analysis of purified Nef7 HIV-1 particles. Supernatants harvested 48 hours after the transfection of 293T cells with the Δ*nef* NL4-3 molecular clone either alone or together with vectors expressing wt Nef or Nef7, were concentrated by ultra-centrifugation and the viral pellets purified through two ultra-centrifugation cycles on 20% sucrose cushions. Twenty ng of each purified viral preparation were analyzed by Western blot using both anti-Nef and anti-p24 Gag as described in the legend of panel A. (C). Western blot analysis of Nef7 incorporating MLV particles. 293T cells were transfected with a molecular clone expressing the whole MLV genome together with vectors expressing either wt or Nef7. Non-transfected cells were used as control (Ctrl). Forty-eight hours thereafter, both cells and supernatants were harvested for the Western blot analysis. Thirty μg of cell lysates were analyzed for the expression of both Nef and actin, and, at the same time, supernatants were concentrated, viral contents measured by RT assay. The amounts equivalent to $5x10^6$ cpm of MLV particles were run and analyzed for the presence of virionic Nef by incubating the filters with anti-Nef polyclonal Abs.

For all panels, the migration of major viral products are indicated on the left side, whereas the molecular marker sizes are reported on the right.

FIG. 2. Nef7 accumulates in rafts at high levels. Western blot analysis of fractions recovered from discontinuous sucrose gradients carried out on lysates from 5×10^7 293T cells transfected with NL4-3 or NL4-3/Nef7 HIV-1 molecular clones, or with

Δ*nef* NL4-3 together with wt Nef or Nef7 expressing vectors. Forty-height hours after transfection, cells were lysed, 50 μg of total proteins analyzed for the Nef expression (A), and the remainder cell lysates run in 4 ml gradients. A total of 12 fractions were harvested, and 50 μl of each fraction were analyzed by Western blot using 1:1000 and 1:500 dilutions of ARP444 anti-Nef Abs and of anti-CD71 mAb, respectively, or by dot-blot using a 1:5000 dilution of HRP-conjugated cholera toxin, subunit B. The results from the analysis of relevant fractions from the lysates of cells expressing Nef *in cis* (B) or *in trans* (C) are reported. In Western blot panels, the molecular marker sizes are reported on the right.

FIG. 3. Nef7-based fusion products are incorporated at high efficiency in HIV-1 VLPs. Western blot analysis for the Nef incorporation in (VSV-G) pseudotyped HIV-1-based VLPs carrying wt Nef or Nef7/GFP (A), and wt Nef or Nef7/TK (B). 293T cells were co-transfected with the HIV-1 packaging construct pCMVΔR8.74 and a vector expressing the VSV-G receptor in the absence (Ctrl) or in the presence of vectors expressing the diverse Nef derivatives. Forty-eight hours after, supernatants were harvested, clarified, concentrated and titrated. Volumes equivalent to 20 ng of each viral preparation were then loaded in 10% SDS-PAGE and analyzed by Western blot for the presence of Nef and p24 Gag-related products. Abs preparations used were quoted in the legend of Fig.1A

For all panels, the migration of major viral products are indicated on the left side, whereas the molecular marker sizes are reported on the right.

FIG. 4. Nef7/GFP is efficiently delivered in cells by VLPs. (A) Effective removing of adsorbed VLPs by trypsin treatment. Ten ng of (VSV-G) Nef7/GFP VLPs were used to challenge 10^5 CEMss cells for 2 hours at 4 °C. Then, cells were treated or not with trypsin for 10 minutes at 4 °C, and analyzed by FACs for the GFP-related fluorescence. (B). Delivery of Nef7/GFP molecules upon VLP challenge. Ten ng of Nef7/GFP VLPs expressing or not the VSV-G receptors were used to infect 10^5 CEMss cells. After extensive washings, cells were incubated at 37 °C in complete medium and, after 3, 6 and 10 hours, harvested, treated with trypsin, and analyzed by FACs for the GFP-related fluorescence. In each panel, the percentages of GFP positive cells are indicated.

FIG. 5. Cell killing upon (VSV-G) Nef7/TK VLP challenge of CEMss cells and GCV cultivation. Growth kinetics of CEMss cells left untreated or challenged with VSV-G pseudotyped Nef7 or Nef/TK VLPs, and cultivated in the presence or absence of GCV. Twenty ng of VLPs were adsorbed to 10^5 cells for two cycles of two hours at 37 °C. Thereafter, cells were seeded in complete medium in the presence or not of 10 μM GCV. At the indicated times, the cell viability was scored by the trypan bleu exclusion dye. The overall cell mortality was below 10%, except than in (VSV-G) Nef7/TK VLP treated cells cultivated in GCV whose values are reported in the pertinent panel. The means values ±sd from five independent experiments are reported.

FIG. 6. Primary human MDM cells are efficiently killed by (VSV-G)Nef7/TK VLPs. Cell viability as evaluated by FACs scatter plot analysis of MDM from two healthy donors (I-II) after challenge with 1 to 5 ng of (VSV-G) Nef7/TK VLPs and cultivation in the presence or not of GCV. As control, cells either unchallenged or challenged with Nef7 (VSV-G) VLPs and cultivated with or without GCV, were also analyzed. 2×10^5 MDM were challenged in 48-well plates by adsorbing increasing doses of (VSV-G) Nef7/TK VLPs in 100 μl of complete medium at 37°C, and then cultivated in 300 m,l of complete medium in the presence or not of GCV. Cells from replicated wells were then collected daily and analyzed for the cell viability. Data refer to the analysis performed at the day 5 after challenging on MDM from six healthy donors.

FIG. 7. The challenge with (VSV-G) Nef7/TK VLPs induces an increase of Annexin-V binding in both replicating and resting cells. Annexin-V binding in CEMss (A) or human MDM (B) after infection with (VSV-G) Nef7/TK VLPs and cultivation in the presence or absence of GCV. CEMss cells and MDM were infected by (VSV-G) Nef7/TK VLP as above described, and cultivated with or without GCV. From 2 till 5 days for CEMss cells, or three days after the challenging for MDM, cells were harvested, labelled with FITC-conjugated Annexin-V and PI, and analyzed by FACs. For MDM, unchallenged cells (Ctrl) cultivated in the presence of GCV were included as an additional control. The FITC-related histograms referring to PI negative cells are reported. The percentages of positive cells are herein indicated.

Results are representative of three independent experiments for both CEMss and MDM.

FIG. 8. The (VSV-G)Nef7/TK VLP challenge induces the decrease of the levels of intracytoplasmic Bcl-2. Two-fluorescence FACs analysis for the detection of cell membrane CD45 and intracytoplasmic Bcl-2 in CEMss cells challenged with (VSV-G) Nef7/TK VLPs and cultivated in the presence or in the absence of GCV. As controls, cell cultures either unchallenged (Ctrl) or challenged with (VSV-G)Nef7 VLPs and maintained in GCV are included. CEMss cells were challenged by VLPs as described in the legend of Fig. 5 and, from 2 to 4 days thereafter, were labelled with a 1:100 dilution of PE-conjugated anti-CD45 mAb, permeabilized, and finally incubated with a 1:30 dilution of FITC-conjugated anti-Bcl-2 mAb.

FIG. 9. Molecular analysis of Nef7-based VLPs. Western blot analysis of different Nef7-based VLPs. 293T cells were co-transfected with the HIV-1 packaging construct pCMVΔR8.74 together with vectors expressing either CD4T, CXCR4 or CCR5, and either Nef7, Nef7/TK or Nef7/GFP. Forty-eight hours thereafter, supernatants were harvested, clarified and concentrated. After a purification cycle on 20% sucrose cushion, 20 ng of VLPs were loaded in 10% SDS-PAGE and analyzed by Western blot for the presence of both Nef- and HIV-1-related products (A-B), CD4 C) , CXCR4 (D), and CCR5 (E) using 1:1000 (for both Nef and HIV-1-related products), 1:100 (for CD4), and1:50 (for both CXCR4 and CCR5) dilutions of the Abs preparations indicated in the Experimental Procedures. Control supernatants from mock-transfected cells processed as for the VLP recovery, were included in all panels. The migration of relevant VLP products are indicated on the left side, whereas the molecular marker sizes are reported on the right.

FIG. 10. Nef7-GFP is efficiently delivered in cells by both (CD4-CXCR4) and (CD4-CCR5) VLPs. Analysis of the internalization of Nef7/GFP molecules upon the challenge of either 8E5 cells with (CD4-CXCR4) VLPs (A), or ADA HIV-1 transfected 293T cells with (CD4-CCR5) VLPs (B). Both cell cultures were preliminarly analyzed by FACs for the membrane expression of HIV-1 gp120 using a 1:100 dilution of the 4G10 anti-gp120 mAb or the same amounts of isotype-matched, specie-specific IgG as control. Fifty ng of Nef7/GFP (CD4-CXCR4) or (CD4-CCR5)

VLPs were used to infect 10^5 of either 8E5 cells or 293T cells 48 hours after the transfection with ADA HIV-1 molecular clone, respectively. As control, the same amounts of Nef7/GFP VLPs expressing CD4 alone were used. After extensive washings, cells were incubated at 37 °C in complete medium and, after 3, 6 and 10 hours, harvested, incubated 5 minutes with trypsin, and analyzed by FACs for the GFP-related fluorescence. The percentages of GFP positive cells are indicated in the respective quadrants.

FIG. 11. T-tropic HIV-1 chronically infected cells are killed upon (CD4-CXCR4) Nef7/TK VLP challenge and GCV treatment. Cell viability expressed as percentages of untreated cells, measured in 8E5 cells seven days after the challenge with Nef/TK VLPs pseudotyped by VSV-G or CD4-CXCR4 receptors, and treated or not with GCV. 10^5 cells were spinoculated with 50 ng of VLPs for three cycles and, thereafter, seeded in complete medium in the presence or not of 10 μM GCV. After seven days, the cell viability was scored by the trypan bleu exclusion dye. As control, mockinfected 8E5 cells cultivated in the presence of GCV were included (Ctrl). The means values ±sd from seven independent experiments are reported.

FIG. 12. Selective elimination of human T cells acutely infected with T-tropic HIV-1 upon challenging with (CD4-CXCR4) Nef7/TK VLPs. Percentages of fluorescent cells as scored by FACs analysis of CEM-GFP cells infected with increasing doses of T-tropic HIV-1 and challenged with (CD4-CXCR4) Nef7/TK VLPs. CEM-GFP cells were infected with 2 to 50 ng of NL4-3 HIV- $1/10^5$ cells by adsorbing the viral inoculum in 50 μl for 2 hours at 37 °C. Thereafter, cells were kept in culture for two days and then challenged with 30 ng of VLPs $/10^5$ cells for two cycles. Finally, cells were re-fed in complete medium in the presence or in the absence of GCV, and monitored for the GFP-associated fluorescence after additional five days. Results are the means values from two independent experiments.

FIG. 13. Blocking of viral spread upon challenge with (CD4-CCR5) Nef7/TK VLPs of MDM acutely infected with M-tropic HIV-1. (A) HIV-1 amounts measured as ng/ml of p24 Gag HIV-1 in supernatants harvested at the indicated days after the infection of MDM with 100 pg/10^5 cells of ADA HIV-1 followed, two days after, by two cycles of challenge with 30 ng/10^5 cells of (CD4-CCR5) Nef7/TK VLPs, and

cultivation in the presence or absence of GCV. Data are representative of independent experiments performed on MDM from three healthy donors. (B) FACs analysis for the cytoplasmic accumulation of Gag-related products in MDM eleven days after the infection with ADA HIV-1, followed by VLP challenge and cultivation with or without GCV. Bars limit the fluorescence intensity detected in uninfected MDM labeled with the anti-Gag mAb. Percentages of positive cells are reported in the respective panels.

FIG. 14. Selective elimination of HIV-1 expressing MDM upon challenge with (CD4-CCR5) Nef7/TK VLPs. 10^5 five days-old MDM were infected with 1 ng of (VSV-G) HIV-1 or left untreated (Ctrl). After 24 hours, infected MDM were challenged or not with 50 ng/10^5 cells of (CD4-CCR5) Nef7/TK VLPs for three cycles, and cultivated in the presence or absence of GCV. After additional four days, the percentages of HIV-1 Gag expressing cells were evaluated by FACs analyses. Percentages of positive cells are reported in the respective panels. Data are representative of the analysis performed on MDM from two healthy donors.

FIG. 15 Sequence ID No: 1.

FIG. 16 CD8+ MAGE-3 specific T cells were cultured with autologous DCs previously infected with HIV VLPs (Nef7/MAGE-3 VSV-G and Nef7/MAGE-3 RD114) and with MLV-VLP (Nef7/MAGE-3). The presence of IFN-γ in the supernatant was measured by ELISA after 24 hours.

Examples

Molecular constructs. Vectors expressing the NL4-3 HIV-1 (52) and its derivatives, i.e. the viral genomes deleted of the Δ*nef* gene (*nef* HIV-1) (53), and expressing the Nef7 mutant (54) have been already described. The pCMVΔR8.74 plasmid (55) was used as HIV-1 packaging construct. VSVG, wt Nef and Nef7 were expressed under the control of the immediate-early cytomegalovirus (ie-CMV) promoter. The construction of vector expressing the Nef7-GFP fusion protein was already described (45). The open reading frame encoding the Nef7/TK fusion protein was obtained

through the overlapping polymerase chain reaction (PCR) procedure. In particular, Nef7 and HSV-TK genes were amplified from, respectively, pcDNA3-Nef7 and TgCMV-Hy/TK expressing vectors, using DyNAzyme EXT polymerase (Finnzymes, Espoo, Finland). Sequences of oligoprimers are the following: Nef7 Forward:5' GCG AAG CTT ATG GGT GGC AAG TGG TCA AAA 3'; Nef7 Reverse: 5' CTG GTC GAA CGC AGA CGC GCA GTT CTT GAA GTA CTC CGG 3'; TK Forward: 5' GAG TAC TTC AAG AAC TGC GCG TCT GCG TTC GAC CAG GCT 3'; TK Reverse: 5' CGC GGA TCC TCA GTT AGC CTC CCC CAT CTC 3'. Both cloning sites (i.e., *Hind* III and *Bam HI*) and overlapping sequences are underscored. Of note, the stop codon was removed in the Nef7 sequence, and the TK gene started with an Alanine residue. Single PCR products were purified from agarose gel, and mixed in a single PCR amplification (100 ng each in 50 μL of final reaction volume) using exclusively the Nef7 Forward and the TK Reverse as primers. The final amplification product was purified, doubly digested with *Hind* III and *Bam HI* restriction sites, and inserted in the homologous sites of the pcDNA3 vector. All PCR products were sequenced by the dideoxy chain termination method.

Cell lines and cultures. 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% decomplemented Fetal Calf Serum (dFCS). CEMss and U937 cells were cultivated in RPMI supplemented with 10% dFCS. Human primary lymphocytes were isolated from peripheral blood mononucleated cells (PBMC) isolated from from 20-40 year-old healthy male blood donors. Lymphocytes were negatively selected from PBMC using the appropriate immunomagnetic-based selection kit from Miltenyi Bioytec (Auburn, CA). PBLs were activated with 2 μg/ml phytohemagglutinin (Sigma-Aldrich, Milan, Italy), and cultivated in RPMI containing 20% of dFCS in the presence of 100 U/ml of recombinant human IL-2 (Roche, Nutley, NJ). Monocytes were isolated by 1 h adherence of PBMC, followed by immunomagnetic depletion of non-monocytic cells carried out through a Miltenyi selection kit. Monocytes were cultivated in 48-well plates in RPMI supplemented with 20% of dFCS. Ganciclovir was from Synthex Laboratories, Inc. (Palo Alto, CA) and was used at the concentration of 10 to 30 μM.

VLP production, titration, and challenges. Preparations of VSV-G pseudotyped VLPs were obtained from supernatants of 293T cells 48-60 h after the calcium

phosphate co-transfection with the pCMVΔR8.74 packaging vector (55), the Nef7 or Nef7-based fusion products expressing vectors, and a plasmid expressing the VSV-G, in a molar ratio of 3:3:1. Supernatants were clarified and concentrated by ultracentrifugation as described (56). In some instances, the concentrated VLPs were purified through additional ultracentrifugation (SW 60 rotor, 30,000 *g*, 4h at 4°C) by loading the concentrated viral particles on a 20% sucrose cushion.

VLP preparations were titrated both by measuring HIV-1 p24 contents by quantitative ELISA (Abbott, Abbott Park, Illinois), and by the reverse transcriptase assay (56). VLPs were adsorbed incubating the cells for 1 h at 37°C. Afterwards, the cells were re-fed by adding fresh medium complemented or not with GCV. In the case of Nef7-GFP VLP infections, cells underwent spinoculation, i.e., the adsorption of VLPs to cells by means of 1 h of centrifugation at 150 *g* at r.t.. Afterwards, the cells were washed to eliminate non adsorbed VLPs, re-fed by adding fresh medium, collected at different times, treated for 5 min with trypsin at r.t., and finally analyzed by FACs.

The evaluation of cell mortality was performed either by labeling cells with the trypan blue exclusion dye, by analyzing the scatter profile by FACs (FACscalibur, Becton-Dickinson, Mountain View, CA), and by scoring the labeled cells by FACs after the treatment with 5 μg/ml of propidium iodide (PI).

Western blot and pulse & chase analyses. Both cells and purified VLP preparations were lysed in PBS, 1% Triton-X100 in the presence of anti-proteolytic agents. For the preparation of cytoplasmic extracts, whole cell lysates were centrifuged at 6,000 *g* for 10 min at 4 °C, and the supernatants frozen at –80 °C. Aliquots of 30 μg of total cell proteins were separated in 10% SDS-PAGE, thereby undergoing the immunoblot analysis (Western blot). The following mono- or polyclonal Abs served for the revelation of both VLP- and cell-associated HIV-1 products: AG3.0 anti-p24 Gag HIV-1 mAb from the NIH AIDS Research and Reference Reagent Program; ARP 444 sheep anti-Nef antiserum (University of Leeds, Leeds, UK); polyclonal anti-VSV-G protein from Immunology Consultant Laboratories (Newberg, OR); and anti-human actin from Amersham (Freiburg, Germany).

The pulse-chase labeling assay was performed by transfecting 293T cells with vectors expressing either wt Nef or Nef7 alone or in the presence of Δ*nef* HIV-1 expressing vector, and, 48 h thereafter, labeling the cells with 1,85 MBq/ml of both ^{35}S-cysteine and -methionine in cysteine/methionine free medium for 4 h in the presence of 10% dialyzed dFCS. Cells were then extensively washed, and re-seeded in complete medium. Cells were sampled at different times, and 100 μg of cell lysates immunoprecipitated overnight with anti-Nef Abs in the presence of protein A-G agarose beads (Pierce, Rockford, IL). Immunoprecipitated proteins were finally resolved in 10% SDS-PAGE and revealed by autoradiography. Specific signals were quantified by means of an Instant Imager software (Packard, Meriden, CT).

Isolation of detergent-resistant membrane (raft) fractions. Plasma membrane rafts were obtained essentially as previously described (57). Briefly, transfected 293T cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed at 4°C in a buffer containing 25 mM morpholineethanesulfonic acid (pH 6.5), 1% vol/vol Triton X-100, and protease inhibitors. The cell lysates were then homogenized and adjusted to 40% sucrose. A discontinuous sucrose gradient (40%-35%-5%) was then loaded on the cell lysate, and the sample ultra-centrifuged at equilibrium for 18 h at 4°C, 200,000 *g*, SW 60 rotor on a Beckman L70-ultracentrifuge. From the top of the gradient, 0.35 ml fractions were collected to yield a total of 12 fractions. Proteins from each fraction were finally quantified through the Bio-Rad assay (Bio-Rad, Hercules, CA), separated by 10% SDS-PAGE, and subjected to immunoblot analysis. Filters were finally incubated with polyclonal anti-Nef ARP444, anti-transferrin receptor (CD71) mAb, clone H68.4 (Zymed, Berlin, Germany), or HRP-conjugated Cholera Toxin (Sigma-Aldrich), subunit B.

Flow cytometry analyses. For the simultaneous detection of Bcl-2 and CD45 in VLP treated cells, 3×10^5 CEMss cells were incubated with a 1:30 dilution of a phycoerythrin (PE)- conjugated anti- CD45 mAb (Dako, Glostrup, Denmark) for 1 h at 4 °C. Thereafter, cells were treated with Permeafix (Ortho Diagnostic, Raritan, NJ) for 30 min at r.t., and labelled for 1 h at r.t. with a 1:30 dilution of a fluorescein isothiocyanate (FITC)-conjugated anti-Bcl-2 mAb (Dako). Cell populations were finally analyzed by FACs

Annexin-V binding assay. The Annexin-V binding assays was performed by re-suspending cells in binding buffer (100 mM Hepes, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4), and adding 1:20 diluted of FITC-conjugated Annexin-V (BD Biosciences, Mountain View, CA) for 30 min at r.t.. Thereafter, 5 μg/ml of PI was added, and, after additional 5 min, cells were analyzed by FACs.

Nef7 is Incorporated in Both Lenti- and Retroviral Particles at High Levels

We cloned a Nef double mutant (the $^{V}153^{L}$, $^{E}177^{G}$, here defined Nef7) having the unique property to undergo virion incorporation at quite high levels. As shown in Fig. 1A, this occurs whatever Nef7 is expressed *in cis* or *in trans* with respect to the HIV-1 genome. Through densitometric analysis, we estimated a 20-50 fold increase of the Nef7 virion incorporation compared with wt Nef (not shown). This was not the consequence of differences in the antibody recognition, as similar results were obtained using different either mono- or polyclonal anti-Nef antibody preparations (not shown). Importantly, these results were confirmed by purifying the virus preparations by two-rounds of centrifugation on 20% sucrose cushions (Fig. 1B). In addition, a similar outcome was obtained by co-transfecting the Nef7 expressing vector with a molecular construct expressing the whole Moloney Leukemia Virus (MLV) genome, thus indicating that HIV- products are not required for the high incorporation of Nef7 into the virions (Fig. 1C).

These results clearly demonstrated that the Nef7 mutant is incorporated at unusually high levels in VLPs such as lenti- and retroviral particles.

The Strong Nef7 Virion Incorporation Correlates with Enhanced Localization in Rafts

Next, we investigated the molecular mechanism underlying the strong viral incorporation of Nef7. The Western blot analyses of cell lysates depicted in Fig. 1 failed to reveal major differences in the steady-state levels between wt Nef and Nef7, that was suggestive of similar intracellular stabilities. This was also formally demonstrated by means of "pulse and chase" experiments carried out by labeling with ^{35}S cysteine/methionine 293T cells transfected with wt Nef or Nef7 expressing

vectors, either in the presence or in the absence of the *nef* HIV-1 genome (data not shown).

HIV egresses at level of the so called "detergent-resistant microdomains" (rafts) (58), that are regions within the cell membrane enriched in cholesterol and sphingolipids. In addition, several authors demonstrated the presence of Nef in rafts (59-62), even if the relative biologic significance has been recently questioned (63). We were interested in determining whether alterations in the localization of Nef7 into the rafts could correlate with its high levels of virion incorporation. For this purpose, wt Nef or Nef7 were expressed by transfecting 293T cells as part of the HIV-1 genome or together with a vector expressing the Δ*nef* HIV-1 genome. Forty-eight h thereafter, cells were lysed, tested for the Nef expression (Fig. 2A), and the remaining cell lysates ultra-centrifuged in a discontinuous sucrose gradients. Fractions recovered hereafter were analyzed by Western blot for the presence of Nef, GM1 (a raft marker), and CD71 (i.e., the transferrin receptor, a non-raft marker). Whatever expressed *in cis* (Fig. 2B) or *in trans* (Fig. 2C), Nef7 was reproducibly found in rafts at higher levels in comparison to wt Nef. Of note, the same result was obtained by expressing Nef7 alone (not shown), indicating that its accumulation in rafts does not require the presence of additional HIV-1 products. This may account for the highly efficient incorporation of Nef7 also in MLV, a murine retrovirus assembling at the lipid rafts as well (55).

Interestingly, we also noticed that an altered cell membrane disposition of Nef7, as in CD8/Nef7, where the N-terminal CD8 moiety localizes the fusion product in non-raft regions, led to a virion incorporation activity turning to the levels of the wild-type counterpart (not shown). This enforces the idea that the cell membrane raft localization is important for the high levels of Nef7 virion incorporation.

Recovery and Characterization of Nef7-Based (VSV-G)VLPs

To test whether the unexpectedly high levels of Nef7 virion incorporation could be exploited as a new system of protein delivery, we attempted to produce VLPs incorporating Nef7 tagged with either GFP or HSV-TK. Both Nef7/GFP and Nef7/TK VLPs were recovered upon concentration of supernatants of 293T cells co-

transfected with the pCMVΔR8.74 HIV-1 packaging construct (55), a VSV-G expressing vector, and the vectors expressing the respective Nef-derivative. By titrating the VLP preparations by both RT assay and Gag p24 ELISA, we assessed that the RT activity/p24 Gag content ratios did not differ significantly among empty, Nef7/GFP and Nef7/TK carrying VLPs (i.e., 2.5-3.3×10^4 cpm/ng p24), suggesting that the inclusion of Nef7-based fusion products had no major negative effects on the lentiviral particle assembling.

A Western blot-based molecular characterization was then performed to prove the real inclusion of Nef7-based molecules in VLPs. Of interest, both Nef7/GFP (Fig. 3A) and Nef7/TK (Fig. 3B) were found incorporated at high levels in empty virions, allowing to proceed with the biologic characterization of Nef7-based VLPs.

VLP-Associated Nef7 is Efficiently Delivered into the Cells

We then were interested in establishing whether and how efficiently Nef7-based fusion products undergo internalization in VLP-challenged cells. To this end, 10 ng of (VSV-G) Nef7/GFP or, as control, Nef7-GFP VLPs deprived of receptors ("null" VLPs), were used to challenge 10^5 human T CEMss cells. Thereafter, cells were washed, treated with trypsin 5 minutes at room temperature (r.t.), and the extents of Nef7/GFP entry evaluated by flow cytometry on cells harvested from 3 to 10 h after the challenge. For instance, the efficiency of the trypsin treatment in eliminating non-specifically bound VLPs was demonstrated by the strong reduction of the positive events within the cells challenged with (VSV-G) Nef7/GFP VLPs for 1 h at +4°C (Fig. 4A).

The challenge at 37 °C with 10 ng/10^5 cells of (VSV-G) Nef7/GFP, but not with the "null" ones, clearly led to the appearance of a strongly fluorescent cell sub-population (Fig. 4B), whose extent decreased over time likely as the consequence of intracellular degradation. Of note, the possibility that GFP-related pseudo-transduction (i.e., the delivery of GFP-related products non-specifically trapped in lentiviral particles) can at least in part contribute to the final outcome was excluded by the observation that fluorescence levels similar to those of control conditions have been detected upon cell challenging with the same amounts of wt Nef/GFP VLPs (not shown).

We concluded that VLPs, including pseudotyped VLPs, can efficiently deliver Nef7-based fusion proteins, thus prompting us to assay the biologic consequences of the Nef7/TK delivery in human cells.

Both Cycling and Resting Cells are Killed by Nef7/TK (VSV-G) VLPs

Next, we tested the efficiency of Nef7/TK VLPs in killing target cells. We preliminarily verified that TK preserved its activity also upon fusion with Nef7 by transfecting 293T cells with the Nef7/TK expression vector and cultivating them with GCV (not shown). Then, human T lymphoblastoid CEMss cell were challenged with two cycles of 5 ng/10^5 cells of Nef7/TK VLPs. Fig. 5 shows the anti-cellular effect of the (VSV-G)Nef7/TK VLPs challenge in CEMss cultivated in the presence of GCV. Conversely, no apparent effects on the cell growth were detectable in both mock-infected cells and cells infected with (VSV-G) VLPs incorporating Nef7 alone, even in the presence of GCV. Similar results were obtained using either the human promonocytic U937 cells or PHA-stimulated human PBLs (not shown). The specificity of the killing effect was confirmed by pre-treating VLPs with anti-VSV-G polyclonal antibodies. As expected, blocking VSV-G receptors meant strongly inhibiting the effect of (VSV-G) Nef7/TK VLPs (not shown). Of note, bystander effects likely did not contribute to the massive cell killing we observed, as we did not detect toxic effects on unchallenged CEMss cells upon co-cultivation with cells previously challenged with (VSV-G) Nef7/TK VLPs (not shown).

There are mounting evidences that the GCV cytotoxic effect is not restricted to duplicating cells. In fact, it was reported that resting cells also can be susceptible to the HSV-TK/GCV induced apoptosis (64,65), possibly as the consequence of lethal damages induced on mitochondrial DNA (66-68). To investigate the effects of (VSV-G) Nef7/TK VLPs challenge in non-replicating cells, monocyte-derived macrophages (MDM) from different donors were purified and cultured for 5 days. Thereafter, 2×10^5 cells were challenged with increasing amounts of VLPs, i.e. from 1 to 5 ng, and re-cultured with or without GCV. The cell viability was evaluated by analyzing both the cell shape (Fig. 6) and the PI staining (not shown) by FACs. Noteworthy, MDM appeared extremely responsive to the (VSV-G) Nef7/TK VLP challenge and GCV

treatment, due that a single input of 2 ng of VLPs specifically and reproducibly induced cell death within 4-6 days, depending on the donor source.

Taken together, our results indicate that VLPs comtaining Nef7 based fusion products of the present inventin can efficiently kill both replicating and resting cells.

Apoptotic Events Couple with the Nef7/TK (VSV-G) VLP-Induced Cell Death

We were interested in establishing whether the cell death we originally induced upon HSV-TK delivery recognized mechanisms similar to those already described in HSV-TK engineered cells. We focused our investigations on two hallmarks of the apoptotic process, i.e., the Annexin-V binding and the Bcl-2 intracellular levels. To this end, CEMss were treated with (VSV-G) Nef7/TK VLPs and cultured in the presence or in the absence of GCV. Cells were then harvested from 2 to 5 days after the challenge, doubly labeled with Annexin-V-FITC and PI, and analyzed by FACs. As shown in Fig. 7A, (VSV-G) Nef7/TK VLP treated cells significantly increased the Annexin-V binding over time, but only when cultivated in the presence of GCV. Of interest, overlapping results were obtained by analyzing MDM from two healthy donors three days after the challenge with 2 ng of VLPs (Fig. 7B).

The decline of Bcl-2 in GCV treated, HSV/TK expressing cells has been previously described as an hallmark of mitochondrial damages contributing to the development of apoptotic events (66). To evaluate the Bcl-2 levels in VLP treated cells, CEMss cells cultivated in the presence of GCV were harvested at days 2, 3 and 4 after the challenge, and analyzed by FACs for the levels of both Bcl-2 and CD45, the latter being an ubiquitous cell membrane phosphatase we used as a marker of healthy cells. The results from the time course shown in Fig. 8 clearly indicate a both gradual and significant decrease of intracytoplasmic Bcl-2 levels, in the absence of significant reductions of CD45, supporting the idea that the cell delivery of Nef7/TK induces mitochondrial damages that are likely part of the overall apoptotic effect. Of note, the analysis at later time points showed a sudden drop of CD45 levels, correlating with progressive loss of cell viability (not shown). Similar results were obtained in human MDM (not shown).

Taken together, our data are consistent with the hypothesis that the cell death we reproducibly induced by challenging either replicating or resting cells relied on apoptotic processes, as already described in GCV-treated cells expressing ectopic HSV-TK.

EXAMPLE 2

Cell cultures. 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% decomplemented Fetal Calf Serum (dFCS). CEMss and CEM-GFP (51) were cultivated in RPMI 1640 (Life Technologies, Milan, Italy) supplemented with 10% dFCS. The same medium was used for H9/HTLVIII (70) and 8E5 cells (71), i.e. human T lymphoblastoid cell lines expressing a *nef* deleted infectious HIV-1, and a RT-defective, non-infectious HIV-1, respectively. PBMC were isolated from the buffy coat obtained from 20 to 40-years old healthy male donors. Monocytes were isolated by 1 h adherence of PBMC followed by a immune-magnetic depletion of non monocytic cells carried out by means of a selection kit from Miltenyi (Auburn, CA) used following the manufacturer's recommendations. The purity of recovered cell populations was assayed by FACS analysis by means of phycoerithrine (PE)- conjugated anti-CD14 mAb (Becton Dickinson, Mountain View, CA) labeling. Cell preparations staining below 95% positive for CD14 (a cell surface marker specific for monocyte-macrophage cell populations) were discarded. Monocytes were cultured in 48 well plates in RPMI 1640 supplemented with 20% dFCS. Ganciclovir was from Synthex Laboratories, Inc., Palo Alto, CA, and was used at the concentration of 10 to 30 μM.

VLP and HIV-1 production, infection, and detection. Preparations of VLPs pseudotyped with the CD4 and CXCR4 or CCR5 HIV cell receptors were obtained from the supernatants of 293T cells 48-60 h after the calcium phosphate co-transfection of molar equivalent amounts of the pCMVΔR8.74 packaging vector (55), and vectors expressing, respectively, Nef7, Nef7/GFP (72) or Nef7/TK, the human CD4 receptor truncated in its intracytoplasmic domain (73), and either human CXCR4 (74) or CCR5. Supernatants were clarified and concentrated by ultracentrifugation on SW 41 rotor, 45,000 *g*, 1.30 h at 4 °C. In some instances, the concentrated VLPs

were purified through additional ultra-centrifugation (SW 60 rotor, 65,000 *g*, 4 h at 4°C) on 20% sucrose cushions.

VLP preparations were titrated both by measuring HIV-1 Gag p24 contents by quantitative ELISA (Abbott, Abbott Park, Illinois) and by the reverse transcriptase assay (56). Challenges were performed by spinoculation, i.e., the adsorption of VLPs to cells by means of 1 h of centrifugation at 150 *g* at r.t.. Afterwards, the cells were re-fed by adding fresh medium complemented or not with GCV. In Nef7-GFP VLP internalization experiments, cells were washed to eliminate non adsorbed virions, and seeded in complete medium. At different intervals, cells were harvested, treated with trypsin for 5 min at r.t., and finally analyzed by FACs.

The evaluation of cell mortality was performed by both the trypan blue exclusion dye, and scoring the percentages of labeled cells by FACs after treatment with 5 μg/ml of propidium iodide (PI).

Infectious HIV-1 viral preparations were obtained from the supernatants of 293T cells 48 hours after the transfection with vectors expressing the T-tropic NL4-3 (75) or the M-tropic ADA (76) HIV-1 genomes. Supernatants were clarified and concentrated by ultracentrifugation as described (72). HIV-1 titrations were performed as for VLP preparations. For the recovery of pseudotyped HIV-1, 293T cells were co-transfected with the pNL4-3 molecular clone and a VSV-G expressing vector in a 5:1 molar ratio. Supernatants were harvested 48 h thereafter, clarified and concentrated by ultra-centrifugation

Western blot analysis. Both cells and purified VLP preparations were lysed in PBS, 1% Triton-X100 in the presence of anti-proteolytic agents. For the preparation of cytoplasmic extracts, whole cell lysates were centrifuged at 6,000 *g* for 10 min at 4 °C, and the supernatants frozen at –80 °C. Aliquots of 30 μg of total cell proteins were run in 10% SDS-PAGE, thereby undergoing to immunoblotting analysis. The following mono- or polyclonal Abs served for the revelation of VLP associated products: a pool of strongly reactive HIV-1 positive sera, ARP 444 sheep anti-Nef 14 antiserum, (University of Leeds, Leeds, UK), anti human CD4 mAb, clone 1F6 (Novacastra, Newcastle, UK), anti-human CXCR4 mAb clone C-20 (Santa Cruz

Biotechnology, Santa Cruz, CA), and anti-human CCR5 mAb clone H-185 (Santa Cruz Biotechnology).

Flow cytometry analyses. For the detection of HIV-1 Env gp120, 3×10^5 cells were incubated with a 1:100 dilution of the 4G10 anti-gp120 mAb or, as control, with a isotype-matched, specie-specific irrelevant mAb for 1 h at 4 °C. Thereafter, cells were washed, and labeled for 1 h at r.t. with a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG. Cell populations were finally analyzed by FACs. For the detection of HIV-1 Gag-related products, MDM were treated with Permeafix (Ortho Diagnostic, Raritan, NJ) for 30 min at r.t., and then labelled for 1 h at r.t. with a 1:50 dilution of phycoerythrin (PE)- conjugated anti-p24 HIV-1 Gag KC-57 mAb (Coulter Corp. Hialeah, FL).

Recovery and Characterization of (CD4-CXCR4) and (CD4-CCR5) Nef7/GFP and Nef7/TK VLPs

We have already shown in Example 1 that the VLP-mediated intracellular delivery of Nef7/TK led to cell death in the presence of GCV. We attempted to exploit such a phenomenon in a new anti-HIV therapeutic strategy by creating VLPs specifically directed to HIV infected cells. This was carried out by inserting HIV cell receptors, i.e. CD4 and CXCR4 or CCR5, in VLPs that, in this manner, would enter HIV Env expressing cells through the inverse fusion process. To this end, both CD4-CXCR4 and CD4-CCR5 pseudotyped HIV-1 based VLPs were recovered from the supernatants of 293T cells transiently transfected with the respective expression vectors as detailed in the experimental procedures. In order to assess the internalization efficiency of VLPs by the cells, Nef7/GFP incorporating VLPs were also produced.

In a first instance, the VLPs were molecularly characterized by Western blot analyses performed on 50 ng of VLPs purified on 20% sucrose cushion. We proved that VLPs indeed incorporated amounts of Nef7-based fusion products comparable to Nef7 alone (Fig. 9A-B) as well as the expected cell receptors (Fig. 9C-E). Titers of recovered VLPs reached 1-3 ng/ml in supernatants, and 500-1,500 ng/ml after 500× concentration. Thus, the transient transfection on 293T cells appeared a suitable

method for producing the amounts of both (CD4-CXCR4) and (CD4-CCR5) Nef7-based VLPs required for both molecular and biological characterizations.

Both (CD4-CXCR4) and (CD4-CCR5) Nef7/GFP VLPs are Efficiently Internalized in Cells Expressing T or M-Tropic HIV-1

We next sought to establish whether both CD4-CXCR4 and CD4-CCR5 pseudotyped VLPs were able to enter HIV infected cells. To this end, HIV-1 chronically infected 8E5 human T cells (whose Env expression was preventively monitored by FACs, Fig. 10A) were infected with (CD4-5 CXCR4) or, as control, (CD4) Nef7/GFP VLPs. Cells were then sampled at 3, 6 and 10 hours post infection, treated with trypsin to eliminate VLPs still bound to the cell membrane, and analyzed by FACs. Clearly, a GFP-fluorescent cell sub-population was detected in cells challenged with (CD4-CXCR4) VLPs, but not with (CD4) VLPs (Fig. 10A). The percentages of fluorescent cells reproducibly decreased over time likely as the consequence of the intracellular degradation of Nef7/GFP protein. Overlapping results were obtained using H9/HTLVIII cells (not shown).

The assay on (CD4-CCR5) Nef7/GFP VLPs was carried out on 293T cells transfected with the M-tropic ADA HIV-1 expressing molecular clone. The levels of Env expression were monitored 48 hours thereafter (Fig. 10B) to ensure that the large majority of cells expressed the HIV-1 receptor. Thereby, the cells were challenged with (CD4-CCR5) Nef7-GFP VLPs or, as control, with the counterpart carrying the CD4 receptor alone. Similarly to that observed for the T-tropic infected cells, GFP-fluorescent cells were detected within ADA HIV-1 expressing cells challenged with (CD4-CCR5) but not with (CD4) Nef7/GFP VLPs (Fig. 10B), thus indicating that (CD4-CCR5) VLPs specifically entered M-tropic Env expressing cells. Of note, differently to that observed in (CD4-CXCR4) VLP-treated 8E5 cells, the percentage of fluorescent cells decreased till to control levels 10 hours post infection, probably the consequence of differences in the efficiency of the intracellular protein degradation. Likely due to relatively low titers of Nef7/GFP-based VLPs we recovered (i.e., 500 ng/ml), we were not able to obtain higher percentages of GFP positive cells.

In conclusion, the "inverse fusion" process seemed nicely operative in HIV-1 infected cells challenged with either (CD4-CXCR4) or (CD4-CCR5) VLPs. This prompted us to investigate the biological consequences of the challenge of HIV-1 infected cells with Nef7/TK-based VLPs.

HIV-1 Chronically Infected Cells are Efficiently Killed by (CD4-CXCR4) Nef7/TK VLPs

To gain the proof-of-principle that (CD4-CXCR4) Nef/TK VLPs can specifically kill HIV expressing cells, the effects on two cell lines chronically infected by T-tropic HIV-1 variants, i.e., 8E5 and H9/HTLV-III, were analyzed. 10^5 cells were challenged with 50 ng of (CD4-CXCR4) Nef7/TK VLPs for three cycles, and cultured with or without GCV. As control, cells were challenged also with the VSV-G pseudotyped Nef7/TK VLPs. The cell cultures were monitored at the day seven post VLP challenge, i.e. the time we previously observed to be required for the killing of the cells upon the challenge with (VSV-G) Nef7/TK VLPs (not shown). We observed that more than 90% of both (CD4-CXCR4) VLP-challenged cells were killed when re-cultured with GCV (Fig. 11), a result very close to that observed using (VSV-G) VLPs. Importantly, no effects were detected in cells challenged with VLPs and cultivated in the absence of GCV, as well as in GCV treated cells challenged with VLPs carrying either VSV-G or CD4-CXCR4 receptors, but incorporating Nef7 alone (not shown). Similarly, the challenge with (CD4-CXCR4) VLPs had no effects in GCV treated HIV-1 negative cells (data not shown).

In sum, these results support the notion that the inclusion of CD4 and CXCR4 receptors in Nef7/TK VLPs is a suitable strategy for killing T-tropic HIV-1 infected cells.

HIV-1 Acutely Infected Human T Lymphocytes are Targeted by (CD4-CXCR4) Nef7/TK VLPs

We next investigated the ability of (CD4-CXCR4) VLPs in targeting HIV-1 acutely infected cells. To this end, CEM-GFP, i.e., a human T lymphoblastoid cell line expressing the GFP exclusively in the presence of Tat, were infected with increasing

amounts of the T-tropic NL4-3 HIV-1 strain. Two days thereafter, i.e. the time required for the appearance of HIV-1 Env markers on the cell membrane (not shown), the cells were extensively washed and challenged twice with 30 ng/10^5 cells of (CD4-CXCR4) VLPs. Thereafter, cells were cultivated in the presence or in the absence of GCV, and after additional five days, scored by FACs for the percentages of fluorescent (i.e. HIV infected) cells. In this way, the anti-HIV effect of Nef7/TK VLPs can be evaluated in terms of the reduction of the percentages of fluorescent cells. Fig. 12 shows that the challenge with (CD4-CXCR4) VLPs coupled with the GCV treatment led to a lack of infected cells till the HIV-1 input of 5 ng of HIV-1/10^5 cells. By increasing the HIV-1 input, despite HIV-1 infected cells became detectable, the relative percentages appeared constantly reduced compared with control conditions. Of note, VLP-unchallenged HIV-1-infected cells cultured with or without GCV, supported the HIV-1 replication as well as the VLP-challenged cells re-cultured in the absence of GCV (not shown).

These results indicate that also cells acutely infected with T-tropic HIV-1 can be efficiently targeted by (CD4-CXCR4) VLPs.

HIV-1 Spread is Inhibited in Human Primary Monocyte/Macrophages Treated with (CD4-CCR5) Nef7/TK VLPs

Besides activated T lymphocytes, monocyte/macrophages, dendritic cells and resting T cells are natural host cells for HIV. These are non-replicating cells where the virus establishes a latent/persistent infection leading to the *in vivo* formation of privileged sites where the virus efficiently resists the HAART therapies. Being the presence of "sanctuaries" sites a major bottleneck towards the HIV eradication, it appears of major importance finding new anti-HIV therapeutic approaches efficiently targeting non-replicating, infected cells. In this respect, we sought to evaluate the efficiency of Nef7/TK VLPs in inhibiting the viral spread in a rather relevant *in vitro* model, i.e. the HIV-1 infected human primary monocyte-derived macrophages (MDM). Monocytes were purified from the peripheral blood of healthy donors, cultured for 5 days, and infected with 100 pg/10^5 cells of the ADA M-tropic HIV-1 strain. Two days thereafter, cells were washed, challenged twice with 30 ng of (CD4-CCR5) Nef7/TK VLPs, and cultivated in the presence of GCV. Supernatants were harvested

from 7 days post-HIV infection and the Gag p24 levels herein measured. As shown in Fig. 13A, the challenging with CD4-CCR5 VLPs efficiently prevented the viral spread within the MDM cultures cultivated with GCV (Fig. 5A). The FACs analysis carried out by labeling infected cultures with anti-Gag p24 mAb at day 11 post-infection, showed that the decrease of HIV-1 release in the supernatants correlated with a reduced number of infected cells (Fig. 13B).

By increasing the HIV-1 input 5-fold, we observed that VLPs still had an antiviral effect, even if with a reduced potency likely due to the overwhelming amounts of infecting HIV-1 (not shown). VLP-untreated MDM re-cultured with or without GCV replicated HIV-1 as efficiently as the (CD4-CCR5) VLP-treated MDM re-cultured in the absence of GCV (data not shown).

Typically, only a minority of *in vitro* infected MDM sustains active HIV replication, even using high MOIs. In order to substantiate our previous observations, we circumvented such a technical limitation by reproducing the HIV-1 infection/VLP challenging protocol using VSV-G pseudotyped HIV-1. In this way, we already showed that the large majority of MDM becomes infected and expresses HIV-1 proteins within 24 hours (77). Consistently with the previous approach, we observed that the challenging with (CD4-CCR5) Nef7/TK VLPs and cultivation with GCV strongly reduced the number of infected cells (Fig. 14), thus enforcing the idea that (CD4-CCR5) VLPs specifically target and kill HIV-1 Env-expressing cells.

In sum, (CD4-CCR5) VLPs appeared effective in clearing HIV-1infected MDM. Hence, such VLPs may be considered a new potential therapeutic tool for combating persistent/latent HIV infections in view of an effective strategy of HIV eradication.

EXAMPLE 3

Production of the Nef7-MAGE/A3 VLPs

The HIV-1 Nef7 mutant was previously inserted in the pCNefsg25GFP construct expressing the Nef-GFP fusion product by replacing the wt Nef open reading frame (78). The GFP moiety of the Nef7-GFP construct was then replaced by the MAGE-

A3 open reading frame PCR amplified from the LM3TN plasmid using the following primers: forward 5' CTT GTA GCT AGC CCT CTT GAG CAG AGG AGT CAG CAC 3', and reverse 5'GTA TCT AGA TCA CTC TTC CCC CCT CTC 3'. In this manner, the MAGE-A3 ATG start codon was eliminated, and two unique sites were created at the 5' (Nhe I) and 3' (Xba I) ends. The fully digested MAGE-A3 PCR product was then inserted in the NheI/Xba I unique sites of the Nef7-GFP plasmid upon GFP deletion, leading to the formation of a unique Nef7-MAGE-A3 open reading frame.

Preparations of Nef7-MAGE-A3 VLPs were obtained from supernatants of 293T cells 48-60 h after the calcium phosphate co-transfection with the pCMV□R8.74 packaging vector, the Nef7-MAGE-A3, and a plasmid expressing either the VSV-G or the RD114 receptors, in a molar ratio of 3:3:1. Supernatants were filtered on 0.45μm pore size membrane, and concentrated by ultra-centrifugation on a 20% sucrose cushion. VLP preparations were then titrated both by measuring HIV-1 p24 contents by quantitative ELISA, and by the reverse transcriptase assay.

DC generation

Dendritic cells were generated from peripheral blood mononuclear cells (5x107 cells/T25 flask) by 1h adherence at 37°C. After that the adherent cells were cultivated in the presence of IL-4 (500 U/ml) and GM-CSF (800 U/ml) in RPMI 1640 medium 10% FBS for 5 days.

VLPs loading

DCs (2x105) were collected and infected with 50 ng of VLPs (Nef7/MAGE-3 VSV-G HIV-VLP, Nef7/MAGE-3 MLV-VLP and Nef7/MAGE-3 RD114 HIV-VLP) in PBS by spinoculation (30' of centrifugation at 2000rpm at room temperature). Afterward, the cells were re-fed by adding fresh medium and were incubated for 12h at 37°C

IFN-γ assay

Autologous DCs (3x104) loaded with VLPs as described above, were washed with PBS and seeded in 96-microwell plates with the anti-MAGE-3 CTL (104 cells/well) in IMDM 10% human plasma in the presence of IL-2 (50 U/ml).

After 12h of coculture, the supernatant was collected and the content of IFN-g was measured by ELISA.

Efficiency of dendritic cells loading of Nef7-MAGE/A3 VLPs

To verify the ability of VLPs containing the fusion protein Nef7/MAGE-3, to effectively loading human monocyte-derived dendritic cells (DCs), DCs were loaded as described above and used to stimulate a MAGE-3-specific CTL. A specific IFN-γ release was observed in the presence of the loaded DCs regardless of the VLPs origin (MLV, HIV), while unloaded DCs were not recognized.

These results strongly suggest that both MLV and HIV-derived VLPs are able to load DCs with a comparable efficiency and more importantly that the Nef7/MAGE-3 fusion protein is effectively processed and presented by DCs to immune effectors.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

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Claims

1. A fusion protein comprising a Nef double mutant (V 153 L, E 177 G) polypeptide (hereinafter Nef7) as shown in Seq ID No: 1 associated with a heterologous polypeptide.

2. A fusion protein according to claim 1 wherein the heterologous polypeptide is associated with the C terminus of Nef7.

3. The fusion protein according to claim 1 or 2 wherein the heterologous polypeptide is a therapeutic or diagnostic polypeptide.

4. The fusion protein according to claim 3 wherein the therapeutic polypeptide is a tumor antigen selected from the group consisting of BAGE-1, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GnTVf, HERV-K-MEL, KM-HN-1, LAGE-1, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A9, MAGE-A10, MAGE-A12, MAGE-C2, MART-1, mucink, NA-88, NY-ESO-1/LAGE-2, SAGE, Sp17, SSX-2, SSX-4TRP2-INT2g tumor antigens.

5. The fusion protein according to claim 3 wherein the therapeutic polypeptide is the tumor antigen MAGE-A3.

6. The fusion protein according to claim 3 wherein the therapeutic polypeptide has antitumor properties.

7. The fusion protein according to claim 3 wherein the therapeutic polypeptide is an enzyme capable of converting a non-toxix prodrug into an active cytotoxic drug selected from the group consisting of

a) Herpes Simplex Virus derived Thimidine Kinase (TK)

b) E.coli derived cytosine deaminase

c) Mammalian cytocrome P450.

8. The fusion protein according to claim 7 wherein the therapeutic polypeptide is TK.

9. The fusion protein according to claim 3 wherein the therapeutic polypeptide is a viral antigen.

10. The fusion protein according to claim 9 wherein the therapeutic polypeptide is selected from the group consisting of:

a) Human Papilloma Virus derived oncoproteins E6 and E7 and derivatives thereof

b) Human Hepatitis C derived NS3, NS5, E1, E2, Core Antigen, and derivatives thereof

c) HIV derived Tat and Env and derivative thereof

11. The fusion protein according to claim 3 wherein the diagnostic peptide is a detectable moiety.

12. A polynucleotide encoding the protein of any preceding claim.

13. An expression vector comprising the polynucleotide of claim 12.

14. A viral particle comprising the protein of any one of claims 1 to 11.

15. The viral particle of claim 14 wherein the viral particle is derivable from a retrovirus or lentivirus.

16. The viral particle of claim 9 wherein the virus is HIV or MLV.

17. The viral particle of any one of claims 14-16 wherein the VLP is pseudotyped.

18. The viral particle of claim 17 wherein the viral particle is pseudotyped with viral envelope protein VSV-G, RD114 glycoprotein, MLV glycoprotein or a chimeric protein comprising the extracellular and transmembrane domain of RD114 and the cytoplasmic domain of MLV glycoproteins.

19. The viral particle of claim 17 wherein the viral particle is pseudotyped with HIV cell receptors.

20. The viral particle of claim 19 wherein the viral particle is pseudotyped with CD4 and CXCR4 or CD4 and CCR5 HIV cell receptors.

21. The viral particle of claim 17 wherein the viral particle is pseudotyped with a tumor cell surface antigen.

22. The viral particle of claim 17 wherein the viral particle is pseudotyped with a ligand of a tissue marker.

23. A cell comprising the protein of any one of claims 1-11, the polynucleotide of claim 12, the expression vector of claim 13 or the viral particle of any one of claims 14-22.

24. The cell of claim 23 wherein the cell is a host cell.

25. The cell of claim 24 wherein the cell is a tumor cell.

26. The cell of claim 24 wherein the cell is a dendritic cell, a lymphocyte or a monocyte.

27. A method comprising exposing a cell obtainable from an individual to the protein of any one of claims 1-11 or the viral particle of any one of claims 14-23.

28. The method of claim 27 wherein the cell is a dendritic cell, a lymphocyte or a monocyte.

29. A cell obtainable from the method of claim 27 or 28.

30. A pharmaceutical composition comprising the protein of any one of claims 1-11, the cell of any one of claims 23-26 or 29 or the viral particle of any one of claims 14-22, together with a pharmaceutically acceptable carrier, excipient or diluent.

31. The protein of any one of claims 1-11, the polynucleotide of claim 12, the expression vector of claim 13, the cell of any one of claims 23-26 or 29 or the viral particle of any one of claims 14-22 for use in medicine.

32. Use of the protein of any one of claims 1-11, the polynucleotide of claim 12, the expression vector of claim 13, the cell of any one of claims 23-26 or 29 or the viral particle of any one of claims 14-22 for the preparation of a medicament for the treatment of cancer.

33. Use of the protein of any one of claims 1-11, the polynucleotide of claim 12, the expression vector of claim 13, the cell of any one of claims 23-26 or 29 or the viral particle of any one of claims 14-22 for the preparation of a medicament for the treatment of infectious diseases.

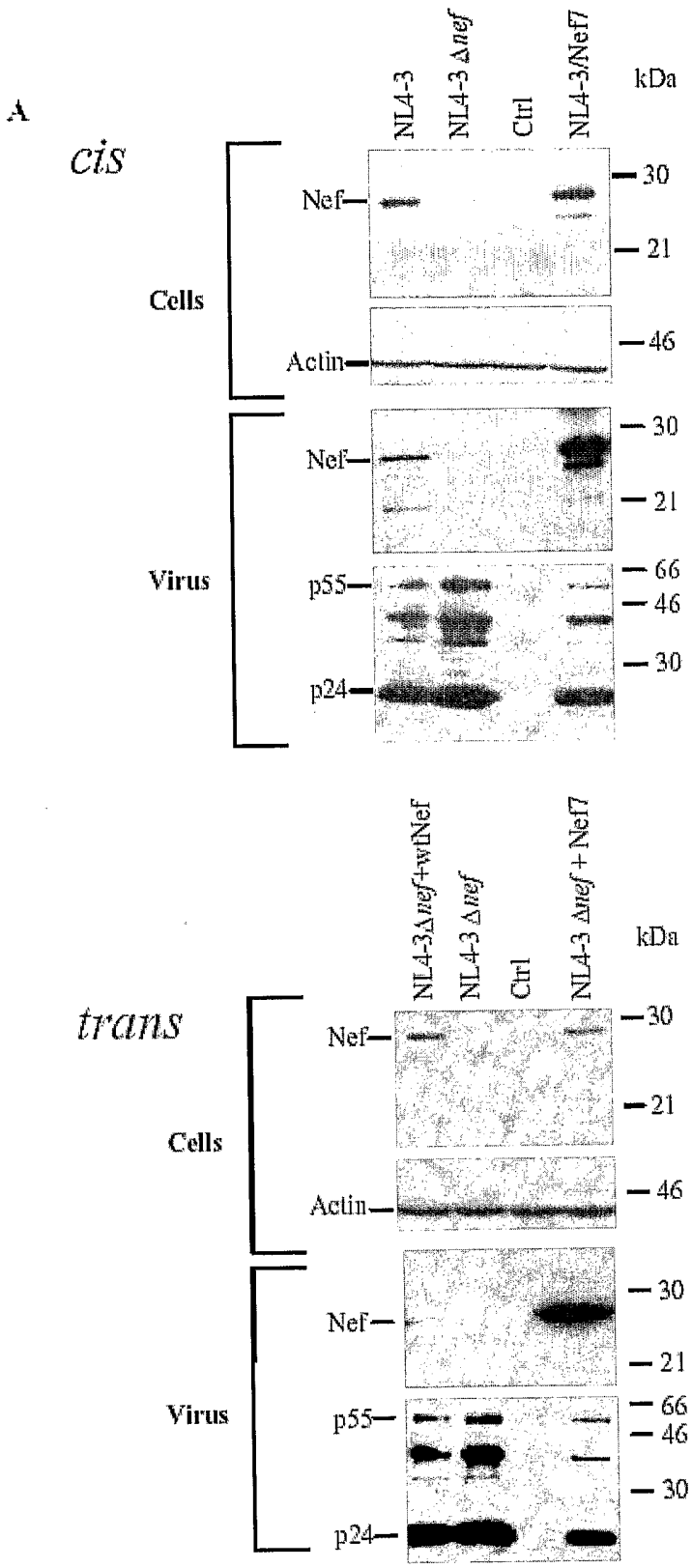
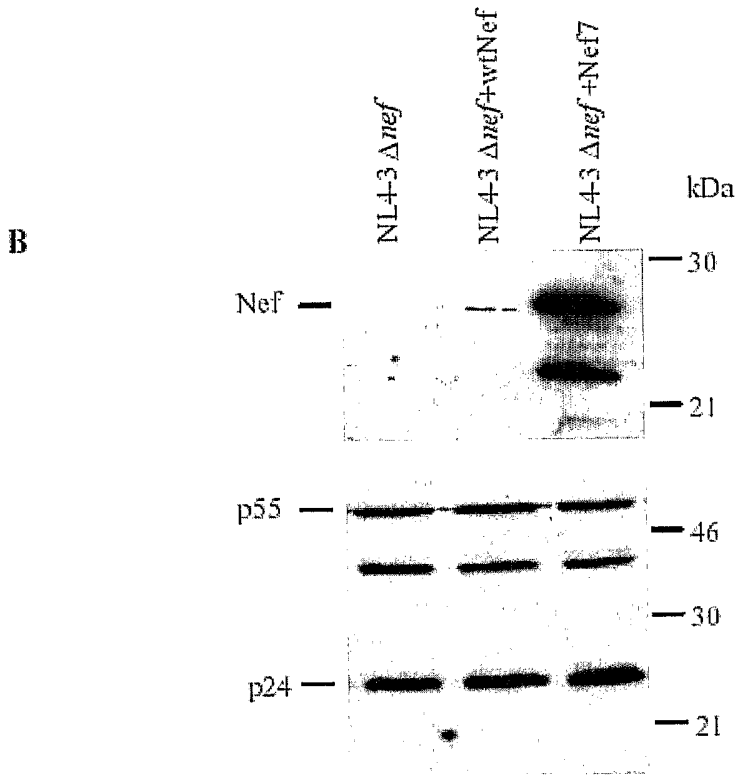

A
cis
NL4-3
NL4-3 Δnef
Ctrl
NL4-3/Nef7
kDa
Cells
Nef
Actin
Virus
Nef
p55
p24
30
21
46
66
trans
NL4-3Δnef+wtNef
NL4-3 Δnef
Ctrl
NL4-3 Δnef + Nef7
kDa
Cells
Nef
Actin
Virus
Nef
p55
p24
30
21
46
66

Fig 1A


B
NL4-3 Δnef
NL4-3 Δnef+wtNef
NL4-3 Δnef +Nef7
kDa
Nef
30
21
p55
46
30
p24
21

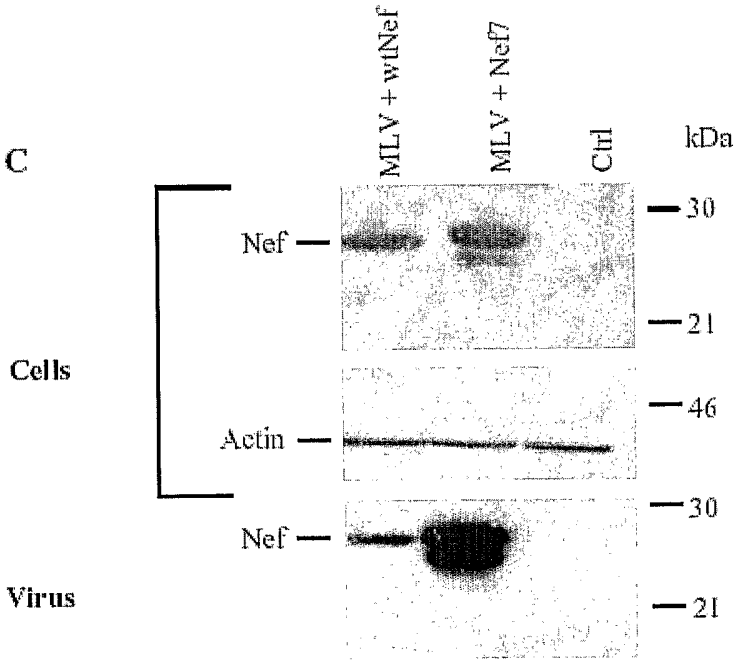

C
MLV + wtNef
MLV + Nef7
Ctrl
kDa
Nef
30
21
Cells
Actin
46
Nef
30
Virus
21

Fig 1B, C

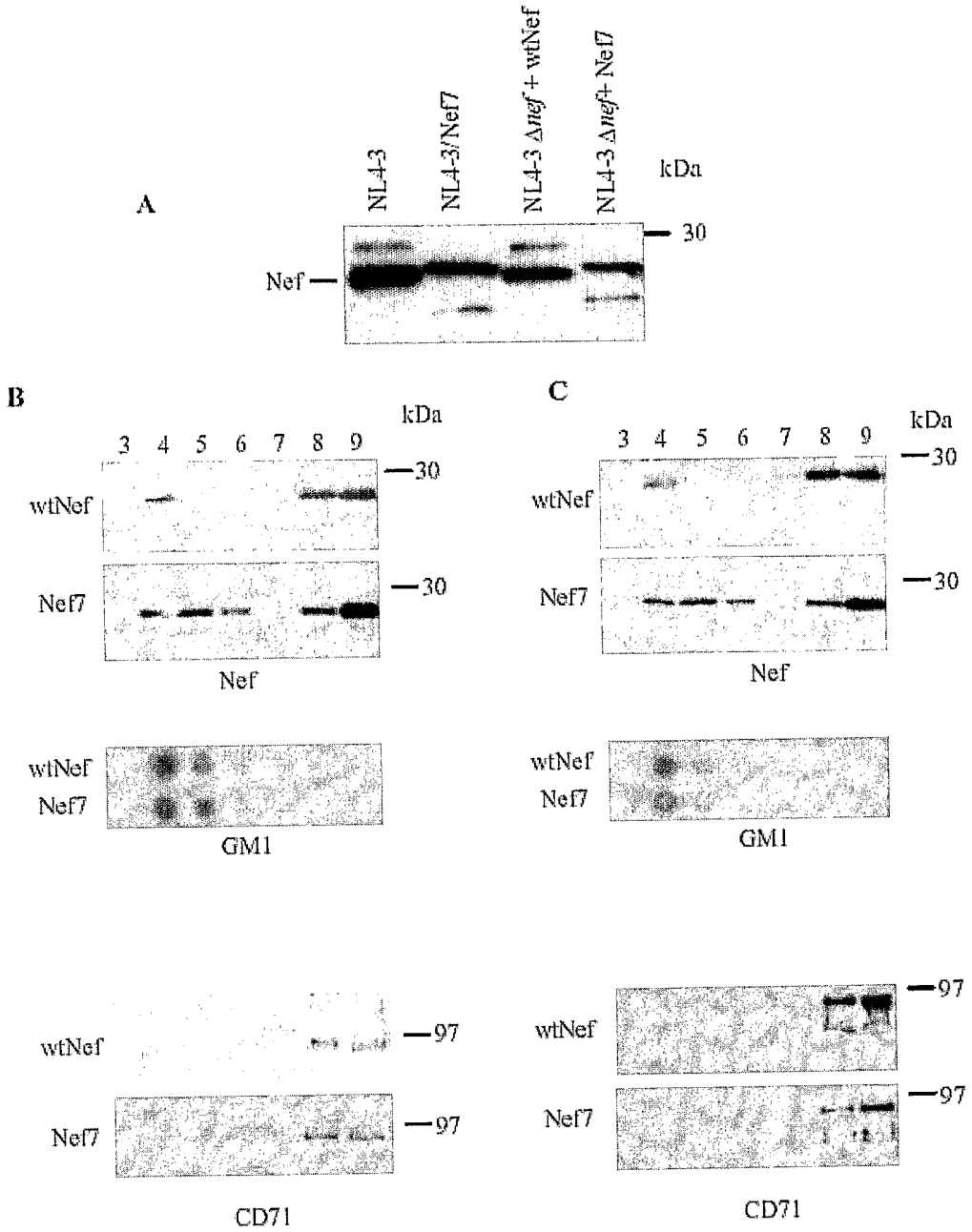
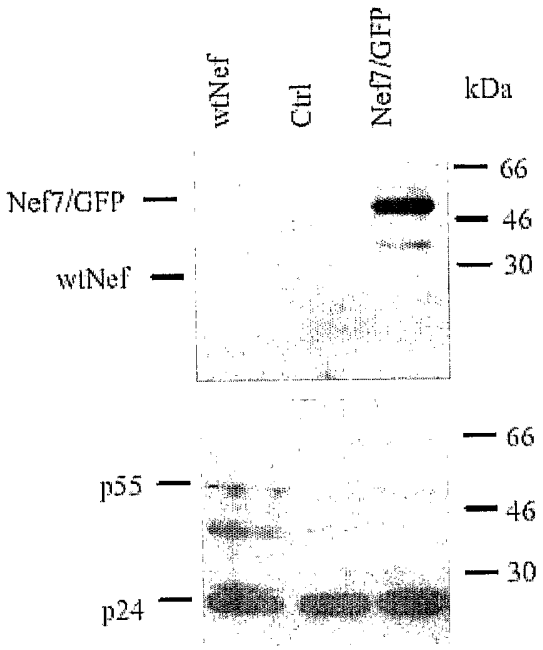

A
NL4-3
NL4-3/Nef7
NL4-3 Δnef + wtNef
NL4-3 Δnef+ Nef7
kDa
30
Nef
B
kDa
3 4 5 6 7 8 9
30
wtNef
30
Nef7
Nef
wtNef
Nef7
GM1
97
wtNef
97
Nef7
CD71
C
kDa
3 4 5 6 7 8 9
30
wtNef
30
Nef7
Nef
wtNef
Nef7
GM1
97
wtNef
97
Nef7
CD71

Fig. 2

A


wtNef
Ctrl
Nef7/GFP
kDa
Nef7/GFP
wtNef
66
46
30
p55
p24
66
46
30

B

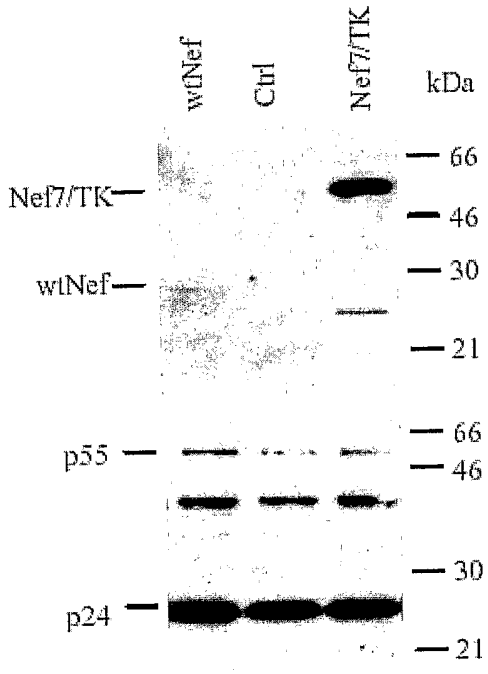

wtNef
Ctrl
Nef7/TK
kDa
Nef7/TK
wtNef
66
46
30
21
p55
p24
66
46
30
21

Fig. 3

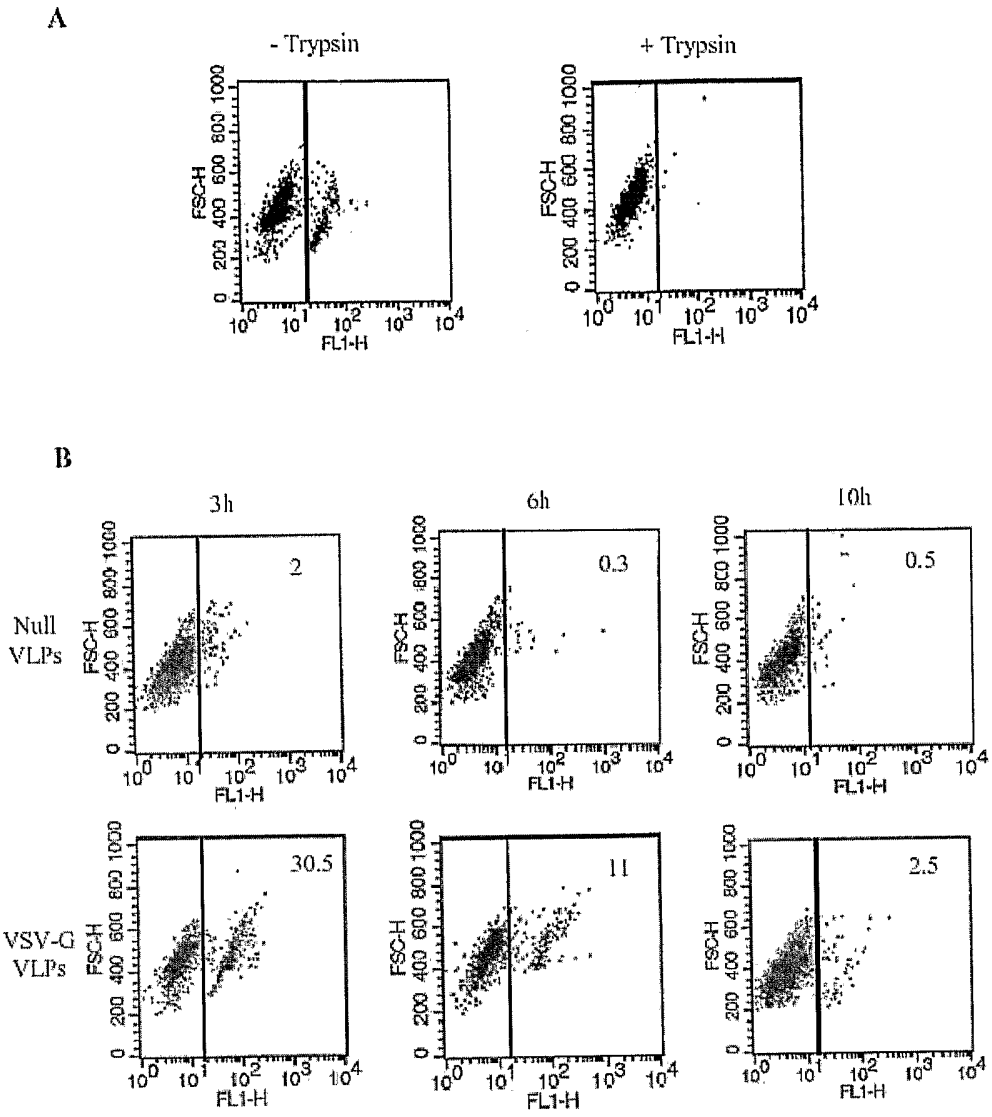

A
- Trypsin
+ Trypsin
FSC-H
FL1-H
B
3h
6h
10h
Null VLPs
VSV-G VLPs
2
0.3
0.5
30.5
11
2.5

Fig. 4

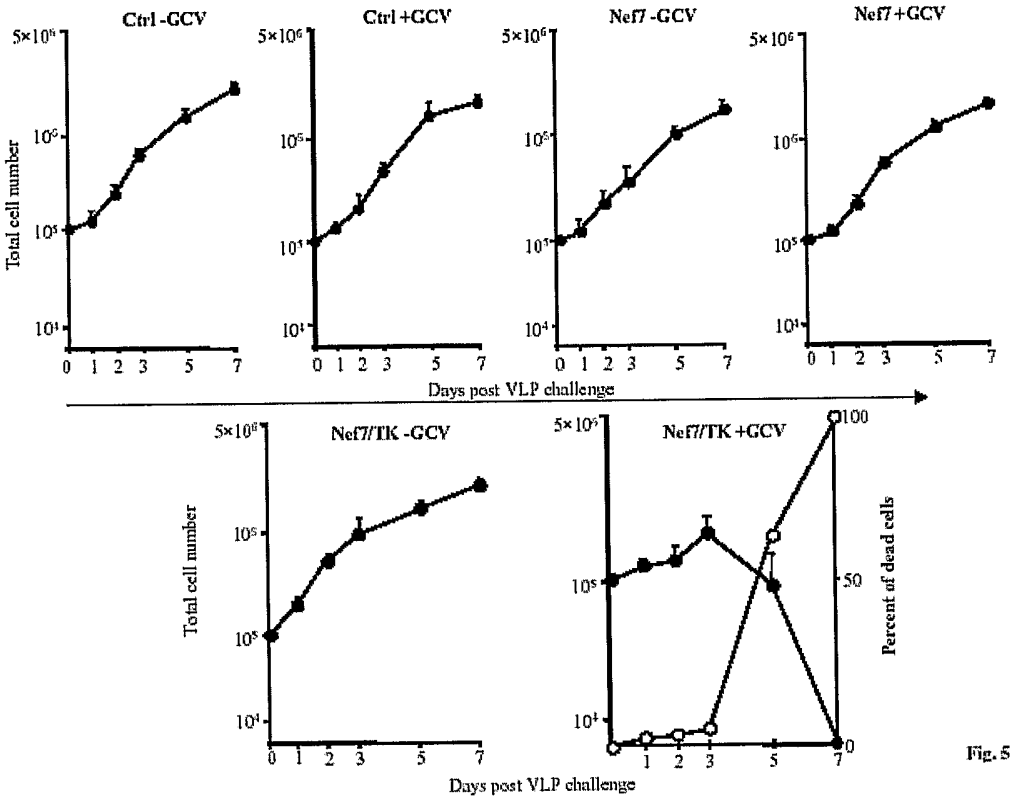

Ctrl -GCV
Ctrl +GCV
Nef7 -GCV
Nef7 +GCV
Total cell number
Days post VLP challenge
Nef7/TK -GCV
Nef7/TK +GCV
Percent of dead cells

Fig. 5

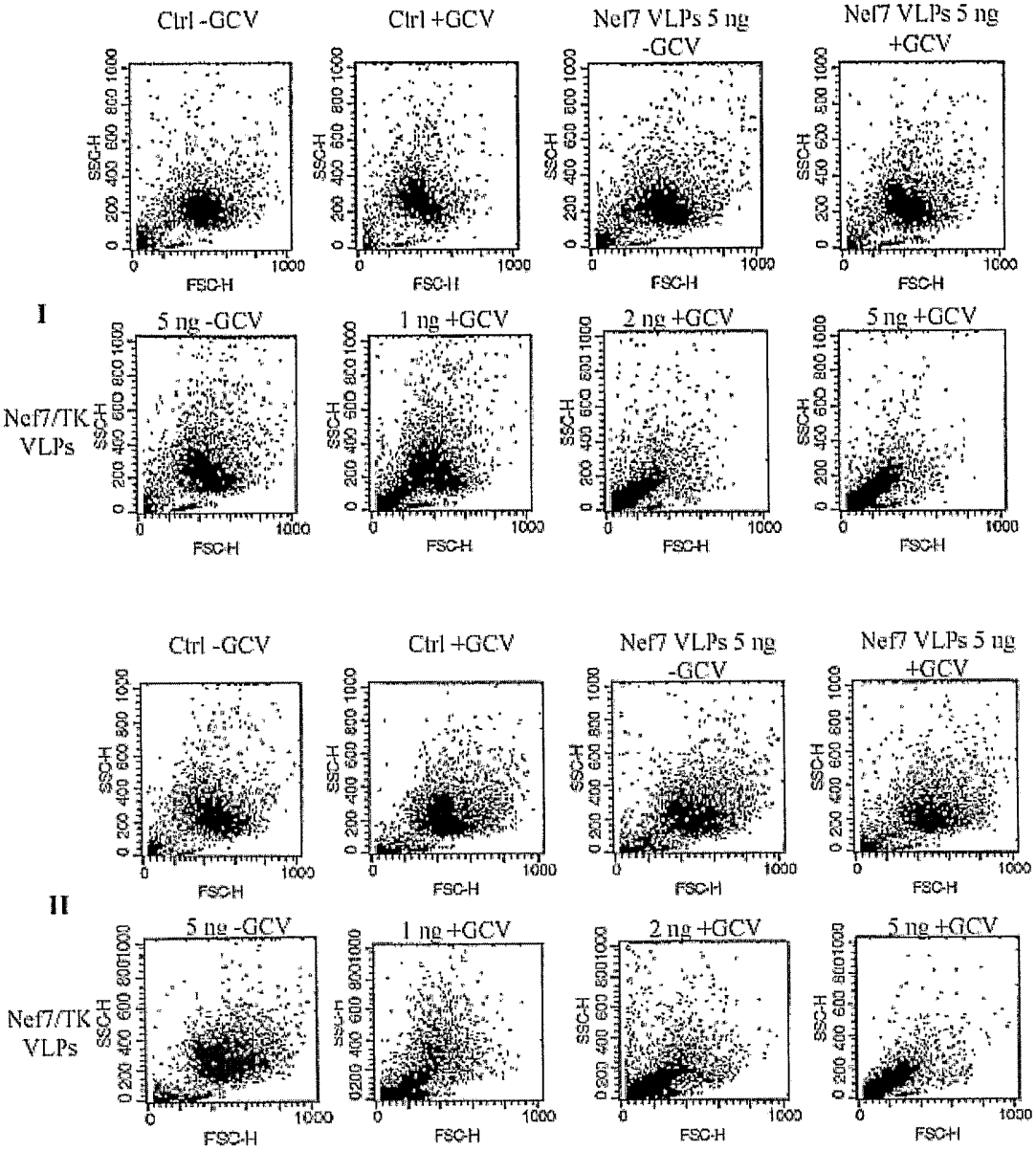

Ctrl -GCV
Ctrl +GCV
Nef7 VLPs 5 ng -GCV
Nef7 VLPs 5 ng +GCV
I
Nef7/TK VLPs
5 ng -GCV
1 ng +GCV
2 ng +GCV
5 ng +GCV
Ctrl -GCV
Ctrl +GCV
Nef7 VLPs 5 ng -GCV
Nef7 VLPs 5 ng +GCV
II
Nef7/TK VLPs
5 ng -GCV
1 ng +GCV
2 ng +GCV
5 ng +GCV
SSC-H
FSC-H

Fig. 6

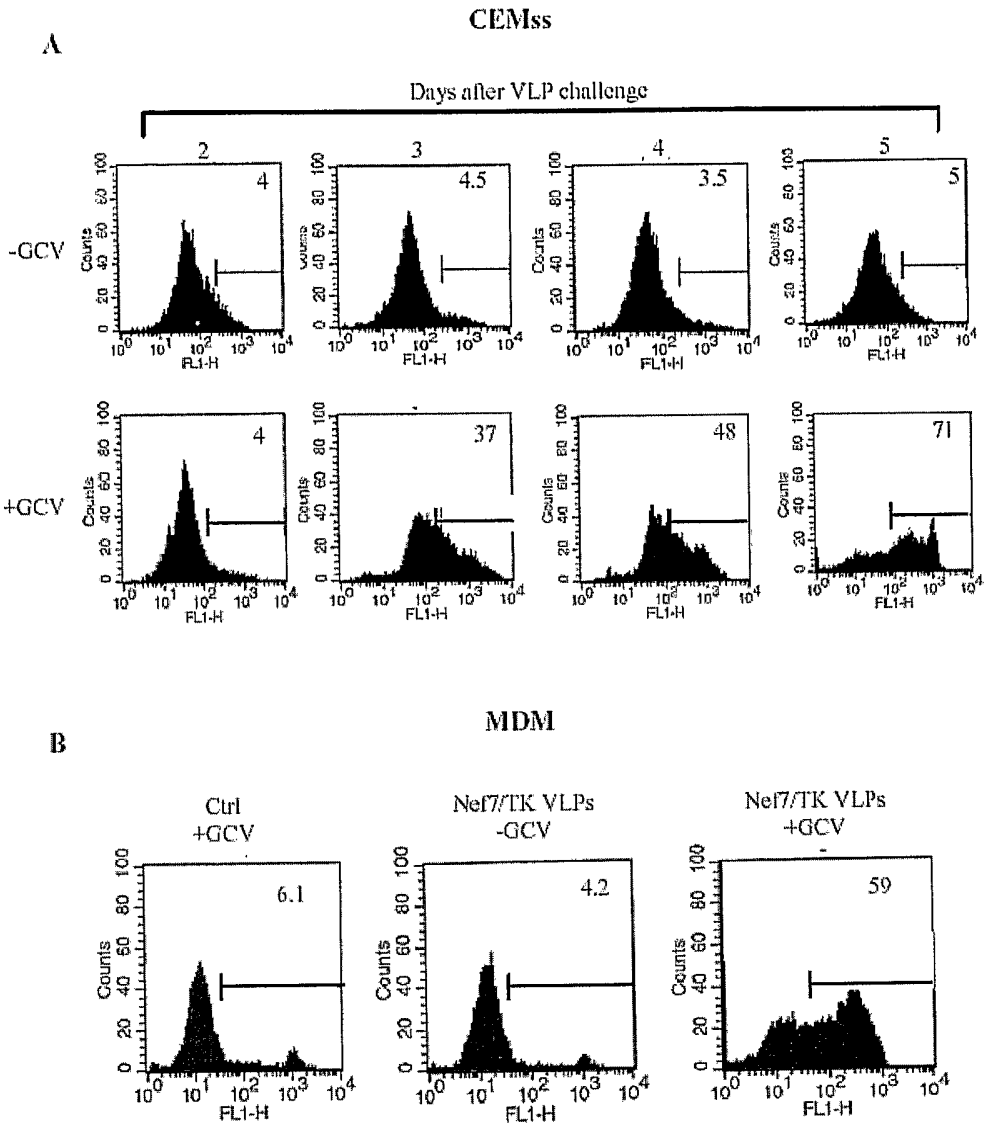

CEMss
A
Days after VLP challenge
2
3
4
5
-GCV
4
4.5
3.5
5
+GCV
4
37
48
71
Counts
FL1-H
MDM
B
Ctrl
+GCV
Nef7/TK VLPs
-GCV
Nef7/TK VLPs
+GCV
6.1
4.2
59

Fig. 7

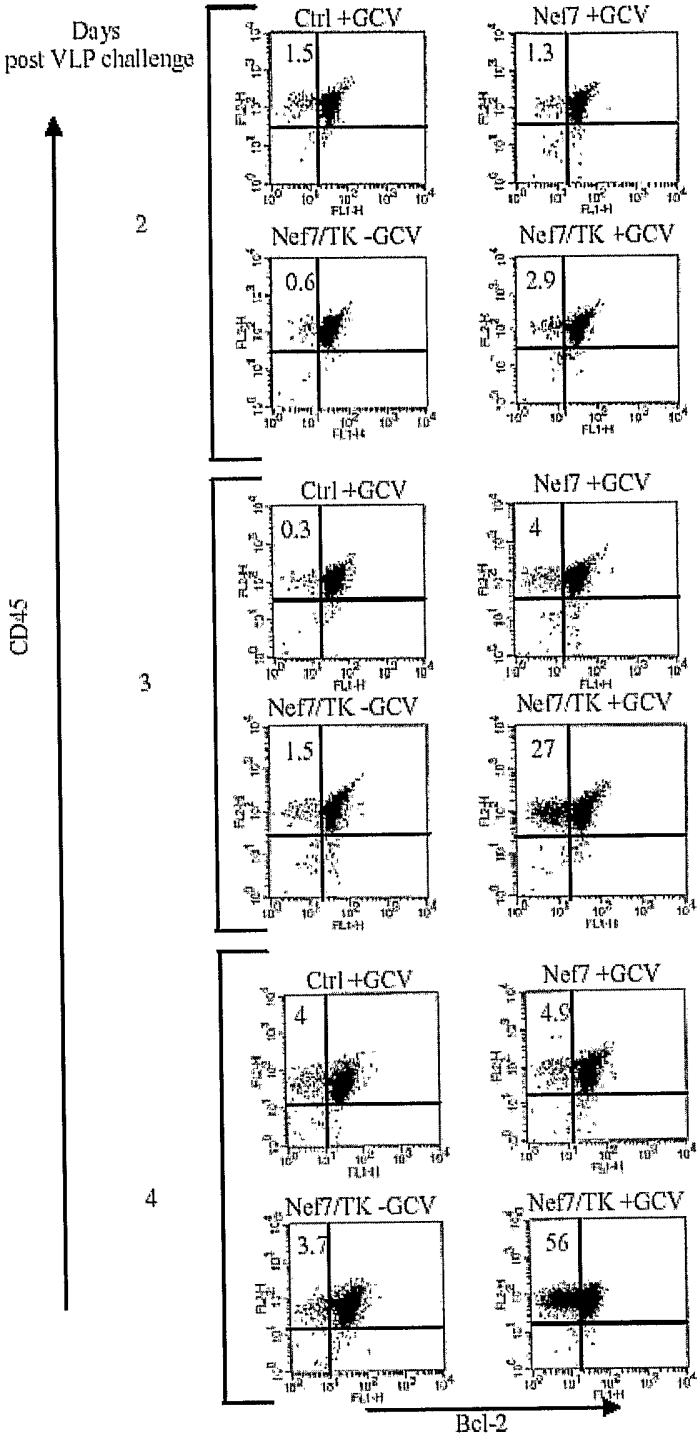

Days
post VLP challenge
2
3
4
CD45
Bcl-2
Ctrl +GCV
1.5
Nef7 +GCV
1.3
Nef7/TK -GCV
0.6
Nef7/TK +GCV
2.9
Ctrl +GCV
0.3
Nef7 +GCV
4
Nef7/TK -GCV
1.5
Nef7/TK +GCV
27
Ctrl +GCV
4
Nef7 +GCV
4.9
Nef7/TK -GCV
3.7
Nef7/TK +GCV
56

Fig. 8

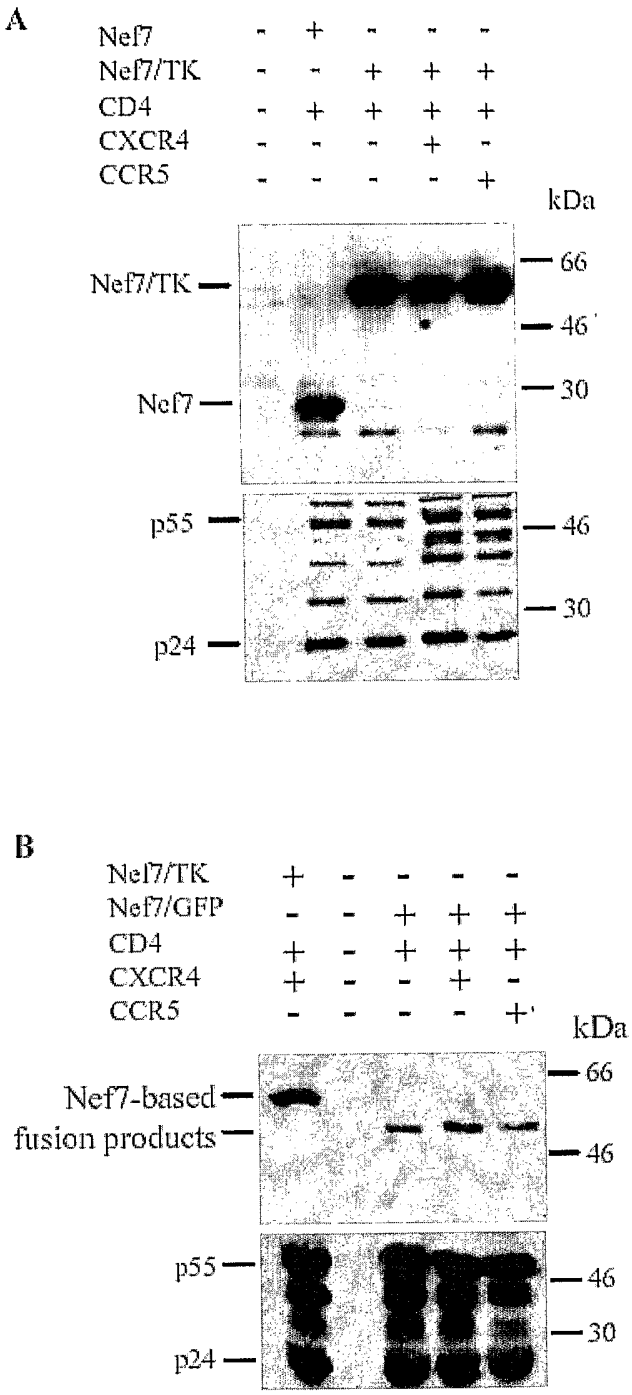

A
Nef7 - + - - -
Nef7/TK - - + + +
CD4 - + + + +
CXCR4 - - - + -
CCR5 - - - - +
kDa
Nef7/TK
Nef7
p55
p24
66
46
30
46
30
B
Nef7/TK + - - - -
Nef7/GFP - - + + +
CD4 + - + + +
CXCR4 + - - + -
CCR5 - - - - +
kDa
Nef7-based fusion products
p55
p24
66
46
46
30

Fig. 9A, B

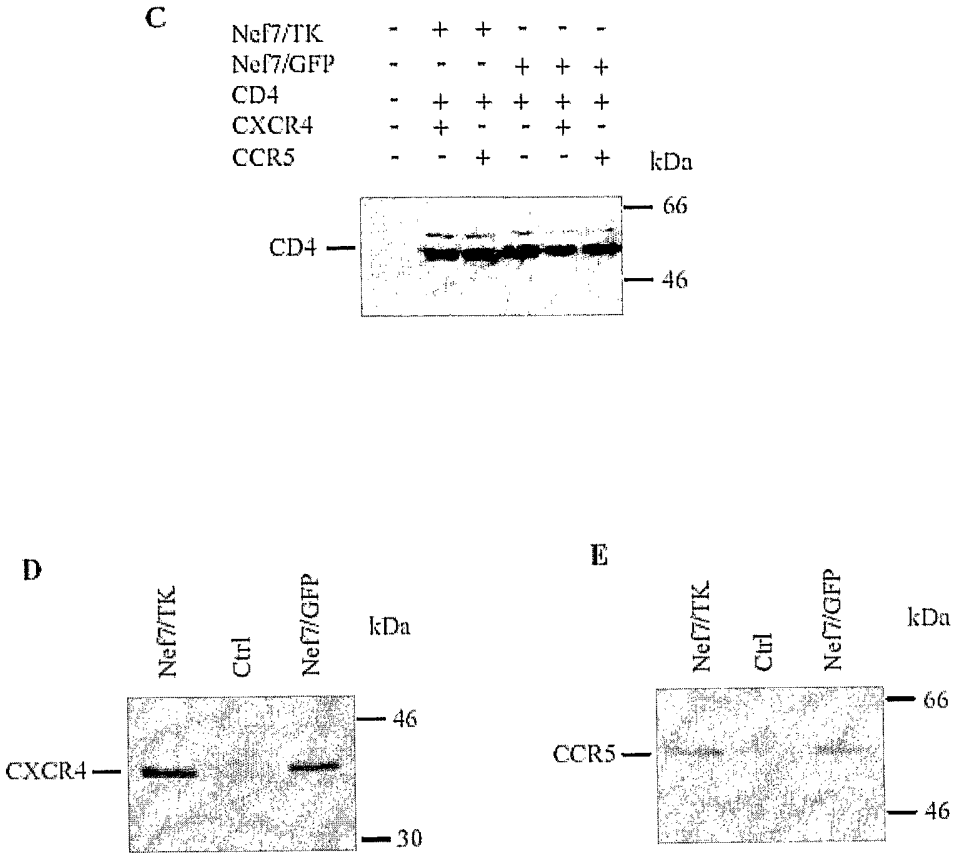

C
Nef7/TK - + + - - -
Nef7/GFP - - - + + +
CD4 - + + + + +
CXCR4 - + - - + -
CCR5 - - + - - +
kDa
CD4
66
46
D
Nef7/TK
Ctrl
Nef7/GFP
kDa
CXCR4
46
30
E
Nef7/TK
Ctrl
Nef7/GFP
kDa
CCR5
66
46

Fig. 9C-E

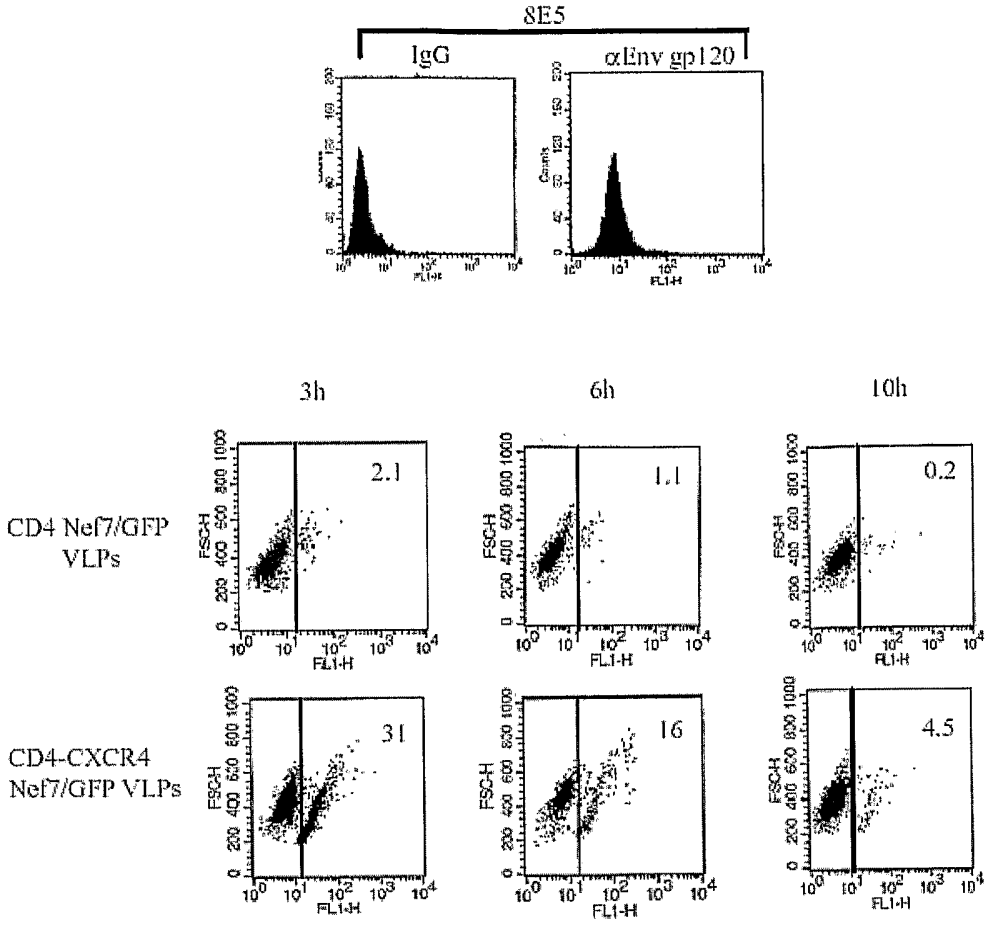

8E5
IgG
αEnv gp120
3h
6h
10h
CD4 Nef7/GFP VLPs
CD4-CXCR4 Nef7/GFP VLPs
2.1
1.1
0.2
31
16
4.5
FSC-H
FL1-H

Fig. 10A

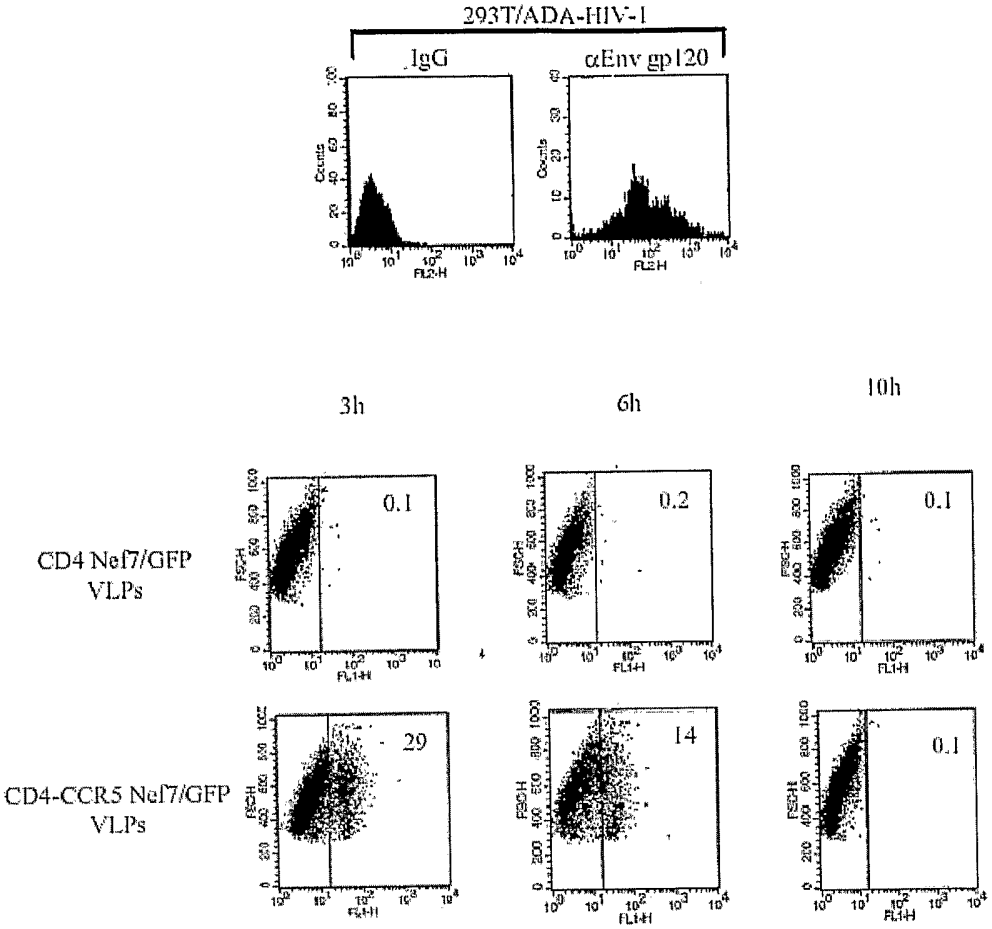

293T/ADA-HIV-1
IgG
αEnv gp120
3h
6h
10h
CD4 Nef7/GFP VLPs
CD4-CCR5 Nef7/GFP VLPs
0.1
0.2
0.1
29
14
0.1

Fig. 10B

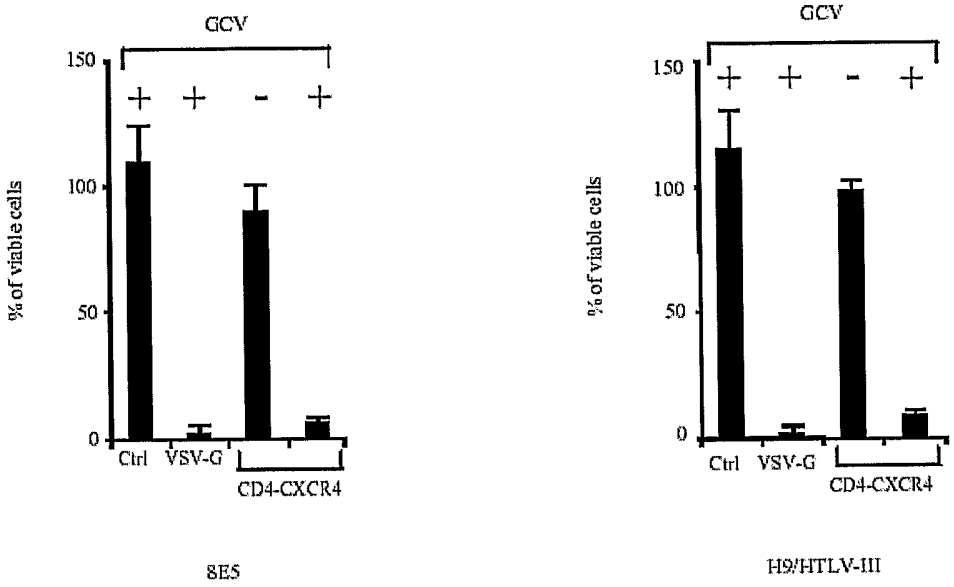

GCV
150
100
50
0
% of viable cells
+ + - +
Ctrl
VSV-G
CD4-CXCR4
8E5
GCV
150
100
50
0
% of viable cells
+ + - +
Ctrl
VSV-G
CD4-CXCR4
H9/HTLV-III

Fig. 11

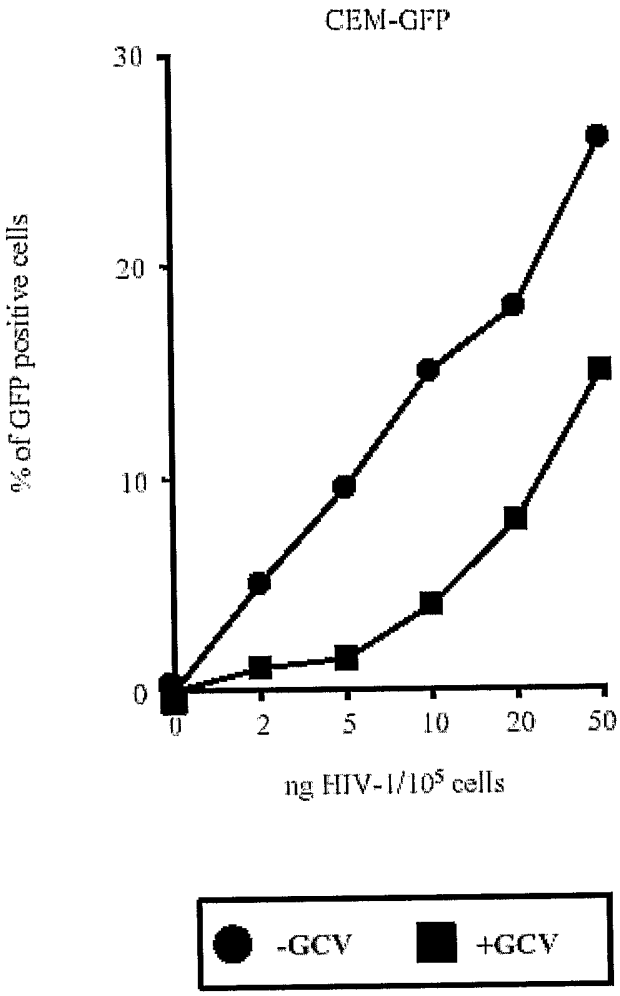
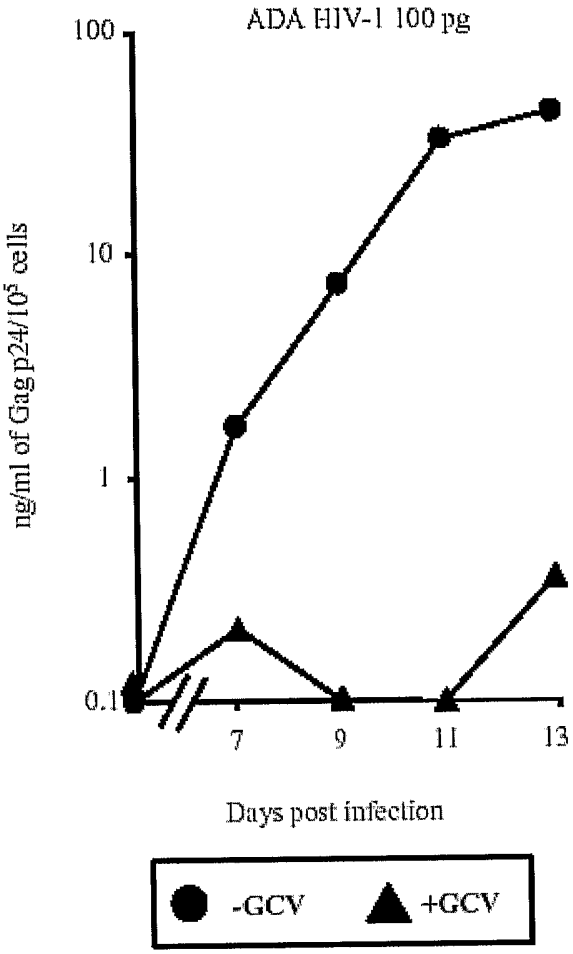

CEM-GFP
% of GFP positive cells
30
20
10
0
0
2
5
10
20
50
ng HIV-1/10^5 cells
-GCV
+GCV

Fig. 12

A


ADA HIV-1 100 pg
100
10
1
0.1
ng/ml of Gag p24/10^5 cells
7
9
11
13
Days post infection
-GCV
+GCV

B

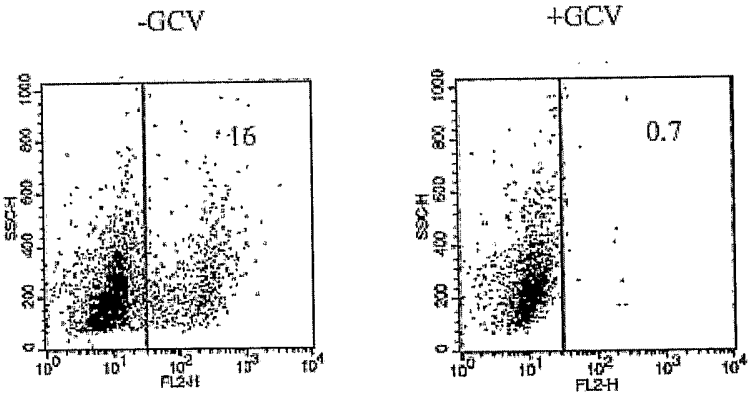

-GCV
+GCV
16
0.7
SSC-H
FL2-H

Fig. 13

Fig. 14

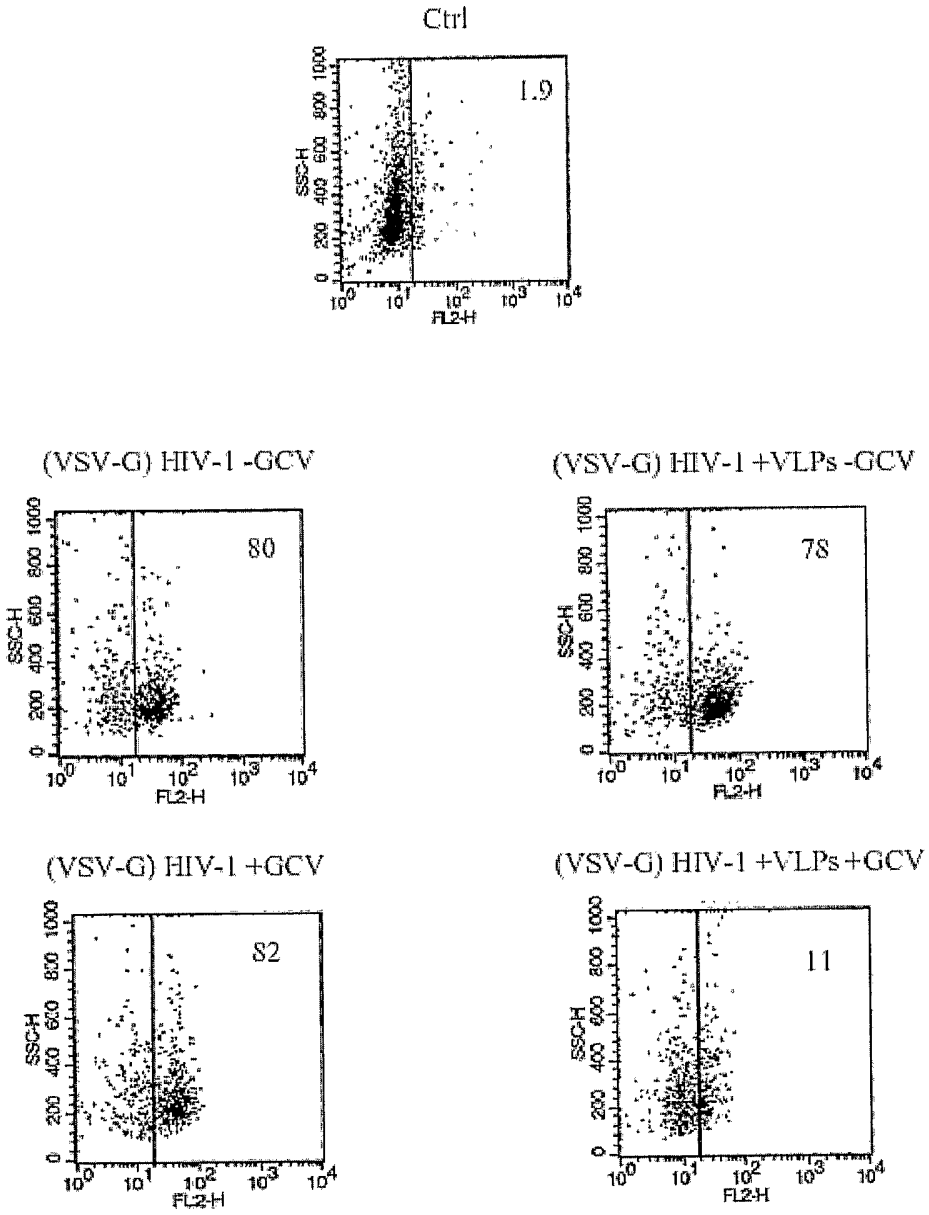

Ctrl
1.9
(VSV-G) HIV-1 -GCV
80
(VSV-G) HIV-1 +VLPs -GCV
78
(VSV-G) HIV-1 +GCV
82
(VSV-G) HIV-1 +VLPs +GCV
11
SSC-H
FL2-H

FIG 15 - NEF 7 sequence

8341 Atg ggt ggc aag tgg tca aaa agt agt gtg **G**tt gga tgg cct **G**ct gta agg gaa aga a

8401 tg aga cga gct gag cca gca gca gat **G**gg gtg gga gca gca tct cga gac ct**A** gaa aaa c

8461 at gga gca atc aca agt agc aat aca gca gct acc aat gct g**A**t tgt gcc tgg cta gaa g

8521 ca caa gag gag gag gag gtg ggt ttt cca gtc aca cct cag gta cct tta aga cca atg a

8581 ct tac aag gca gct gta gat ctt agc cac ttt tta aaa gaa aag ggg gga ctg gaa ggg c

8641 ta att cac tcc caa **C**ga aga caa gat atc ctt gat ctg tgg atc tac cac aca caa ggc t

8701 ac ttc cct gat t**G**g cag aac tac aca cca gg**A** cca ggg gt**T** aga tat cca ctg acc ttt g

8761 ga tgg tgc tac aag cta gta cca gtt gag cca ga**G** aag Tta gaa ga**A** gcc aa**C** aaa gga g

8821 ag aac acc agc ttg tta cac cct gtg agc ctg cat gg**A** atg gat gac ccg gGg aga gaa g

8881 tg tta gag tgg agg ttt gac agc cgc cta gca ttt cat cac gtg gcc cga gag ctg cat c

8941 cg gag tac ttc aag aac tgc tga

Not functional or not significant mutations are indicated in **capitals**.

The three critical amino acid mutation of F12 (G140E, V153L. E177G) are highlighted in shading, the first residue (position 140) is back mutated (E140G) in Nef7 mutant.

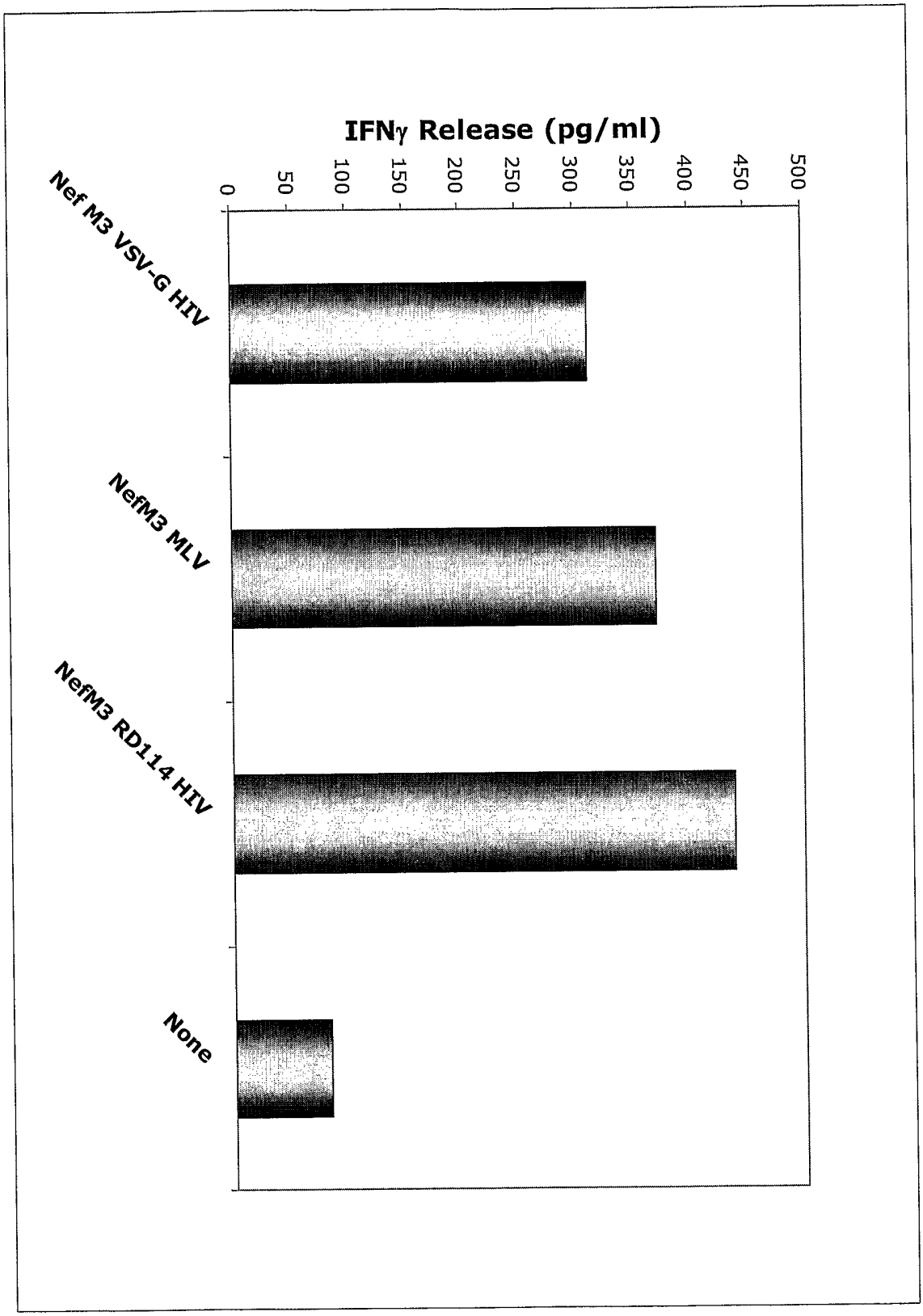

IFNγ Release (pg/ml)
0
50
100
150
200
250
300
350
400
450
500
Nef M3 VSV-G HIV
NefM3 MLV
NefM3 RD114 HIV
None

Figure 16