Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 2916789 C 2022/07/05

(11)(21) 2 916 789

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2014/06/26
(87) Date publication PCT/PCT Publication Date: 2014/12/31
(45) Date de délivrance/Issue Date: 2022/07/05
(85) Entrée phase nationale/National Entry: 2015/12/23
(86) N° demande PCT/PCT Application No.: CA 2014/050614
(87) N° publication PCT/PCT Publication No.: 2014/205579
(30) Priorité/Priority: 2013/06/26 (US61/839,798)

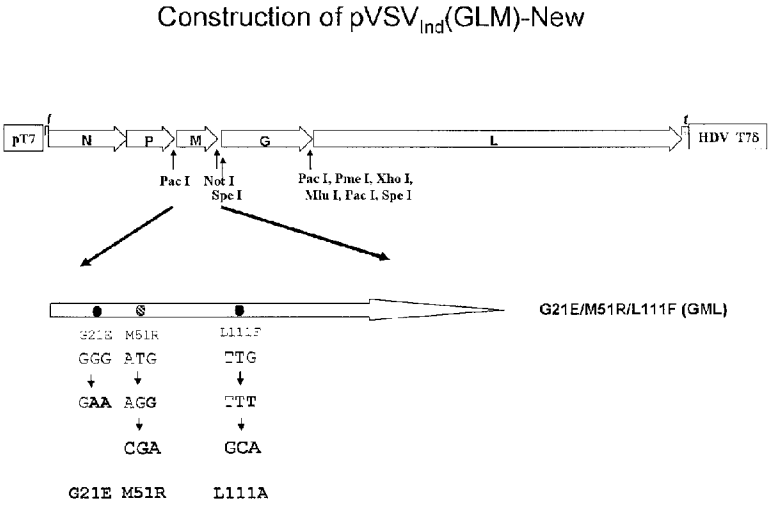
(51) Cl.Int./Int.Cl. *C12N 15/47* (2006.01),
A61K 39/205 (2006.01), *A61K 39/21* (2006.01),
A61K 39/295 (2006.01), *A61K 39/39* (2006.01),
A61P 31/14 (2006.01), *A61P 37/04* (2006.01),
C07K 14/145 (2006.01), *C12N 7/01* (2006.01)

(72) Inventeurs/Inventors:
KANG, CHIL-YONG, CA;
KIM, GYOUNG NYOUN, CA

(73) Propriétaire/Owner:
THE UNIVERSITY OF WESTERN ONTARIO, CA

(74) Agent: KRUPNIK, EDUARDO

(54) Titre : PROTEINES DE MATRICE MODIFIEES DU VIRUS DE LA STOMATITE VESICULAIRE
(54) Title: MODIFIED MATRIX PROTEINS OF VESICULAR STOMATITIS VIRUS



(57) Abrégé/Abstract:
The present invention relates to vesicular stomatitis virus (VSV) matrix (M) protein mutants. One mutant M protein includes a glycine changed to a glutamic acid at position (21), a leucine changed to alanine at position (111) and a methionine changed to an arginine at position (51). Another M protein mutant includes a glycine changed to a glutamic acid at position (22) and a methionine changed to an arginine at positions (48) and (51). These new rVSVs having the mutant M are significantly attenuated and lost virulence, including neurovirulence, and are capable of inducing an immune responses against an antigen of interest. In addition, a rVSV serotype Indiana having the first described M mutant is capable of efficient replication at 31°C, and of poor replication or incapable of replication at about 37°C or higher.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 December 2014 (31.12.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/205579 A4

(51) International Patent Classification:
C12N 15/47 (2006.01) *A61P 31/14* (2006.01)
A61K 39/205 (2006.01) *A61P 37/04* (2006.01)
A61K 39/21 (2006.01) *C07K 14/145* (2006.01)
A61K 39/295 (2006.01) *C12N 7/01* (2006.01)
A61K 39/39 (2006.01)

(21) International Application Number:
PCT/CA2014/050614

(22) International Filing Date:
26 June 2014 (26.06.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/839,798 26 June 2013 (26.06.2013) US

(71) Applicant: **THE UNIVERSITY OF WESTERN ONTARIO** [CA/CA]; 100 Collip Circle, Suite 105, London, Ontario N6G 4X8 (CA).

(72) Inventors: **KANG, Chil-Yong**; 111 Glenridge Crescent, London, Ontario N6G 4X9 (CA). **KIM, Gyoung Nyoun**; 251 Platt's Lane, Apt 714, London, Ontario N6H 4P4 (CA).

(74) Agent: **KRUPNIK, Eduardo**; 2010-255 Queens Avenue, London, Ontario N6A5R8 (CA).

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with amended claims (Art. 19(1))*

Date of publication of the amended claims: 12 February 2015

(54) **Title**: MODIFIED MATRIX PROTEINS OF VESICULAR STOMATITIS VIRUS

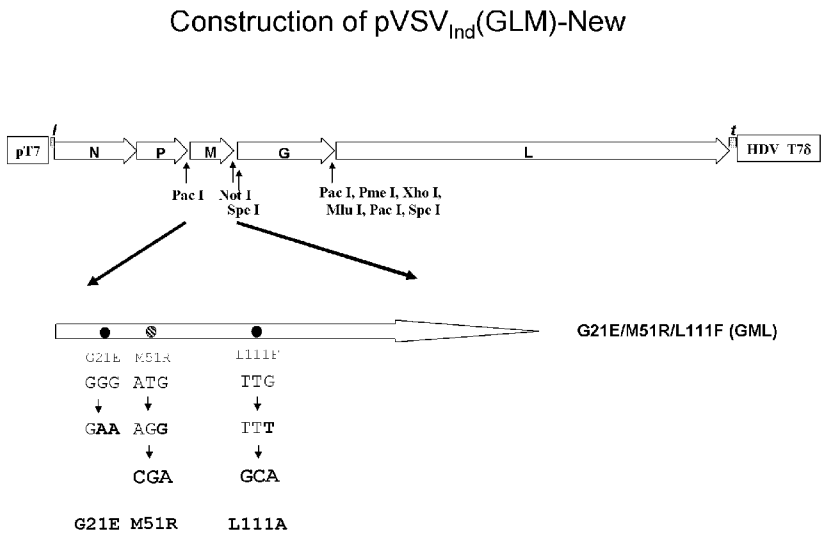


FIGURE 36

(57) **Abstract**: The present invention relates to vesicular stomatitis virus (VSV) matrix (M) protein mutants. One mutant M protein includes a glycine changed to a glutamic acid at position (21), a leucine changed to alanine at position (111) and a methionine changed to an arginine at position (51). Another M protein mutant includes a glycine changed to a glutamic acid at position (22) and a methionine changed to an arginine at positions (48) and (51). These new rVSVs having the mutant M are significantly attenuated and lost virulence, including neurovirulence, and are capable of inducing an immune responses against an antigen of interest. In addition, a rVSV serotype Indiana having the first described M mutant is capable of efficient replication at 31°C, and of poor replication or incapable of replication at about 37°C or higher.

WO 2014/205579 A4

MODIFIED MATRIX PROTEINS OF VESICULAR STOMATITIS VIRUS

FIELD OF THE INVENTION

The present invention relates, in general, to biotechnology and immunology. In particular, the present invention relates to modified matrix (M) proteins of vesicular stomatitis virus (VSV), to attenuated recombinant VSVs expressing the modified M proteins and to VSV based prime-boost vaccines that induce long-lasting humoral, cell-mediated and mucosal immune responses against foreign antigens expressed by the recombinant VSV carrying foreign genes.

BACKGROUND OF THE INVENTION

Vesicular stomatitis virus (VSV) is a negative stranded RNA virus which infects most mammalian cells and expresses viral proteins up to 60% of total proteins in infected cells [Kim, G. N., and C. Y. Kang. Virology 357:41, 2007]. In nature VSV infects pigs, cattle, and horses, and causes vesicular disease around the mouth and foot. Although human infection by VSV has been reported, VSV does not cause any serious symptoms in humans [Fields, B. N., and K. Hawkins. N Engl J Med 277:989, 1967; Johnson, K. M. et al. Am J Trop Med Hyg 15:244, 1966].

VSV encodes five proteins, nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), surface glycoprotein (G), and RNA dependent RNA polymerase (L). The N, P, and L proteins of VSV are required for synthesis of positive sense and negative sense genomic RNAs and mRNA, which are necessary for the synthesis of VSV proteins.

Blocking the host cellular protein synthesis by VSV matrix (M) protein induces cell death. Changing a methionine residue at position 51 of the M protein to arginine (M51R) in the vesicular stomatitis virus Indiana serotype (VSV_{Ind}), and changing methionines at position 48 (Met48) and 51 (Met51) to Arg in the vesicular stomatitis virus New Jersey serotype (VSV_{NJ}) M gene could negate this function of VSV M protein (Kim, G. and Kang, C., Virology, 357:41-53, 2007). While the VSVs with these Met to Arg mutations in the M protein have significantly reduced cytopathic effects, they still replicate and produce progeny viruses in the cell and in the infected animal. One of the temperature sensitive (*ts*) M gene mutants of VSV_{Ind} Orsay strain, *ts*O23 has shown limited replication in a mouse glial cell line at 37°C and 39°C

(Rabinowitz, S. et al. Infection and Immunity 33:120-125, 1981). It has been demonstrated that the temperature sensitivity of *ts*O23 was the result of the improper or lack of the initiation of the viral assembly with the characteristic bullet shaped structure at 39°C (Flood, E. et al. Virology 278:520-533, 2000; Lyles, D. et al. Virology, 217:76–87, 1996). In addition, the *ts*O23 lost its neurovirulence in mice even after direct inoculation into the brain (Rabinowitz, S. et al. Infection and Immunity, 33:120-125, 1981).

There are three amino acid differences between wild type M of VSV Orsay strain and M of *ts*O23, which are glycin (G) to glutamic acid (E) at 21st amino acid (G21E), leucine (L) to phenylalanine (F) at 111th amino acid (L111F), and histidine (H) to tyrosine (Y) at 227th amino acids (H227Y) (Morita, K. et al. J. Virol. 61:256-263, 1987). The single amino acid reversion at F111 to L in revertants implicated that the F111 appears to play a major role in the temperature sensitivity of the *ts*O23. However, the other two mutations may also have some role because the single reversion from the F to L at 111th position did not recover the virus completely to its wild type phenotype (Morita, K. et al. J. Virol. 61:256-263, 1987; Li, Y. et al. J. Virol. 62:3729-3737, 1988).

Given the single amino acid reversions at F111 to L in revertants, it may be advantageous to create mutations that are both less susceptible to reversion and that play a role in the temperature sensitivity of the *ts*O23.

Currently, research groups have developed replication competent, assembly defective VSV having a G glycoprotein (G) gene deleted (ΔG) or both G and M genes deleted (ΔMG) as safer vaccine vectors (Kahn, J. et al. J. Virol. 75:11079-11087, 2001; Schwartz, J. et al., Virology 366:166-73, 2007). However, having the G gene deleted or M and G gene deleted, the VSV vector requires the supply of G or both M and G proteins *in trans* for the production of the assembly-defective VSV. In order to reduce the cost of producing vaccines, it is necessary to generate a system, which can produce the viral vaccine vectors that can replicate in high titre. Therefore, what is needed is a VSV vector system, which may be a full-length VSV vector, which has lost its virulence (avirulent) and still can replicate to a high titer *in vitro* at 31°C cannot assemble properly at non-permissible temperatures, induce good immune responses against the gene of interest that it expresses, and having less chance of reverting back to a wild type phenotype.

Further and other objects of the invention will be realized from the following Summary of the Invention, the Discussion of the Invention and the embodiments and Examples thereof.

SUMMARY OF THE INVENTION

The inventors have generated new matrix (M) protein mutants of vesicular stomatitis virus (VSV), including VSV Indiana serotype (VSV_{Ind}) and VSV New Jersey serotype (VSV_{NJ}). These new VSVs having the mutant M proteins are essentially non-cytolytic, significantly attenuated and avirulent, including neurovirulence (safer). Furthermore, these new VSVs having the mutant M proteins are capable of inducing immune responses against an antigen or epitope of interest. The immune response may be humoral, cellular and mucosal immune responses. In addition, the VSV_{Ind} having the M mutant can assemble and be released from the infected cells normally at 31°C, but they can not assemble properly at 37°C or higher and the release of the viruses from infected cells is reduced significantly relative to wild type VSV_{Ind}. In addition, the M protein mutants of the present invention may be less susceptible to reversion relative to known M protein mutants of the prior art.

As such, in one embodiment, the present application relates to a modified matrix (M) protein of a vesicular stomatitis virus (VSV). In one embodiment, the modified M protein of the present invention includes an amino acid sequence selected from the group consisting of: (i) SEQ ID NO: 3 including at least the following substitutions: G21E/L111A/M51R; and (ii) SEQ ID NO: 8 including at least the following substitutions: G22E/M48R/M51R. In one aspect of the present invention, SEQ ID NO:8 includes at least the following substitutions: G22E/L110A/M48R/M51R.

In one embodiment of the present invention, the modified M protein of (i) includes an amino acid sequence SEQ ID NO:4 and the modified M protein of (ii) includes the amino acid sequence SEQ ID NO:9 or SEQ ID NO:10.

In one embodiment of the present invention, the E at position 21 in (i) and the E at position 22 in (ii) are encoded by a gaa codon, and wherein the R at position 51 in (i) and (ii) and the R at position 48 in (ii) is encoded by a cga codon.

In one embodiment of the present invention, the A at position 111 of SEQ ID NO:4 and the A at position 110 of SEQ ID NO:10 is encoded by a gca codon.

In one embodiment of the present invention, the amino acid sequence of (i) is encoded by a gene comprising SEQ ID NO:2, and the amino acid sequence of (ii) is encoded by a gene comprising SEQ ID NO:6 or SEQ ID NO:7.

In another embodiment, the present invention provides for a nucleotide sequence or a gene that encodes a modified matrix (M) protein of a vesicular stomatitis virus (VSV), wherein the nucleotide sequence or gene includes a nucleotide sequence selected from SEQ ID NO:2, SEQ ID NO:6 and SEQ ID NO: 7.

In another embodiment the present invention provides for a recombinant VSV (rVSV). The rVSV of the present invention, in one embodiment, includes a nucleotide sequence or gene comprising a nucleotide sequence selected from SEQ ID NOs:2, 6 and 7.

In another embodiment the present invention provides for a recombinant vesicular stomatitis virus (rVSV). The rVSV of the present invention includes a modified matrix (M) protein according to any of the previous embodiments.

In one embodiment of the present invention, the rVSV is a recombinant vesicular stomatitis virus Indiana serotype (rVSVInd), and the modified M protein includes the amino acid sequence of SEQ ID NO: 3 including at least the following substitution: G21E/L111A/M51R.

In another embodiment of the present invention, the modified M protein of the $rVSV_{Ind}$ includes the amino acid sequence of SEQ ID NO: 4.

In another embodiment of the present invention, the modified M protein of the $rVSV_{Ind}$ is encoded by a gene comprising a nucleotide sequence of SEQ ID NO: 2.

In another embodiment of the present invention, the rVSVInd is capable of producing VSV_{Ind} particles at permissible temperatures and incapable of producing the particles at non-permissible temperatures.

In another embodiment of the present invention, the rVSV is a recombinant vesicular stomatitis virus New Jersey serotype ($rVSV_{NJ}$), and the modified M protein includes the amino acid sequence of SEQ ID NO: 8 including at least the following substitutions: G22E/M48R/M51R. In one aspect of this embodiment, SEQ ID NO:8 includes at least the following substitutions: G22E/L110A/M48R/M51R.

In one embodiment, the modified M protein of the $rVSV_{NJ}$ is encoded by a gene including a nucleotide sequence of SEQ ID NO: 6 or SEQ ID NO:7.

In one embodiment, the modified M protein of the $rVSV_{NJ}$ includes the amino acid sequence SEQ ID NO: 9 or SEQ ID NO:10.

In another embodiment of the present invention, the rVSV is a chimeric rVSV that expresses a protein of a foreign pathogen.

In one embodiment of the present invention, the pathogen is a viral, fungal, bacterial or parasitic pathogen.

In another embodiment, the rVSV of the present invention is essentially non-cytolytic and avirulent.

In one embodiment, the present invention provides for a vaccine. The vaccine, in one embodiment, includes an effective amount of a modified matrix (M) protein, the modified M protein being encoded by a nucleotide sequence comprising a sequence selected from: SEQ ID NO:2, SEQ ID NO:6 and SEQ ID NO:7.

In another embodiment, the present invention provides for a vaccine. The vaccine, in one embodiment, includes an effective amount of one or more attenuated recombinant vesicular stomatitis virus (rVSV), the one or more attenuated rVSVs including a modified matrix (M) protein, the modified M protein comprising an amino acid sequence selected from the group consisting of: (i) SEQ ID NO: 3 including at least the following substitutions: G21E/L111A/M51R, and (ii) SEQ ID NO: 8 including at least the following substitutions: G22E/M48R/M51R. In one aspect, SEQ ID NO:8 further includes the substitution L110A.

In one embodiment of the vaccine of the present invention, the rVSV is a recombinant vesicular stomatitis virus Indiana serotype ($rVSV_{Ind}$), and the modified M protein includes the amino acid sequence of SEQ ID NO: 3 including at least the following substitution: G21E/L111A/M51R.

In another embodiment of the vaccine of the present invention, the rVSV is a recombinant vesicular stomatitis virus Indiana serotype ($rVSV_{Ind}$), and the modified M protein includes the amino acid sequence of SEQ ID NO: 4.

In another embodiment of the vaccine of the present invention, the modified M protein is encoded by a gene comprising a nucleotide sequence of SEQ ID NO: 2.

In another embodiment of the vaccine of the present invention, the $rVSV_{Ind}$ is capable of producing $rVSV_{Ind}$ particles at permissible temperatures and incapable of producing the particles at non-permissible temperatures.

In another embodiment of the vaccine of the present invention, the $rVSV_{Ind}$ is a full length $rVSV_{Ind}$.

In another embodiment of the vaccine of the present invention, the rVSV is a recombinant vesicular stomatitis virus New Jersey serotype ($rVSV_{NJ}$), and the modified M protein comprises the amino acid sequence of SEQ ID NO: 8 including at least the following substitutions: G22E/M48R/M51R. In one aspect of this embodiment, SEQ ID NO:8 further includes the substitution L110A.

In one embodiment of the vaccine of the present invention, the rVSV is a recombinant vesicular stomatitis virus New Jersey serotype ($rVSV_{NJ}$), and wherein the modified M protein comprises the amino acid sequence SEQ ID NO: 9 or SEQ ID NO:10.

In one embodiment of the vaccine of the present invention, the modified M protein is encoded by a gene having a nucleotide sequence of SEQ ID NO: 6 or SEQ ID NO:7.

In another embodiment of the vaccine of the present invention, the $rVSV_{NJ}$ is a full length $rVSV_{NJ}$.

In another embodiment of the vaccine of the present invention, the rVSV is a chimeric rVSV that expresses a protein of a foreign pathogen, and wherein said chimeric rVSV is capable of inducing an immune response to said protein.

In another embodiment of the vaccine of the present invention, the vaccine comprises a mixture of attenuated chimeric rVSVs, wherein at least two chimeric rVSVs in the mixture express a different protein of the foreign pathogen.

In another embodiment of the vaccine of the present invention, the pathogen is a viral, a fungal, a bacterial or a parasitic pathogen.

In another embodiment of the vaccine of the present invention, the pathogen is a lentivirus.

In another embodiment of the vaccine of the present invention, the lentivirus is a HIV and the protein of the foreign pathogen is a HIV protein.

In another embodiment of the vaccine of the present invention, the pathogen is HCV and the protein of the foreign pathogen is a HCV protein.

In another embodiment of the vaccine of the present invention, the E at position 21 in (i) and the E at position 22 are encoded by a gaa codon, and the R at position 51 and the R at position 48 is encoded by a cga codon.

In another embodiment of the vaccine of the present invention, the A at position 111 and the A at position 110 is encoded by a gca codon.

In another embodiment of the vaccine of the present invention, vaccine is capable of inducing a humoral, cellular and mucosal immune response.

In another embodiment of the vaccine of the present invention, the vaccine further includes an adjuvant.

In one embodiment, the present invention provides for a prime boost combination vaccine. The prime boost combination vaccine, according to one embodiment, includes: (a) an effective amount of a vaccine comprising an attenuated recombinant vesicular stomatitis virus (rVSV) of one serotype having a first modified M protein comprising the amino acid sequence of SEQ ID NO:4; and (b) an effective amount of a vaccine comprising a rVSV of another serotype having a second modified M protein comprising the amino acid sequence of SEQ ID NO:9 or comprising the amino acid sequence of SEQ ID NO:10.

In one embodiment of the prime boost combination vaccine, SEQ ID NO:4 is encoded by a gene comprising SEQ ID NO:2.

In another embodiment of the prime boost combination vaccine, SEQ ID NO:9 is encoded by a gene comprising SEQ ID NO:6 and SEQ ID NO:10 is encoded by a gene comprising SEQ ID NO:7.

In another embodiment of the prime boost combination vaccine, (a) is a priming vaccine and (b) is a booster vaccine.

In another embodiment of the prime boost combination vaccine, (b) is a priming vaccine and (a) is a booster vaccine.

In another embodiment of the prime boost combination vaccine of the present invention, the two attenuated rVSVs are chimeric rVSVs that express a protein of a

foreign pathogen, and wherein the two chimeric rVSVs are capable of inducing an immune response to the protein.

In another embodiment of the prime boost combination vaccine of the present invention, the pathogen is a viral, a fungal, a bacterial or a parasitic pathogen.

In another embodiment of the prime boost combination vaccine of the present invention, the pathogen is a lentivirus.

In another embodiment of the prime boost combination vaccine of the present invention, the lentivirus is a HIV and the protein is a HIV protein.

In another embodiment of the prime boost combination vaccine of the present invention, the rVSV of one serotype and the rVSV of the other serotype include a surface glycoprotein (G) gene and a RNA dependent RNA polymerase (L) gene, and wherein a gene for expressing the HIV protein is inserted in between the G gene and the L gene.

In another embodiment of the prime boost combination vaccine of the present invention, wherein the HIV gene is selected from the group of HIV genes consisting of env, gag and pol.

In another embodiment of the prime boost combination vaccine of the present invention, the pathogen is HCV and the epitope is a HCV protein.

In another embodiment of the prime boost combination vaccine of the present invention, the rVSV of one serotype and the rVSV of the other serotype include a surface glycoprotein (G) gene and a RNA dependent RNA polymerase (L) gene, and a gene for expressing the HCV protein is inserted in between the G gene and the L gene.

In another embodiment of the prime boost combination vaccine of the present invention, the HCV protein is a structural or a non-structural HCV protein.

In another embodiment of the prime boost combination vaccine of the present invention, each one of the two vaccines includes a mixture of the attenuated chimeric rVSVs, and at least two of the attenuated chimeric rVSVs in the mixture have a different protein of the pathogen.

In another embodiment of the prime boost combination vaccine of the present invention, each one of the two vaccines is capable of inducing humoral, cellular and mucosal immune responses.

In another embodiment of the prime boost combination vaccine of the present invention, the serotype of the rVSV of vaccine (a) is Indiana and the serotype of the rVSV of vaccine (b) is New Jersey.

In another embodiment of the prime boost combination vaccine of the present invention, each one of vaccine (a) and vaccine (b) further comprises an adjuvant.

According to another embodiment, the present invention provides for a kit. The kit, in one embodiment, includes: (a) at least one dose of an effective amount of a vaccine comprising a recombinant vesicular stomatitis virus Indiana serotype ($rVSV_{Ind}$) having a modified M protein comprising the amino acid sequence of SEQ ID NO:4, and (b) at least one dose of an effective amount of a vaccine comprising a recombinant vesicular stomatitis virus New Jersey serotype ($rVSV_{NJ}$) having a modified M protein comprising the amino acid sequence of SEQ ID NO:9 or the amino acid sequence of SEQ ID NO:10.

In one embodiment of the kit of the present invention (a) and (b) are formulated in a pharmaceutically acceptable carrier.

In another embodiment of the kit of the present invention SEQ ID NO:4 is encoded by a gene comprising SEQ ID NO:2.

In another embodiment of the kit of the present invention SEQ ID NO:9 is encoded by a gene comprising SEQ ID NO:6 and SEQ ID NO:10 is encoded by a gene comprising SEQ ID NO:7.

In another embodiment, the present invention provides for an isolated peptide comprising an amino acid sequence selected from the group of amino acid sequences listed as SEQ ID NOs: 4, 9 and 10. In one embodiment the isolated peptide is provided in purified form.

In another embodiment, the present invention provides for an isolated nucleotide sequences comprising a nucleotide sequence selected from the group SEQ ID NO:2, SEQ ID NO:6 and SEQ ID NO:7. In one embodiment the isolated nucleotide sequences is provided in purified form.

In one embodiment, the present invention provides for a vaccine of the present invention for use to induce an immune response in a subject.

In one embodiment, the present invention relates to the prime boost combination vaccine of the present invention for use to induce an immune response in a subject.

In another embodiment, the present invention relates to a method of inducing an immune response in a subject. The method, according to one embodiment, includes administering to the subject: (a) an effective amount of a vaccine comprising an attenuated recombinant vesicular stomatitis virus (rVSV) of one serotype having a first modified M protein, the first modified M protein comprising the amino acid sequence of SEQ ID NO: 3 including at least the following substitutions: G21E/L111A/M51R; and (b) an effective amount of another vaccine comprising an attenuated rVSV of another serotype having a second modified M protein, the second modified M protein comprising the amino acid sequence of SEQ ID NO: 8 including at least the following substitutions: G22E/M48R/M51R. In one aspect of this embodiment, SEQ ID NO:8 further includes the substitution L110A.

In one embodiment of the method of inducing an immune response in a subject of the present invention (a) is administered to the subject before (b) is administered to the subject.

In another embodiment of the method of inducing an immune response in a subject of the present invention (b) is administered to the subject more than one time over the course of inducing.

In another embodiment of the method of inducing an immune response in a subject of the present invention (a) is administered to the subject and (b) is administered to the subject at about weeks three, eight and sixteen post-administration of (a).

In another embodiment of the method of inducing an immune response in a subject of the present invention (b) is administered to the subject before (a) is administered to the subject.

In another embodiment of the method of inducing an immune response in a subject of the present invention (a) is administered to the subject more than one time over the course of inducing.

In another embodiment of the method of inducing an immune response in a subject of the present invention (b) is administered to the subject and (a) is administered to the subject at about weeks three, eight and sixteen post-administration of (b).

In another embodiment of the method of inducing an immune response in a subject of the present invention the two rVSVs are chimeric rVSVs that express a protein of a foreign pathogen, and wherein the two rVSVs are capable of inducing an immune response to the protein.

In another embodiment of the method of inducing an immune response in a subject of the present invention the pathogen is a viral, a fungal, a bacterial or a parasitic pathogen.

In another embodiment of the method of inducing an immune response in a subject of the present invention the pathogen is a lentivirus.

In another embodiment of the method of inducing an immune response in a subject of the present invention the lentivirus is a human immunodeficiency virus (HIV) and the protein is a HIV protein.

In another embodiment of the method of inducing an immune response in a subject of the present invention the rVSV of one serotype and the rVSV of the other serotype include a surface glycoprotein (G) gene and a RNA dependent RNA polymerase (L) gene, and a gene for expressing the HIV protein is a HIV gene inserted in between the G gene and the L gene.

In another embodiment of the method of inducing an immune response in a subject of the present invention the HIV gene is selected from the group of HIV genes consisting of gag, env and pol.

In another embodiment of the method of inducing an immune response in a subject of the present invention the pathogen is hepatitis C virus (HCV) and the protein is a HCV protein.

In another embodiment of the method of inducing an immune response in a subject of the present invention the rVSV of one serotype and the rVSV of the other serotype include a surface glycoprotein (G) gene and a RNA dependent RNA polymerase (L) gene, and wherein a gene for expressing the HCV protein is inserted in between the G gene and the L gene of the rVSV.

In another embodiment of the method of inducing an immune response in a subject of the present invention the two vaccines comprise a mixture of attenuated chimeric rVSVs, and at least two of the attenuated chimeric rVSVs in the mixture express a different protein of the pathogen.

In another embodiment of the method of inducing an immune response in a subject of the present invention each one of the two vaccines (a) and (b) induces humoral, cellular and mucosal immune responses.

In another embodiment of the method of inducing an immune response in a subject of the present invention the one serotype of vaccine (a) is Indiana and the other serotype of vaccine (b) is New Jersey.

In another embodiment of the method of inducing an immune response in a subject of the present invention each of vaccine (a) and vaccine (b) further includes an adjuvant.

BRIEF DESCRIPTION OF THE FIGURES

Embodiments will now be described, by way of example only, with reference to the drawings, in which:

FIG. 1 illustrates gene organization of the vesicular stomatitis virus (VSV), Indiana (Ind) serotype with mutations in the M gene.

FIG. 2 illustrates gene organization of vesicular stomatitis virus (VSV), New Jersey (NJ) with mutations in the M gene.

FIG. 3 illustrates a reverse genetics system for the recovery of VSV from cDNA.

FIG. 4 illustrates the burst size effect of mutation L111F in the M protein of $rVSV_{Ind}$ at permissive temperature [panels A) and C)] and at semi-permissive temperature [panels B) and D)], in baby hamster kidney (BHK) cells infected with recombinant VSVs [panels A) and B)] and in human neuroblastoma cells (SH-SY5Y) infected with the rVSV.

FIG. 5 is a graph representing the burst size effect of mutations M48R+M51R, G22E, G22E/M48R+M51R, G22E/L110F, G22E/L110F/M48R+M51R in the M protein of $rVSV_{NJ}$ at 31 °C and 37 °C in BHK21 cells.

FIG. 6 A) is a Western blot analysis illustrating the level of rVSV protein expression of $rVSV_{Ind}$ 1. WT, 2. M51R, 3. G21E 4. G21E/M51R 5. G21E/L111F and 6. G21E/L111F/M51R.

FIG. 6 B) is a Western blot analysis illustrating the level of rVSV protein expression of $rVSV_{NJ}$ 1. WT, 2. M48R+M51R, 3. G22E 4. G22E/M48R+M51R 5. G22E/L110F and 6. G22E/L110F/M48R+M51R.

FIG. 7 includes photographs of BHK21 cells infected with $rVSV_{Ind}$ having a wild type M gene (panels B and I) and with $rVSV_{Ind}$ having different mutations in the M gene (panels A, D, E, F, G, H, K, L, M and N) and photographs of uninfected BHK21 cells (panels C and J). Cells were incubated at 31°C (panels A to G) or 37°C (panels H to N).

FIG. 8 includes photographs of SH-SY5Y cells infected with $rVSV_{Ind}$ having different mutations in the M gene (panels C, D, E, F, I, J, K and L) or wild type M gene (panels A and G) and photographs of uninfected SH-SY5S cells (panels B and H). Cells were incubated at 31°C (panels A to F) or 37°C (panels G to L).

FIG. 9 includes photographs of BHK21 cells infected with $rVSV_{NJ}$ having different mutations in the M gene (panels A, D, E, F, G, H, K, L, M and N) or wild type M gene (panels B and I), and photographs of uninfected BHK21 cells (panels C and J). Cells were incubated at 31°C (panels A to G) or 37°C (panels H to N).

FIG. 10 includes photographs of SH-SY5Y cells infected with $rVSV_{NJ}$ having different mutations in the M gene (panels A, D, E, F, G, H, K, L, M, and N) or having wild type M gene (panels B and I), and photographs of uninfected SH-SY5Y cells (panels C and J). Cells were incubated at 31°C (panels A to G) or 37°C (panels H to N).

FIG. 11 includes graphs illustrating neurovirulence studies in Swiss Webster mouse by intralateral ventricular injection: A) UV-irradiated $rVSV_{Ind}$ G21E/L111F/M51R, B) $rVSV_{Ind}$ WT, C) $rVSV_{Ind}$ M51R and D) $rVSV_{Ind}$ G21E/L111F/M51R.

FIG. 12 includes graphs illustrating neurovirulence studies in Swiss Webster mouse by intralateral ventricular injection: A) $rVSV_{NJ}$ WT, B), $rVSV_{NJ}$ M48R+M51R, C) $rVSV_{NJ}$ G22E/M48R+M51R and D) $rVSV_{NJ}$ G22E/L110F/M48R+M51R.

FIG. 13 (SEQ ID NOS: 11 - 15) A) illustrates the cloning of HIV-1 Gag-En genes into the cDNA clone of rVSV. FIG. 13 B) is a Western blot analysis of BHK cells infected with rVSVs expressing Gag-En and having different mutations to the M gene, and incubated at 31°C. FIG. 13 C) is a Western blot analysis of BHK cells infected with rVSVs expressing Gag-En and having different mutations to the M gene, and incubated at 37°C.

FIG. 14 depicts a vaccination regime and a table of vaccination groups.

FIG. 15 depicts a graph of the frequency of peptide specific CD8+ IFN gamma+ T cells among the different vaccination groups illustrated in the insert stimulated with dimethyl sulfoxide (DMSO).

FIG. 16 is a graph illustrating the frequency of VSV N peptide specific CD8+ IFN gamma+ T cells among the different vaccination groups illustrated in the insert stimulated with VSV N.

FIG. 17 is a graph illustrating the frequency of HIV-1 Gag peptide specific CD8+ IFN gamma+ T cells among the different vaccination groups illustrated in the insert stimulated with HIV-1 Gag.

FIG. 18 is a graph illustrating HIV-1 Gag specific antibody responses among the different vaccination groups illustrated in the insert.

FIG. 19 is a graph illustrating the frequency of VSV N peptide specific CD8+ IFN gamma+ T cells among different vaccination groups vaccinated with various doses of rVSV of Table 1.

FIG. 20 is a graph illustrating the frequency of HIV-1 Gag peptide specific CD8+ IFN gamma+ T cells among different vaccination groups vaccinated with various doses of rVSV of Table 1.

FIG. 21 is a graph illustrating HIV-1 Gag specific antibody responses among different vaccination groups vaccinated with various doses of rVSV of Table 1.

FIG. 22 (SEQ ID NOS: 16 - 26) illustrates the cloning of HIV-1 *gag* gene linked to nucleotides encoding human B cell and T cell epitopes of gp120 and gp41 into the cDNA clone of $rVSV_{Ind}$ G21E/L111F/M51R and $rVSV_{NJ}$ G22E/M48R+M51R.

FIG. 23 (SEQ ID NOS: 11-15 and 27 -31, respectively) illustrates the cloning of HIV-1 *gag* gene linked to nucleotides encoding human B cell and T cell epitopes of

gp41, nef, gp120, RT, Tat and Rev into the cDNA clone of $rVSV_{Ind}$ G21E/L111F/M51R and $rVSV_{NJ}$ G22E/M48R+M51R. It also illustrates the cloning of HIV-1 *pol* and *env* genes into the cDNA clone of $rVSV_{Ind}$ G21E/L111F/M51R and $rVSV_{NJ}$ G22E/M48R+M51R.

FIG. 24 illustrates a Western blot analysis of BHK21 cells infected with $rVSV_{Ind}$ G21E/L111F/M51R expressing Gag A, B, C, En, and RT and incubated at 31°C and 37°C.

FIG. 25 illustrates a Western blot analysis of BHK21 cells infected with $rVSV_{NJ}$ G22E/M48R+M51R expressing Gag A, B, C, En, and RT and incubated at 31°C and 37°C.

FIG. 26 illustrates a Western blot analysis of BHK21 cells infected with $rVSV_{Ind}$ G21E/L111F/M51R or $rVSV_{NJ}$ G22E/M48R+M51R expressing HIV-1 *pol* gene.

FIG. 27 illustrates a Western blot analysis of BHK21 cells infected with $rVSV_{Ind}$ G21E/L111F/M51R or $rVSV_{NJ}$ G22E/M48R+M51R expressing HIV-1 *gp160mss* gene.

FIG. 28 includes graphs illustrating VSV N and HIV-1 protein peptide specific CD8+ T cell responses among the different vaccination groups of Table 6: G1, G2, G3, G4, G5 and G5 without Env P18.

FIG. 29 is a graph illustrating VSV N and HIV-1 protein peptide specific CD8+ T cell responses in vaccination group G6 of Table 6. The amino acid sequences of VSV N peptide and peptides from HIV-1 proteins that are used to stimulated the splenocytes are shown (SEQ ID NOS: 32 - 37).

FIG. 30 illustrates the humoral immune responses against HIV-1 Gag (panel A) and Gp120 (panel B) induced in vaccination groups of Table 6, which were determined by Enzyme Linked Immunosorbant Assay (ELISA).

FIG. 31 depicts the HCV structural protein genes cloned into $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) and $rVSV_{NJ}$(GLM).

FIG. 32 illustrates Western blot analyses of the expression of HCV Core (panel A), E1 (panel B), E2 (panel C), and NS4B (panel D) proteins from the $rVSV_{Ind}$(GLM), $rVSV_{NJ}$(GM), $rVSV_{NJ}$(GLM).

FIG. 33 depicts the HCV non-structural protein genes cloned into $rVSV_{Ind}$(GLM), $rVSV_{NJ}$(GM), and $rVSV_{NJ}$(GLM).

FIG. 34 Western blot analyses of the expression of HCV NS3 at 37°C and 31°C (panel A), NS5Aat 37°C (panel B), and NS5B at 37°C and 31°C (panel C) from the $rVSV_{Ind}$(GLM).

FIG. 35 demonstrates the expression of HCV NS3, NS4B, NS5A, and NS5B from $rVSV_{NJ}$(GM) at 37°C (panel A) and the expression of HCV NS5AB at 37°C (panel B). The protein expression was detected by Western blot analysis.

FIG. 36 illustrates construction of a cDNA plasmid for the full length genomic RNA of $rVSV_{Ind}$(GLM)-new.

FIG. 37 illustrates construction of a cDNA plasmid for the full length genomic RNA of $rVSV_{NJ}$(GMM)-new.

FIG. 38 illustrates $rVSV_{Ind}$(GLM) with additional nucleotide changes shows the same temperature sensitivity as original $rVSV_{Ind}$(GLM)-new.

DETAILED DESCRIPTION OF THE INVENTION

Overview

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Also, unless indicated otherwise, except within the claims, the use of "or" includes "and" and vice versa. Non-limiting terms are not to be construed as limiting unless expressly stated or the context clearly indicates otherwise (for example "including", "having" and "comprising" typically indicate "including without limitation"). Singular forms including in the claims such as "a", "an" and "the" include the plural reference unless expressly stated otherwise. "Consisting essentially of" means any recited elements are necessarily included, elements that would materially affect the basic and novel characteristics of the listed elements are excluded, and other elements may optionally be included. "Consisting of" means that all elements other than those listed are excluded. Embodiments defined by each of these terms are within the scope of this invention.

All numerical designations, e.g., dimensions and weight, including ranges, are approximations that typically may be varied (+) or (-) by increments of 0.1, 1.0, or 10.0, as appropriate. All numerical designations may be understood as preceded by the term "about".

The term "administering" includes any method of delivery of a compound of the present invention, including a pharmaceutical composition, vaccine or therapeutic agent, into a subject's system or to a particular region in or on a subject. The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration. "Parenteral administration" and "administered parenterally" means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intra-articular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

The term "amino acid" is known in the art. In general the abbreviations used herein for designating the amino acids and the protective groups are based on recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (see Biochemistry (1972) 11:1726-1732). For the amino acids relevant to the present invention the designations are: M: methionine, R: arginine, G: glycine, E: glutamic acid, L: leucine, F: phenylalanine. In certain embodiments, the amino acids used in the application of this invention are those naturally occurring amino acids found in proteins, or the naturally occurring anabolic or catabolic products of such amino acids which contain amino and carboxyl groups. Particularly suitable amino acid side chains include side chains selected from those of the following amino acids: glycine, alanine, valine, cysteine, leucine, isoleucine, serine, threonine, methionine, glutamic acid, aspartic acid, glutamine, asparagine, lysine, arginine, proline, histidine, phenylalanine, tyrosine, and tryptophan.

The term "antibody" as used herein is intended to include whole antibodies, e.g., of any isotype (IgG, IgA, IgM, IgE, etc), including polyclonal, monoclonal,

recombinant and humanized antibodies and fragments thereof which specifically recognize and are able to bind an epitope of a protein. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner. Thus, the term includes segments of proteolytically-cleaved or recombinantly-prepared portions of an antibody molecule that are capable of selectively reacting with a certain protein. Nonlimiting examples of such proteolytic and/or recombinant fragments include Fab, F(ab')2, Fab', Fv, and single chain antibodies (scFv) containing a V[L] and/or V[H] domain joined by a peptide linker. The scFvs may be covalently or non-covalently linked to form antibodies having two or more binding sites.

As used herein, the term "epitopes" refers to sites or fragments of a polypeptide or protein having antigenic or immunogenic activity in an animal, preferably in a mammal. An epitope having immunogenic activity is a site or fragment of a polypeptide or protein that elicits an immune response in an animal. An epitope having antigenic activity is a site or fragment of a polypeptide or protein to which an antibody immunospecifically binds as determined by any method well-known to one of skill in the art, for example by immunoassays.

As used herein, the term "fragment" in the context of a proteinaceous agent refers to a peptide or polypeptide comprising an amino acid sequence of at least 2 contiguous amino acid residues, at least 5 contiguous amino acid residues, at least 10 contiguous amino acid residues, at least 15 contiguous amino acid residues, at least 20 contiguous amino acid residues, at least 25 contiguous amino acid residues, at least 40 contiguous amino acid residues, at least 50 contiguous amino acid residues, at least 60 contiguous amino residues, at least 70 contiguous amino acid residues, at least 80 contiguous amino acid residues, at least 90 contiguous amino acid residues, at least 100 contiguous amino acid residues, at least 125 contiguous amino acid residues, at least 150 contiguous amino acid residues, at least 175 contiguous amino acid residues, at least 200 contiguous amino acid residues, or at least 250 contiguous amino acid residues of the amino acid sequence of a peptide, polypeptide or protein. In one embodiment, a fragment of a full-length protein retains activity of the full-length protein, e.g., immunogenic activity. In another embodiment, the fragment of the full-length protein does not retain the activity of the full-length protein, e.g., non-immunogenic activity.

As used herein, the term "fragment" in the context of a nucleic acid refers to a nucleic acid comprising an nucleic acid sequence of at least 2 contiguous nucleotides, at least 5 contiguous nucleotides, at least 10 contiguous nucleotides, at least 15 contiguous nucleotides, at least 20 contiguous nucleotides, at least 25 contiguous nucleotides, at least 30 contiguous nucleotides, at least 35 contiguous nucleotides, at least 40 contiguous nucleotides, at least 50 contiguous nucleotides, at least 60 contiguous nucleotides, at least 70 contiguous nucleotides, at least contiguous 80 nucleotides, at least 90 contiguous nucleotides, at least 100 contiguous nucleotides, at least 125 contiguous nucleotides, at least 150 contiguous nucleotides, at least 175 contiguous nucleotides, at least 200 contiguous nucleotides, at least 250 contiguous nucleotides, at least 300 contiguous nucleotides, at least 350 contiguous nucleotides, or at least 380 contiguous nucleotides of the nucleic acid sequence encoding a peptide, polypeptide or protein. In a preferred embodiment, a fragment of a nucleic acid encodes a peptide or polypeptide that retains activity of the full-length protein, e.g., immuogenic activity. In another embodiment, the fragment of the full-length protein does not retain the activity of the full-length protein, e.g., non-immunogenic activity.

The term "essentially noncytolytic" as used herein means that the recombinant vesicular stomatitis virus (rVSV) does not significantly damage or kill the cells it infects.

The term "HIV" is known to one skilled in the art to refer to Human Immunodeficiency Virus. There are two types of HIV: HIV-1 and HIV-2. There are many different strains of HIV-1. The strains of HIV-1 can be classified into three groups: the "major" group M, the "outlier" group O and the "new" group N. These three groups may represent three separate introductions of simian immunodeficiency virus into humans. Within the M-group there are at least ten subtypes or clades: e.g., clade A, B, C, D, E, F, G, H, I, J, and K. A "clade" is a group of organisms, such as a species, whose members share homologous features derived from a common ancestor. Any reference to HIV-1 in this application includes all of these strains.

The term "non-infectious" means of reduced to non-existent ability to infect.

As used herein, the terms "subject" or "patient" are used interchangeably. As used herein, the terms "subject" and "subjects" refers to either a human or non-human animal.

The term "pharmaceutical delivery device" refers to any device that may be used to administer a therapeutic agent or agents to a subject. Non-limiting examples of pharmaceutical delivery devices include hypodermic syringes, multichamber syringes, stents, catheters, transcutaneous patches, microneedles, microabraders, and implantable controlled release devices. In one embodiment, the term "pharmaceutical delivery device" refers to a dual-chambered syringe capable of mixing two compounds prior to injection.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides;

and (22) other non-toxic compatible substances employed in pharmaceutical formulations.

The terms "polynucleotide", "nucleic acid sequence" and "nucleic acid" are used interchangeably to refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. The term "recombinant" polynucleotide means a polynucleotide of genomic, cDNA, semi-synthetic, or synthetic origin, which either does not occur in nature or is linked to another polynucleotide in a non-natural arrangement. An "oligonucleotide" refers to a single stranded polynucleotide having less than about 100 nucleotides, less than about, e.g., 75, 50, 25, or 10 nucleotides.

The terms "polypeptide", "peptide" and "protein" (if single chain) are used interchangeably herein to refer to polymers of amino acids. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component.

In certain embodiments, polypeptides of the invention may be synthesized chemically, ribosomally in a cell free system, or ribosomally within a cell. Chemical synthesis of polypeptides of the invention may be carried out using a variety of art recognized methods, including stepwise solid phase synthesis, semi-synthesis through the conformationally-assisted re-ligation of peptide fragments, enzymatic ligation of cloned or synthetic peptide segments, and chemical ligation. Native

chemical ligation employs a chemoselective reaction of two unprotected peptide segments to produce a transient thioester-linked intermediate. The transient thioester-linked intermediate then spontaneously undergoes a rearrangement to provide the full length ligation product having a native peptide bond at the ligation site. Full length ligation products are chemically identical to proteins produced by cell free synthesis. Full length ligation products may be refolded and/or oxidized, as allowed, to form native disulfide-containing protein molecules (see e.g., U.S. Pat. Nos. 6,184,344 and 6,174,530; and T. W. Muir et al., Curr. Opin. Biotech. (1993): vol. 4, p 420; M. Miller, et al., Science (1989): vol. 246, p 1149; A. Wlodawer, et al., Science (1989): vol. 245, p 616; L. H. Huang, et al., Biochemistry (1991): vol. 30, p 7402; M. Schnolzer, et al., Int. J. Pept. Prot. Res. (1992): vol. 40, p 180-193; K. Rajarathnam, et al., Science (1994): vol. 264, p 90; R. E. Offord, "Chemical Approaches to Protein Engineering", in Protein Design and the Development of New therapeutics and Vaccines, J. B. Hook, G. Poste, Eds., (Plenum Press, New York, 1990) pp. 253-282; C. J. A. Wallace, et al., J. Biol. Chem. (1992): vol. 267, p 3852; L. Abrahmsen, et al., Biochemistry (1991): vol. 30, p 4151; T. K. Chang, et al., Proc. Natl. Acad. Sci. USA (1994) 91: 12544-12548; M. Schnlzer, et al., Science (1992): vol., 3256, p 221; and K. Akaji, et al., Chem. Pharm. Bull. (Tokyo) (1985) 33: 184).

"VSV" is used to refer to vesicular stomatitis virus.

"rVSV" is used to refer to a recombinant vesicular stomatitis virus.

The term "Indiana", and "IND" are used to refer to the VSV serotype Indiana (VSV_{Ind}).

The term "New Jersey", and "NJ" are used to refer to the VSV serotype New Jersey (VSV_{NJ}).

"MWT" "M(WT)" are used to refer to a wild type M protein. The nucleotide sequence of wild type M gene of the VSV_{Ind} may comprise the nucleotide sequence represented by SEQ ID NO: 1. The amino acid sequence of wild type M protein of the VSV_{Ind} may comprise the amino acid sequence represented by SEQ ID NO: 3. The nucleotide sequence of wild type M gene of the VSV_{NJ} may comprise the nucleotide sequence represented by SEQ ID NO: 5. The amino acid sequence of wild type M protein of the VSV_{NJ} may comprise the amino acid sequence represented by SEQ ID NO: 8. "M51R" is used to refer to an M(WT) in the VSV_{Ind} having a

methionine changed to an arginine at position 51. "G21E" is used to refer to an M(WT) in VSV_{Ind} having a glycine changed to a glutamic acid at position 21. "L111A" is used to refer to an M(WT) in VSV_{Ind} having a leucine (L) changed to alanine (A) at position 111. "G22E" is used to refer to an M(WT) in VSV_{NJ} having a glycine (G) changed to glutamic acid (E) at position 22. "L110A" is used to refer to an M(WT) in VSV_{NJ} having a leucine (L) changed to alanine (A) at position 110. "M48R+M51R" or "M48R/M51R" is used to refer to an M(WT) in VSV_{NJ} having a methionine (M) changed to an arginine (R) at positions 48 and 51. "$rVSV_{Ind}$ M(G21E/L111A/M51R)" or "$rVSV_{Ind}$ (GLM)-new" are used to refer to a $rVSV_{Ind}$ having an M(WT) having a glycine changed to a glutamic acid at position 21, a leucine changed to alanine at position 111 and a methionine changed to an arginine at position 51. "$rVSV_{NJ}$ M(G22E/M48R/M51R)" "$rVSV_{NJ}$ M(G22E/M48R+M51R)" or "$rVSV_{NJ}$ (GM)" are used to refer to $rVSV_{NJ}$ having an M(WT) having a glycine changed to a glutamic acid at position 22 and a methionine changed to an arginine at positions 48 and 51. "$rVSV_{NJ}$(GM)-new" refers to $rVSV_{NJ}$(GM) with the codons of the present invention. "$rVSV_{NJ}$ M(G22E/L110F/M48R/M51R)" or "$rVSV_{NJ}$ M(G22E/L110F/M48R+M51R)" or "$rVSV_{NJ}$ (GLM)" are used to refer to $rVSV_{NJ}$ having an M(WT) having a glycine changed to a glutamic acid at position 22, a leucine changed to a phenylalanine at position 110 and a methionine changed to an arginine at positions 48 and 51. "$rVSV_{NJ}$ M (G22E/L110A/M48R/M51R)", "$rVSV_{NJ}$ M (G22E/L110A/M48R+M51R)" or "$rVSV_{NJ}$ (GLM)-new" is used to refer to $rVSV_{NJ}$ having an M(WT) having a glycine changed to a glutamic acid at position 22, a leucine changed to alanine at position 110 and a methionine changed to an arginine at positions 48 and 51.

Overview

The inventors generated novel M proteins and novel attenuated rVSVs capable of producing the novel M proteins. The novel proteins of the present invention may include: M(G21E/L111A/M51R), and M(G22E/M48R/M51R). The novel attenuated rVSVs of the present invention may be used as protein expression and vaccine vectors and in methods for preventing or treating infections. The rVSV of the present invention may be applied to make vaccines for the infectious diseases of human and other animals to induce cellular and humoral immune responses.

Isolated Proteins and M Proteins

In one embodiment, the present invention relates to isolated proteins and to nucleotide sequences that encode the isolated proteins. As such, in one embodiment the present invention relates to an isolated peptide comprising an amino acid sequence selected from amino acid sequences listed as SEQ ID NOs: 4 and 9. In another embodiment, the present invention relates to isolated nucleotide sequences comprising a nucleotide sequence selected from the polynucleotides listed as SEQ ID NOs: 2 and 6. The isolated peptides and nucleotide sequences may be provided in purified form. The nucleotides and amino acid sequences of the present invention may be artificially made or synthesized by known methods in the art.

In one embodiment, the present invention relates to novel VSV M proteins having at least one of the following substitutions: M(G21E/L111A/M51R), M(G22E/M48R/M51R) or M(G22E/L110A/M48R/M51R).

The E at positions 21 or 22 as the case may be, is encoded by the codon gaa and R at positions 48 and 51, as the case may be, is encoded by codon cga. The A at position 111 may preferably be encoded by the codon gca.

In one aspect the present invention relates to a VSV M protein comprising an amino acid sequence selected from the amino acid sequences listed as SEQ ID NOs: 4, 9 and 10. In another embodiment the present invention relates to nucleotide sequences which encode for the novel VSV M proteins of the present invention. The nucleotide sequences may be selected from the group of sequences listed as SEQ ID NOs: 2, 6 and 7.

Methods of Preventing or Treating an Infection

Provided are methods of inducing an immune response, preventing or treating infections. In one embodiment, the methods may include administering to a subject: (a) an effective amount of a vaccine comprising an attenuated rVSV of one serotype having (i) a first modified M protein, the first modified M protein comprising the amino acid sequence of SEQ ID NO: 3 including the following substitutions: G21E/L111A/M51R, and (ii) an epitope of the pathogen; and (b) an effective amount of another vaccine comprising an attenuated rVSV of another serotype having: (i) a second modified M protein, the second modified M protein comprising the amino acid sequence of SEQ ID NO: 8 including the following substitutions:

G22E/M48R/M51R, and (ii) the epitope of the pathogen. In embodiments of the present invention, the methods may include administering to a subject (a) an effective amount of $rVSV_{Ind}$ M(G21E/L111A/M51R), and (b) an effective amount of $rVSV_{NJ}$ M(G22E/M48R/M51R) or M(G22E/L110A/M48R/M51R) in a prime-boost immunization modality. The E at positions 21 or 22 as the case may be, is encoded by the codon gaa and R at positions 48 and 51, as the case may be, is encoded by codon cga. The A at position 111 or 110 may preferably be encoded by the codon gca.

The term "effective amount" as used herein means an amount effective and at dosages and for periods of time necessary to achieve the desired result.

In certain embodiments, (a) is administered to the subject before (b) is administered to the subject.

In certain embodiments, (b) is administered to the subject more than one time over the course of treating or preventing.

In certain embodiments, (a) is administered to the subject in need thereof and (b) is administered to the subject in need thereof at about weeks three, eight and sixteen post-administration of (a).

In certain embodiments, (b) is administered to the subject before (a) is administered to the subject.

In certain embodiments, (a) is administered to the subject more than one time over the course of treating or preventing.

In certain embodiments, (b) is administered to the subject in need thereof and (a) is administered to the subject in need thereof at about weeks three, eight and sixteen post-administration of (b).

Recombinant Virus

In certain embodiments, present invention relates to a recombinant vesicular stomatitis virus (rVSV) which may be a full length VSV, essentially non-cytolytic, avirulent, capable of inducing an immune response in a subject, capable of reproducing virus particles to a high tire at permissive temperatures, reproducing virus particles to a low titre at semi-permissive temperatures and which may be incapable of producing virus at non-permissive temperatures, and that can express an

epitope of a foreign pathogen. The rVSV of the present invention may be capable of inducing humoral, cellular and mucosal immune responses.

In one embodiment, the present invention relates to $rVSV_{Ind}$ and $rVSV_{NJ}$. The $rVSV_{Ind}$ may be a full length, essentially noncytolytic $rVSV_{Ind}$ M(G21E/L111A/M51R) capable of producing virus particles at a permissible temperature of about 31°C, and which may be incapable of or poorly capable of producing virus particles at a semi-permissive temperatures of about 37°C and incapable of producing virus particles at non-permissive temperatures above 37°C, for example 39°C. In certain embodiments, the $rVSV_{Ind}$ may include a M(G21E/L111A/M51R). In certain embodiments, the $rVSV_{Ind}$ may include an M gene comprising a nucleotide sequence SEQ ID NO: 2.

In certain embodiments, the rVSV is a full-length, essentially noncytolytic $rVSV_{NJ}$ M (G22E/M48R/M51R) or M(G22/L110A/M48R/M51R). In certain embodiments, the rVSV is an essentially noncytolytic $rVSV_{NJ}$ including an M gene, wherein the nucleotide sequence of the M gene is selected from SEQ ID NO: 6 and SEQ ID NO: 7.

The rVSVs of the present invention can be prepared using techniques known in the art. In one embodiment, the rVSVs may be introduced in a host cell under conditions suitable for the replication and expression of the rVSV in the host. Accordingly, the present invention also provides a cell having a $rVSV_{Ind}$ wherein the amino acid sequence of the virus' M protein is modified to provide an essentially non-cytotoxic which also allows the $rVSV_{Ind}$ to effectively replicate at permissible temperature but may not replicate at non-permissible temperature.

As such, the present invention relates also to a cell having one or more of the recombinant VSVs of the present invention.

Vaccines or Immunogenic Compositions of the Invention

The present invention further features vaccines or immunogenic compositions comprising one or more of the rVSVs of the present invention. In one embodiment, the present invention features vaccines or immunogenic compositions comprising an $rVSV_{Ind}$ and vaccines or immunogenic compositions comprising an $rVSV_{NJ}$, as described above.

In one embodiment, the vaccines may include rVSVs expressing an epitope of a pathogen. In another embodiment, the vaccines may include a mixture or cocktail of rVSVs expressing different epitopes of a pathogen (see Table 6, vaccination groups 5 and 6).

The vaccine or immunogenic compositions of the invention are suitable for administration to subjects in a biologically compatible form *in vivo*. The expression "biologically compatible form suitable for administration *in vivo*" as used herein means a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances maybe administered to any animal or subject, preferably humans. The vaccines of the present invention may be provided as a lyophilized preparation. The vaccines of the present invention may also be provided as a solution that can be frozen for transportation. Additionally, the vaccines may contain suitable preservatives such as glycerol or may be formulated without preservatives. If appropriate (i.e. no damage to the VSV in the vaccine), the vaccines may also contain suitable diluents, adjuvants and/or carriers.

The dose of the vaccine may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regime may be adjusted to provide the optimum therapeutic response. The dose of the vaccine may also be varied to provide optimum preventative dose response depending upon the circumstances.

Kits

The present invention provides kits, for example for preventing or treating an infection. For example, a kit may comprise one or more pharmaceutical compositions or vaccines as described above and optionally instructions for their use. In still other embodiments, the invention provides kits comprising one or more pharmaceutical compositions or vaccines and one or more devices for accomplishing administration of such compositions.

Kit components may be packaged for either manual or partially or wholly automated practice of the foregoing methods. In other embodiments involving kits, this invention contemplates a kit including compositions of the present invention, and optionally instructions for their use. Such kits may have a variety of uses, including, for example, imaging, diagnosis, therapy, and other applications.

Advantages and Unique Features of the rVSVs of the present invention

Novel and Unusual features of the invention:

Normal assembly and release of $rVSV_{Ind}$ M(G21E/L111A/M51) at permissive temperature (about 31°C) made it possible to amplify the new mutant rVSVs at the permissible temperature to a high titre to make viral stock. The assembly defectiveness of $rVSV_{Ind}$ M(G21E/L111A/M51) at non-permissive temperature (about 37°C (around body temperature) increased the safety of the using the rVSV in human and other animals by significantly reducing the number of progeny infectious viruses at the non-permissive temperature. The addition of these mutations to the pre-existing M51R mutation in the M protein of $rVSV_{Ind}$ further attenuated the virulence of VSV_{Ind}.

Prior to the present invention it was unknown that including the substitution L111A would result in rVSV having normal assembly at permissive temperatures and assembly defectiveness at non-permissive temperatures. The advantage of having a L111A substitution instead of L111F is that the former has less chance of reverting back to a wild type form.

The E at positions 21, which may be encoded by the codon gaa and R at positions 48 and 51, as the case may be, being encoded by codon cga decreases the chances of the mutants reverting back to wild type.

Previously the inventors developed attenuated $rVSV_{Ind}$ with the mutations of G21E, M51R, and L111F in the *M* gene. The amino acid changes were accomplished by changing nucleotides. Nucleotide codon for Glycine 21, GGG was changed to GAA for Glutamic acid with 2 nucleotide changes. Nucleotide codon for Methionine51, ATG was changed to AGG for Arginine with 1 nucleotide change. Nucleotide codon for Leucine, TTG was changed to TTT for Phenylalanine with 1 nucleotide change. The initial nucleotide changes to mutate M51 to R and L111 to F were only one in each amino acid. However, it is very likely that, if we change more nucleotides to change amino acids, will diminish the chances of the changed amino acids reverting back to the original amino acids. Therefore, the iventors mutated AGG of M51R to CGA to have the nucleotide codon changed completely in all 3 nucleotides. TTT of Phenylalanine was mutated to GCA of Alanine to have 3 nucleotides were completely changed. The resulting mutations in the M protein of $rVSV_{Ind}$ (GLM)-

New are G21E/L111A/M51R with nucleotide changes of GAA(E)/GCA(A)/CGA(R). More nucleotides changes in a codon for a amino acid change will increase the stability of the temperature sensitive mutations in the $rVSV_{Ind}$(GLM). Likewise more nucleotides for M48R and M51R in the $rVSV_{NJ}$(GMM) mutant. The initial changes in the nucleotide codon for the mutations G22E/M48R/M51R were GAA(E)/AGG(R)/AGG(R). These nucleotide codons were further changed to GAA(E)/CGA(R)/CGA(R) to have all three nucleotides for the Arginine changed.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

EXAMPLES

The present invention is further illustrated by the following examples which should not be construed as limiting in any way.

Example 1 – – Introduction of Mutations into the M genes of $rVSV_{Ind}$ and $rVSV_{NJ}$ and Recovery of Recombinant VSV by Reverse Genetics

Mutations were introduced into the M gene of VSV_{Ind} (FIG. 1) and VSV_{NJ} (FIG. 2). Nucleotide sequences encoding the amino acids at each position were mutated by the mega-primer PCR method. Each mutation is expressed as a substitution of an amino acid at a specific position (e.g., M51 in M51R) with another amino acid (e.g., R in M51R). In order to attenuate further the virulence of VSV, the inventors combined mutations (G21E and L11F) in the *ts*O23 with methionine to arginine mutations (M51R) in the M gene, which reduced inhibitory activity of M protein on the cellular protein synthesis, in addition, reduced the assembly of the VSV particles at non-permissive (39 °C) and semi-permissive (37 °C) temperatures.

Wild type and mutant recombinant vesicular stomatitis viruses (rVSV) were recovered from the cDNA plasmids by reverse genetics (FIG. 3). The VSV reverse genetics employs the BHK21 cells expressing DNA dependant RNA polymerase of

bacteriophage T7 (T7) and a plasmid which encodes full length genomic RNA of VSV (pVSV) and 3 plasmids expressing nucleocapsid protein (pN), phosphoprotein (pP), and VSV polymerase L protein (pL). The transcription of the full length genomic RNA and the messenger RNAs for N, P, and L proteins are under the control of T7 RNA polymerase. Internal ribosome entry site (IRES) at the upstream of each VSV N, P, and L gene enhances the translation of proteins. The plasmids are transfected into BHK-T7 cells with Lipofectamine™ 2000 in concentrations of 10 μg of pN, 10 μg of pP, μ5 g of pL, and 15 μg of pVSV. The culture media from the transfected cells were harvested when the cells showed about 50-70% of CPE.

Example 2 – The mutation, L111F in the M gene significantly reduces the burst sizes of $rVSV_{Ind}$ at semi-permissive temperature, 37°C.

The recovered viruses were purified 3 times by plaque picking and were amplified for a larger volume of stock viruses by infecting BHK21 cells with an MOI of 0.1 at 31°C. The inventors infected BHK21 cells and human neuroblastoma cells, SH-SY5Y with an MOI of 3 of rVSVs. The infected cells were incubated at permissive temperature (31°C) and semi-permissive temperature (37 °C, body temperature) to determine the temperature sensitivity of the new M mutants in the assembly of virus particles. Culture media from the infected cells were collected every 2 hours until 10 hours after the infection, and the number of infectious viral particles in the culture media was determined by plaque assay with Vero E6 cells. The cells infected with the mutant viruses for the plaque assay were incubated at 31°C. Wild type and all mutants replicated equally well and produced similar titre of infectious viruses all along the period of 10 hrs of infection (FIG. 4A and C). However, the mutants of $rVSV_{Ind}$, $rVSV_{Ind}$-G21E/L111F and $rVSV_{Ind}$-G21E/L111F/M51R replicated in significantly lower titre than wild type or M51R mutant of $rVSV_{Ind}$ at 37°C. The differences in producing infectious particles between the wild type and the new M mutant, $rVSV_{Ind}$-G21E/L111F/M51R were as large as four logs (FIG. 4B and D). The error bars in FIG. 4 represent standard error of the mean. The *P* values for viral titers of $rVSV_{Ind}$-G21E/L111F/M51R at 4hrs, 6hrs, 8hrs, and 10hrs postinfection obtained by comparing them to titers of $rVSV_{Ind}$-WT are $p<0.0001$, $p=0.0055$, $p=0.0040$, and $p=0.0015$ in FIG 4B. The *P* values for viral titers of $rVSV_{Ind}$-G21E/L111F at 4hrs, 6hrs, 8hrs, and 10hrs postinfection obtained by comparing them to titers of $rVSV_{Ind}$-WT are $p<0.0001$, $p=0.0055$, $p=0.0041$, and $p=0.0016$ in

FIG 4B. The *P* values in FIG. 4D were <0.005. *P* values were computed by using two-sided independent *t* tests.

Example 3 – Mutations in the M gene of $rVSV_{NJ}$, G22E and L110F do not reduce the burst size of the $rVSV_{NJ}$ at 37 °C.

BHK21 cells were infected with an MOI of 3 of $rVSV_{NJ}$, wild type and M gene mutants, incubated at both 37°C (semi-permissive temperature) and 31°C (permissive temperature) and the culture media was harvested at 10 hrs post infection. The viral titer of each virus in the culture media was determined by plaque assay using Vero E6 cells. The average viral titre from the duplicate samples is shown in the table. Wild type and all M mutants of $rVSV_{NJ}$ replicated equally well at both temperatures 31°C and 37°C (FIG. 5), indicating the introduction of G22E and L110F mutations into the M gene of $rVSV_{NJ}$ did not affect the assembly and release of the virus at 37°C. The error bars in FIG. 5 represent standard error of the mean.

Example 4 – Assembly defectiveness at semi-permissive temperature by L111F mutation in the M gene of $rVSV_{Ind}$ does not affect the expression of VSV proteins

BHK21 cells were infected with an MOI of 6 of $rVSV_{Ind}$ and $rVSV_{NJ}$, wild type and M gene mutants. The infected cells were incubated at both 37°C and 31°C for 6 hrs. The infected cells were lysed at 6 hrs post-infection, 5 µg of total protein was loaded to the SDS-PAGE gel, and rVSV proteins were detected by Western blot analysis using rabbit antiserum against VSV_{Ind} and VSV_{NJ} (1:5000 dilution). The result demonstrate that in spite of the mutations that reduced the burst size of the $rVSV_{Ind}$ at 37°C (L111F in G21E/L111F and G21E/L111F/M51R), the level of VSV protein expression is comparable to the wild type $rVSV_{Ind}$ (FIG. 6A). Wild type and all M mutants of $rVSV_{NJ}$ expressed their proteins in a similar level at both 31°C and 37°C (FIG. 6B).

Example 5 – Combined Mutations, G21E/L111F/M51R in the M gene of $rVSV_{Ind}$ reduced cytopathogenicity significantly in BHK21 cells and human neuroblastoma cells, SH-SY5Y.

In order to examine the effects of the mutations, L111F and M51R of M gene of $rVSV_{Ind}$ on the cytopathogenicity, the inventors infected BHK21 cells (FIG. 7) and human neuroblastoma cells (FIG. 8) with an MOI of 0.1 of $rVSV_{Ind}$. The typical

cytopathic effects by the VSV are rounding-up of infected cells and cell lysis. At 20 hrs after infection, the cytopathic effect caused by the $rVSV_{Ind}$-G21E/L111F/M51R was compared with those by the other $rVSV_{Ind}$. In 20 hrs of infection with the $rVSV_{Ind}$-G21E/L111F/M51R mutant showed the most reduced cytopathic effects, none or small number of round-up cells at 37°C and the combination of the mutations L111F and M51R (FIG. 7N and FIG. 8L) further attenuated the cytopathogenicity of the virus than the single mutation of each in both BHK21 and SH-SY5Y cells.

Example 6 – Reduced cytopathic effects in the BHK21 cell by the $rVSV_{NJ}$ with the M gene mutations, G22E/M48R+M51R and G22E/L110F/M48R+M51R.

In order to examine the effects of the mutations, G22E, L110F and M48R+M51R of M gene of $rVSV_{NJ}$ on the cytopathogenicity, the inventors infected BHK21 cells and human neuroblastoma cells with an MOI of 0.1 of $rVSV_{NJ}$. At 20 hrs after infection, the inventors compared the cytopathic effects (cell round-up and lysis) caused by the $rVSV_{NJ}$ wild type and other M mutants. In 20 hrs of infection with the G22E/M48R+M51R mutant and G22E/L110F/M48R+M51R mutant showed the least cytopathic effects at 37°C and the combination of the mutations, G22E and M48R+M51R further attenuated the cytopathogenicity of the $rVSV_{NJ}$ in both BHK21 (FIG. 9L and 9N) and SH-SY5Y cells (FIG. 10L and 10N).

Example 7 – $rVSV_{Ind}$ with the mutations of G21E/L111F/M51R and $rVSV_{NJ}$ with the mutations of G22E/M48R+M51R and G22E/L110F/M48R+M51R in the M protein was attenuated to a degree that mice injected with the virus into the brain showed no neurological signs or any symptoms such as weight loss.

VSV does not show the neurotropism in host animal during the natural infection through the skin abrasion or sandfly or mosquito bites. Nevertheless, when wild type VSV is injected directly into the nose or brain in mice or monkeys, the animals demonstrate neurological symptoms. In order to examine the neurovirulence of the new M mutants of VSVs, the inventors injected Swiss Webster mice with $1X10^6$ PFU of mutant VSV and $1X10^3$ PFU of wild type VSV into the intralateral ventricle of the brain. The inventors purchased 5-week-old Swiss Webster mice with intralateral ventricular implant from Charles River laboratory. Three mice/group were injected with viruses after one week of arrival to the animal facility. After the viral injection, mice were observed for neurological signs and were weighed every

two days for 4 weeks. The mice injected with $1X10^3$ PFU of wild type $rVSV_{Ind}$ died within four days after injection (FIG. 11B). Two mice injected with $1X10^6$ PFU of $rVSV_{Ind}$-M51R (FIG. 11C) lost about 20% of body weights 6 days after injection and the body weight bounced back to normal weight at about 14 days after injection. One mice injected with the $rVSV_{Ind}$-M51R did not lose the body weight through the experiment. All three mice injected with $rVSV_{Ind}$-G21E/L111F/M51R showed no sign of illness and did not lose their body weight for 4 weeks until the mice were sacrificed (FIG. 11D) indicating the combination of mutations, G21E/L111F and M51R significantly attenuated the $rVSV_{Ind}$ and lost its neurovirulence in mice. One mice injected with $rVSV_{NJ}$-WT showed paralysis in both hind legs and it was sacrificed on day 6 after injection (FIG. 12A). Two other mice injected with $rVSV_{NJ}$-WT and mice injected with $rVSV_{NJ}$-M48R+M51R, $rVSV_{NJ}$-G22E/M48R+M51R, or $rVSV_{NJ}$-G22E/L110F/M48R+M51R showed no sign of illness and weight loss during 4 weeks after injection (FIG. 12B, 12C, and 12D). The error bars in FIG. 11 and 12 represent standard error of the mean.

Example 8 – Generation of rVSVs expressing HIV-1 Gag protein as a gene of interest; HIV-1 Gag proteins are expressed equally well at both 31°C and 37°C.

The newly generated M mutants, G21E/L111F/M51R mutant of $rVSV_{Ind}$ and G22E/M48R+M51R and G22E/L110F/M48R+M51R mutants of $rVSV_{NJ}$ demonstrated the reduced cytopathogenicity and comparable protein expressions to the wild type rVSV at 37 °C. In order to be used as a vaccine vector, the new M mutants of rVSV should induce good immune responses *in vivo*, both humoral and cellular immune responses. When it is expressed alone in cells, HIV-1 gag proteins produce virus like particles and the virus like particles are secreted from the cells. Therefore, the gag protein was suitable protein to express from the new M mutants of rVSV to examine both cellular and humoral immune response. In addition, the CD8+ cytotoxic T cell epitope, H-$2K^d$-restricted peptide (NH_2-AMQMLKETI-COOH) (SEQ ID NO: 33) in the HIV-1 Gag is well studied in the Balb/c mouse. The inventors inserted the full-length HIV-1 *gag* gene linked to conserved human CD8+ T cell epitopes from gp41, gp120, and nef protein of HIV-1 (Gag-En). The Gag-En gene was inserted into the junction of G gene and L gene in the full-length cDNA clones of wild type (WT) and G21E/L111F/M51R (GLM) of $rVSV_{Ind}$ and wild type, G22E/M48R+M51R (GM), and G22E/L110F/M48R+M51R(GLM) of $rVSV_{NJ}$. The

rVSVs were recovered from the cDNA clones by reverse genetics as described in FIG. 3 and the expression of Gag-En from the rVSVs was examined by Western blot analysis using monoclonal antibody against HIV-1 p24. For the Western blot analysis, BHK21 cells were infected with MOI of 6 of rVSVs and incubated at 31°C and 37°C, and cell lysates were prepared at 6 hrs post-infection. Total protein of 5 μg was loaded onto the SDS-PAGE for the Western blot analysis. $rVSV_{Ind}$(GLM)-Gag-En, $rVSV_{NJ}$(GM)-Gag-En, and $rVSV_{NJ}$(GLM)-Gag-En expressed Gag-En protein (64 kDa) well at semi-permissive temperature (37°C, FIG. 13C) and the expression level was similar to that at permissive temperature (31°C, FIG. 13B).

Example 9 – Vaccination Regimen with rVSV in Mice

Six Balb/c mice per group were vaccinated with the prime-boost regimen illustrated in FIG. 14. Mice were grouped according to the vaccine vector type (wild type vs. mutant) and regimen, e. g., priming and boosting with the same serotype of rVSV or alternating the two serotypes for priming and boost. The mice were prime-vaccinated intramuscularly with $5X10^6$ PFU of rVSVs at age of 6 weeks. Three weeks after the priming, mice were boost-vaccinated with the same dose of rVSV as the prime vaccination. A week after the booster injection, splenocytes and sera were collected to detect the HIV-1 Gag specific CD8+ T cell immune responses and humoral immune responses.

Example 10 – Priming with $rVSV_{Ind}$(GLM)-HIV-1 Gag-En and Booster with $rVSV_{NJ}$(GLM)-HIV-1 Gag-En induces strongest CD8+ T cell immune responses against HIV-1 Gag.

The T cells stimulated by the interaction with MHC I molecule on the antigen presenting cells loaded with the peptide enhances the secretion of interferon-γ (IFN-γ), which indicates the antigen specific T cell immune responses. The splenocytes were double stained with FITC-anti-CD8 and APC-anti-IFN-γ for CD8+ T cells with the increased intracellular INF-γ. In order to examine the HIV-1 Gag protein specific CD8+ T cell immune response, splenocytes were isolated and $1X10^6$ cells were stimulated with H-$2K^d$-restricted HIV-1 Gag peptide, NH_2-AMQMLKETI-COOH (SEQ ID NO: 33), the cells were stained with FITC rat anti-mouse CD8 for the CD8 molecules and stained with APC rat anti-mouse IFN-γ for intracellular IFN-γ. The secretion of IFN-γ was blocked with Brefeldin A before staining them. VSV specific

CD8+ T cell immune responses were examined with the use of nucleocapsid specific peptide, IN275: NH2-MPYLIDFGL-COOH (SEQ ID NO: 32). Peptide specific CD8+-IFN-γ+ T cells were counted with FACSCalibur, a flowcytometer. The splenocytes treated with DMSO (solvent for the peptide, FIG. 15) did not stimulate the CD8+ T cells indicating that treating the splenocytes with the VSV N peptides and HIV-1 Gag peptides stimulated specifically CD8+ T cells in FIG. 16 and FIG. 17. Prime and boost immunization by alternating two serotypes of rVSV(WT) or rVSV(GLM) mutants induced stronger CD8+ T cell immune responses against VSV as well as HIV-1 Gag proteins than using a single serotype of rVSV for the prime and boost vaccinations as seen in groups 1, 2, 5, 6, 9, and 10. (FIG. 16 and FIG. 17). With the vaccination with the new M mutants or rVSV, CD8+ T cell responses against HIV-1 Gag protein was induced better when mice were vaccinated with $rVSV_{Ind}$(GLM)-Gag for priming and $rVSV_{NJ}$(GLM)-Gag for boosting than vice versa (FIG 17, group 6 vs. group 10). *P* value on the group 6 in FIG. 17 was computed by using two-sided independent sample *t* test, and the result was compared to that for the group 10. The error bars in FIG. 16 and 17 represent standard error of the mean.

Example 11 – HIV-1 Gag specific humoral immune responses

Generation of HIV-1 Gag specific antibody was examined with the serum collected at a week after the boost immunization. The Gag specific antibody titer was determined by the indirect enzyme-linked immunosorbent assay (ELISA). For the ELISA, 96 well ELISA plate was coated with recombinant p55 at a concentration of 125 ng/well. The mouse serum was diluted 1:100. The antibody bound to the antigen, p55 was detected with secondary antibody, sheep anti-mouse IgG-HRP. The enzymatic activity of HRP was detected by adding substrates, a mixture of hydrogen peroxide and tetramethylbenzidine. The OD of each sample was read at the wavelength of 450 with the microplate reader. The humoral immune responses against HIV-1 Gag (generation of antibody against HIV-1 Gag) was induced well in mice vaccinated with the new M mutants, and the best humoral immune responses against HIV-1 Gag were induced when two serotypes of rVSV(WT) or rVSV(GLM) were alternated for prime and booster injection (FIG 18, groups 5, 6, 9, and 10). As for the utilization of the new M mutants for inducing humoral immune responses against foreign proteins, priming with $rVSV_{Ind}$(GLM)-Gag and booster with

$rVSV_{NJ}$(GLM)-Gag worked better than vice versa (Gig. 18, group 6 vs. group 10). The error bars in FIG. 18 represent standard error of the mean.

Example 12 – Increasing doses of $rVSV_{Ind}$(GLM), $rVSV_{NJ}$(GM), and $rVSV_{NJ}$(GLM) for vaccination induced stronger immune responses in mice

As illustrated in FIG. 17 and FIG. 18, stronger HIV-1 Gag specific CD8+ T cell immune responses and humoral immune responses were induced when mice were prime vaccinated with $rVSV_{Ind}$(GLM)-Gag and booster vaccinated with $rVSV_{NJ}$(GLM)-Gag. However the immune responses were not as strong as those induced with $rVSV_{Ind}$(WT)-Gag and $rVSV_{NJ}$(WT)-Gag. Therefore, the inventors wanted to examine whether they could increase the immune responses, if they increase the doses of the $rVSV_{Ind}$(GLM)-Gag and $rVSV_{NJ}$(GLM)-Gag. Six (6) week old Balb/c mice, 6 mice/group, were vaccinated with $rVSV_{Ind}$(GLM)-Gag for priming and $rVSV_{NJ}$(GLM)-Gag or $rVSV_{NJ}$(GM)-Gag for booster. Because the $rVSV_{NJ}$(GM) was as attenuated as the $rVSV_{NJ}$(GLM) *in vitro* and *in vivo*, the inventors included $rVSV_{NJ}$(GM)-Gag as a booster virus, and compared the immune responses to that induced by $rVSV_{NJ}$(GLM)-Gag. Mice groups were vaccinated with various doses of $rVSV_{Ind}$(GLM)-Gag, $rVSV_{NJ}$(GLM)-Gag, and $rVSV_{NJ}$(GLM)-Gag; $5X10^6$ PFU/mouse, $5X10^7$ PFU/mouse, $5X10^8$ PFU/mouse, and $5X10^9$ PFU/mouse (Table 1).

Mice were vaccinated according to the schedule as seen in FIG. 14 and were sacrificed a week after booster vaccination for splenocytes and serum. The splenocytes were double stained with FITC-anti-CD8 and APC-anti-IFN-γ for CD8+ T cells with the increased intracellular INF-γ. In order to examine the HIV-1 Gag protein specific CD8+ T cell immune response, splenocytes were isolated and $1X10^6$ cells were stimulated with H-2Kd-restricted HIV-1 Gag peptide, NH2-AMQMLKETI-COOH (SEQ ID NO: 33), the cells were stained with FITC rat anti-mouse CD8 for the CD8 molecules and stained with APC rat anti-mouse IFN-γ for intracellular IFN-γ. The secretion of IFN-γ was blocked with Brefeldin A before staining them. VSV specific CD8+ T cell immune responses were examined with the use of nucleocapsid specific peptide, IN275: NH2-MPYLIDFGL-COOH (SEQ ID NO: 32). Peptide specific CD8+-IFN-γ+ T cells were counted with FACSCalibur, a flowcytometer.

CD8+ T cell immune responses against VSV N protein were very similar in all vaccination groups with different doses of $rVSV_{Ind}$(GLM)-Gag, $rVSV_{NJ}$(GLM)-Gag, or $rVSV_{NJ}$(GM)-Gag (FIG. 19). The HIV-1 Gag specific CD8+ T cell immune responses after prime vaccination with $rVSV_{Ind}$(GLM)-Gag and booster with $rVSV_{NJ}$(GLM)-Gag looked increased a little bit when the vaccine doses were increased, but differences were not statistically significant (FIG. 20, groups 2, 3, 4, 5). However, when mice were prime vaccinated with $rVSV_{Ind}$(GLM)-Gag and booster vaccinated with $rVSV_{NJ}$(GM)-Gag, the frequency of the HIV-1 Gag specific CD8+ T cells were increased significantly with the increasing dose of $5X10^8$ PFU. The HIV-1 Gag specific CD8+ T cell response was strongest in a group vaccinated with $5X10^9$ PFU/mouse (FIG 20. groups 6, 7, 8, 9). *P* values on the groups 8 and 9 in FIG. 20 were computed by using two-sided independent sample *t* test, the result of group 8 was compared to that for the group 7, and the result of group 9 was compared to that for the group 8. The error bars in FIG. 19 and 20 represent standard error of the mean.

Generation of antibody against HIV-1 Gag protein was increased with the increasing doses of $rVSV_{Ind}$(GLM)-Gag for priming and booster with $rVSV_{NJ}$(GLM)-Gag or $rVSV_{NJ}$(GM)-Gag (FIG. 21). Booster vaccination with $rVSV_{NJ}$(GLM)-Gag or with $rVSV_{NJ}$(GM)-Gag generated similar level of Gag specific antibodies in various doses of the virus. The error bars in FIG. 21 represents standard error of the mean.

Example 13 – Generation of $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) expressing HIV-1 proteins; Gag, Pol, Env, and RT

For HIV-1 vaccines utilizing the $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) as vaccine vectors, the inventors included HIV genes encoding most of the large polyproteins, which are cleaved into functional proteins-Gag and Env and proteins with enzymatic activities (*pol* gene products). In addition, HIV-1 *gag* gene was linked to nucleotides encoding peptide epitopes for T cells and B cells in humans. The inventors have generated $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) carrying cassettes encoding HIV-1 Gag linked to B-cell epitopes from HIV Env protein derived from multiple viral clades (FIG. 22). The inventors have generated the $rVSV_{Ind}$(GLM)-Gag-En and $rVSV_{NJ}$(GM)-Gag-En, which expresses *gag* gene and T cell peptide epitopes from Nef, Gp120, and Gp41 (FIG. 23). The inventors generated $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) with HIV-1 Gag-RT, which encodes gag protein with peptide epitopes

from RT, Tat, and Rev proteins (FIG. 23). In addition, the inventors generated $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) with *pol* gene and *env* gene, respectively (FIG. 23). Previously, the inventors have demonstrated that replacement of signal peptide of HIV-1 envelope protein with the honeybee melittin signal peptide dramatically increased the rates of Gp120 expression, glycosylation, and secretion (Yan Li et al., PNAS, 93: 9606-9611, 1996). The increased expression and secretion of Gp120 are expected to enhance the induction of the T cell and B cell immune responses against HIV-1 gp120. Therefore, the inventors generated rVSVs with HIV-1 *env* gene with melittin signal sequence (gp160mss). The recombinant VSVs with the HIV-1 proteins were plaque purified and were amplified for the stock viruses. The expression of HIV-1 proteins at 31 °C and 37 °C from the mutant VSV was examined by Western blot analysis. The Gag proteins tagged with T cell and B cell epitopes from various HIV-1 proteins were expressed well from $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) at both 31 °C and 37 °C (FIG. 24 and FIG. 25). The cloned HIV-1 *pol* gene in the rVSV genome encodes a polyprotein, which is cleaved into protease (PR, p11), reverse transcriptase (RT, p66/p51), and integrase (IN, p31). The inventors detected two subunits of RT products, p66 and p51. The p51 is generated from the p66 by proteolytic cleavage of a fragment of p15 at the carboxy terminus. The RT products p66 and p51 were expressed equally well from both $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) and at both 31 °C and 37 °C (FIG 26). The *env* gene encodes glycoprotein Gp160, which is cleaved into Gp120 of receptor binding protein and gp41 of fusion inducing cytoplasmic and transmembrane protein. Gp160 is cleaved by the cellular trans-golgi resident protease furin. The Gp160 expressed in BHK21 cells from the $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) was not cleaved to Gp120 and Gp41 or the cleavage was not very efficient, although the level of expression was good at both 31 °C and 37 °C (FIG. 27). Our previous data on the expression of the Gp160 in BHK21 cells and cleavage into Gp120 and Gp41 demonstrated that the processing was not very efficient (Kunyu Wu et al., Journal of General Virol., 90:1135-1140, 2009).

Example 14 – Immunization studies in mice with the $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) with HIV-1 proteins, Gag, Gp160 and RT

The preliminary prime-boost immunization studies with new M mutant rVSVs, $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) revealed that priming mice with the

$rVSV_{Ind}$(GLM) and boosting with $rVSV_{NJ}$(GM) induced better CD8+ T cell immune response (FIG. 17) and humoral immune responses (FIG. 18) against the foreign gene than the other way around. With the $rVSV_{Ind}$(GLM)-Gag priming and $rVSV_{NJ}$(GM)-Gag boosting, the dosage of $5X10^8$ pfu of each virus induced better Gag specific CD8+ T cell responses than lower dosages, although the B cell responses against Gag was similar to that induced with the lower dosages (FIG. 20 and FIG. 21). The inventors chose $5X10^8$ pfu as a dosage for immunization studies with $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) expressing HIV-1 Gag, Gp160, and RT.

The inventors prime immunized Balb/c mice with $rVSV_{Ind}$(GLM) expressing HIV-1 proteins and boost immunized with $rVSV_{NJ}$(GM) expressing HIV-1 proteins in three weeks after the priming as described in the table 6. The inventors examined how the immune responses against the individual HIV-1 proteins are induced with individual injection or with the mixed injection of three different viruses expressing separate proteins. One week after the boost immunization, the inventors sacrificed the immunized mice for their splenocytes and sera. The $1X10^6$ splenocytes were stimulated with H-$2K^d$-restricted HIV-1 protein specific peptides, which are Gag, Env P18, RT354, RT464, and RT472. The peptide sequences are shown in the FIG. 29. The stimulated splenocytes were double stained for the cell surface CD8 molecules and intracellular IFN-γ of T cells with FITC rat anti-mouse CD8 and APC rat anti-mouse IFN-γ. The regimen, prime-boost immunization with $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) induced peptide specific CD8+ T cell immune responses with different degree depending on the peptides the inventors used for the stimulation. CD8+ T cells against Env peptide stimulated CD8+ T cells the most up to 19%-23% of CD8+ T cells on average (FIG. 28, G3 and G5). About 4.5% or 3% of CD8+ T cells were responsive to the Gag protein in mice immunized with rVSV expressing Gag alone (FIG. 28, G2) or with mixed three rVSVs expressing Gag A, Gag B, and Gag C linked with various B and T cell epitopes of HIV-1 Gp120 and Gp41 (FIG. 29, G6). RT specific CD8+ T cells were generated in about 2% of CD8+ T cells in mice injected with rVSV with *pol* gene (FIG. 28, G4). CD8+ T cell responses against individual HIV-1 protein, Gag and RT were stronger when mice were injected with single rVSV expressing individual protein (FIG. 28, G2 and G4) than when injected with mixed rVSVs expressing single protein (FIG. 28, G5). Env specific CD8+ T cell responses were strongest and were similar in both single injection and mixed

injections. The CD8+ T cells responses against Env peptide epitope are immunodominant in Balb/c mice. Currently, the inventors are uncertain why mixed injections with three different rVSVs expressing Gag, Env, and RT induced comparatively lower CD8+ T cell responses against VSV N, gag, and RT than with the single virus injection. It is possible that the competition of each virus for the antigen presenting cells in the mixed virus injection lowered the CD8+ T cells againt Gag and RT. Because rVSV is common vector for HIV-1 proteins in mice either injected with single rVSV or mixed rVSVs, VSV N supposed to induce similar or better CD8+ T cell responses in the mixed injection. However, the CD8+ T cell responses against VSV N in mice are lower even with the single injections when the Env is expressed (FIG. 28, G3) as in the mixed injection (FIG. 28, G5). Therefore, it may be the result of immune dominance of Env protein in Balb/c mice, which affects negatively to the presentation of other proteins to the CD8+ T cells when Env is expressed together. For the combined immunizations in mice, all three rVSVs were injected at the single site, which may have resulted in the competition of different proteins for the antigen presentation in the same antigen presentation cells. The inventors will test the immunization with the mixed injections at more than one injection sites to induce CD8+ T cell responses.

HIV-1 Gag proteins expressed in cells can form virus like particles (VLP) and the VLP can be released from the cells. The released VLPs can induce humoral immune responses against Gag proteins. HIV-1 *env* gene encodes glycosylated surface proteins, which are processed through ER-golgi network to be properly folded and cleaved into transmembrane subunit Gp41 and surface unit Gp120. The Gp41 and Gp120 are associated by non-covalent bond and mature to form a trimer of Gp41 and Gp120 heterodimer. The matured trimer of Gp41 and Gp120 are exported to the cell surface. The Gp120 tends to fall off from the cell surface because of the weak bondage between Gp41 and Gp120. Therefore, antibody against Gp120 can be induced to the cell surface Gp120 or fallen off Gp120. Antibody titer against HIV-1 Gag protein and Gp120 was determined by ELISA. For the Gag antibody the microplate was coated with 125 ng/well of recombinant p55 (Pierce Biotechnology, RP-4921) and 50 µl of mice sera was tested with the dilution of 1:100, 1:200, and 1:400. Gag antibody was produced in mice injected with rVSV Gag-En alone (FIG. 30A, G2) and with mixed viruses rVSV-Gag A+rVSV Gag B+rVSV Gag C (FIG.

30A, G6). For the Gp120 ELISA, 96 well microplate was coated with 250 ng/well of HIV-1 SF162 Gp140 trimer from clade B (NIH AIDS reagent program, cat# 12026). Mice sera were diluted in 1:100, 1:200, and 1:400 and 50 µl of the diluted serum was added to the microplate for the ELISA. Antibody specific to Gp120 was generated in mice injected with rVSV-HIV-1 Gp160 alone and in mice injected with the mixed viruses expressing each protein of Gag, Gp160, and RT (FIG. 30B, G3 and G5). The titer of Gp120 antibody was slightly lower, although not statistically significant, in mice injected with mixed viruses than in mice injected with single virus expressing Gp160.

The newly generated M mutants of rVSVs, $rVSV_{Ind}$(GLM) and $rVSV_{NJ}$(GM) induced CD8+ T cell and humoral immune responses against various HIV-1 proteins expressed from the vector after injecting mice with an individual virus expressing single virus or with mixed viruses expressing various HIV-1 proteins.

Example 15 – Generation of $rVSV_{Ind}$(GLM), $rVSV_{NJ}$(GM), and $rVSV_{NJ}$(GLM) with Hepatitis C Virus Structural Proteins

Generally, the humoral immune response is the first line of defence mediated by adaptive immune responses against any pathogens. Although humoral immune responses against HCV and its role in the prevention of HCV infection is not well studied compared to the HCV specific cellular immune responses, it is worthy to include vaccines which can induce HCV specific antibodies. It has been demonstrated that nucleocapsid protein core and surface glycoproteins E1 and E2 form virus-like particles (VLP) which can be released from the cells (Blanchard, E. et al., J. Virol. 76:4073-4079, 2002). In addition, it has been demonstrated that HCV protein p7, a viroporin forming an ion channel in the ER membrane, takes part in releasing the HCV particles from the infected cell (Steinmann, E. et al., PLoS Pathogens 3:962-972, 2007). Another HCV transmembrane protein NS4B forms a membranous web structure, which mainly consists of the ER membrane. The membranous web formed by NS4B is a microstructure for the production of progeny HCV. It is not well known what functions NS4B has in the replication of HCV, but it is appealing to include NS4B into vaccine candidates simply because of its nature forming a membranous structure of ER in which other HCV proteins, especially Core, E1, E2, p7 are localized. Therefore, including Core, E1, E2, P7, and NS4B together into the vaccine may induce humoral and cellular immune responses.

For the HCV structural protein vaccines using the new M mutant rVSVs, the inventors inserted the HCV *core* gene, *E1E2P7* and *NS4B genes* together (connected by the VSV intergenic junction sequences), and *CoreE1E2P7* and *NS4B* genes together (connected by the VSV intergenic junction sequences) to the junction of VSV G gene and L (FIG. 31). The rVSVs; $rVSV_{Ind}$(GLM)-FC, $rVSV_{Ind}$(GLM)-CE1E2P7/NS4B, $rVSV_{NJ}$(GM)-FC, and $rVSV_{NJ}$(GLM)-E1E2P7/NS4B were generated by reverse genetics. The inventors had a trouble to generate the $rVSV_{NJ}$(GM) with CE1E2P7/NS4B from the plasmid DNA, therefore, the inventors removed the core and cloned E1E2P7/NS4B into the plasmid of $rVSV_{NJ}$(GLM) and generated the $rVSV_{NJ}$(GLM)-E1E2P7/NS4B. The expression of Core, E1, E2, and NS4B from the recombinant VSV at 31°C and 37°C was examined by Western blot analysis using antibodies against each protein (FIG. 32). The proteins were expressed similarly in quantity at both temperatures. Core (FIG. 32A), E1 (FIG. 32B), and E2 FIG. 32C) were expressed well from the $rVSV_{Ind}$(GLM)-CE1E2P7/NS4B, but the expression of NB4B was lower than the other proteins (FIG. 32D).

Example 16 – Generation of $rVSV_{Ind}$(GLM), $rVSV_{NJ}$(GM), and $rVSV_{NJ}$(GLM)with HCV Non-Structural Proteins

The inventors want to target most of the HCV proteins including core, E1, E2, NS3, NS4A, NS4B, NS5A and NS5B proteins in order to induce HCV specific CD8+ T cell and CD4+ T cell immune responses to multiple proteins. HCV nonstructural (NS) proteins-NS3, NS4A, NS4B, NS5A, and NS5B cover more than half of the HCV polyprotein. The NS proteins are cleaved into individual proteins by NS3 with the help of NS4A. Several studies demonstrate that patients who recover from the acute HCV infection develope strong CD4+ T cell and CD8+ T cell responses against multiple epitopes in the NS3 protein (Diepolder, H. M. J. Virol. 71:6011-6019, 1997; Lamonaca, V. et al. Hepatology 30:1088-1098, 1999; Shoukry, N. H. et al. J. Immunol. 172:483-492, 2004) indicating that including NS3 in the vaccine candidate is important to elicit successful cellular immune responses against HCV. NS3 protein, a serine protease and RNA helicase associates with NS4A and resides on the ER membrane (Sato, S. et al. J. Virol. 69:4255-4260, 1995; Failla, C. et al. J. Virol. 69:1769-1777, 1995). NS5A protein, a phosphoprotein is believed to be involved in HCV RNA synthesis together with NS5B protein, a RNA dependent RNA polymerase (Shirota, Y. et al., J. Biol. Chem., 277:11149-11155, 2002;

Shimakami, et al. J. Virol. 78:2738-2748, 2004). NS5B protein is a RNA dependent RNA polymerase that synthesizes positive sense HCV genomic RNA as well as intermediate negative sense genomic RNA (Beherens, S. E. EMBO J. 15:12-22, 1996). NS5B protein is a tail-anchored protein and associates with ER membrane through its carboxyl terminal 20 amino acids (Yamashita, T. J. Biol. Chem. 273:15479-15486, 1998; Hagedorn, C. H. Curr. Top. Microbiol. Immunol. 242:225-260, 2000).

The inventors cloned HCV NS genes as a gene for a single protein or a gene for a polyprotein of 2 or 3 NS proteins. The NS genes *NS3*, *NS34AB*, *NS5A*, *NS5B*, *NS5AB* were cloned into the junction at G gene and L gene of $pVSV_{Ind}$(GLM) and $pVSV_{NJ}$(GM) (FIG. 33). The inventors generated $rVSV_{Ind}$(GLM)-NS3, $rVSV_{Ind}$(GLM)-NS34AB, $rVSV_{Ind}$(GLM)-NS5A, $rVSV_{Ind}$(GLM)-NS5B, and $rVSV_{Ind}$(GLM)-NS5AB, and the expression of the NS proteins at 31 °C and 37 °C were determined by Western blot analysis using antibodies against each NS protein (FIG. 34). The inventors generated $rVSV_{NJ}$(GM)-NS3, $rVSV_{NJ}$(GM)-NS4B, $rVSV_{NJ}$(GM)-NS34AB, $rVSV_{NJ}$(GM)-NS5A, $rVSV_{NJ}$(GM)-NS5B, and $rVSV_{NJ}$(GM)-NS5AB. The expression of each protein from the $rVSV_{NJ}$ vector at 37 °C was examined by Western blot analysis using antibodies against each NS protein (FIG. 35). Although the expression level of NS proteins from the $rVSV_{Ind}$ and $rVSV_{NJ}$ was different, it was good enough to use them as HCV vaccine.

Example 17 – Introduction of Mutations into the M genes of $rVSV_{Ind}$ and $rVSV_{NJ}$ and Recovery of Recombinant VSV by Reverse Genetics

Mutations were introduced into the M gene of VSV_{Ind} and VSV_{NJ}. Nucleotide sequences encoding the amino acids at each position were mutated by the mega-primer PCR method. Each mutation is expressed as a substitution of an amino acid at a specific position (e.g., M51 in M51R) with another amino acid (e.g., R in M51R). In order to attenuate further the virulence of VSV, the inventors combined mutations (G21E) in the *ts*O23 with methionine to arginine mutations (M51R) and L111A in the M gene, which reduced inhibitory activity of M protein on the cellular protein synthesis, in addition, reduced the assembly of the VSV particles at non-permissive (39 °C) and semi-permissive (37 °C) temperatures. The nucleotide sequences and amino acid changes from wild type to mutants in the M genes of $rVSV_{Ind}$ and $rVSV_{NJ}$ are shown in Tables 2, 3, 4 and 5. The changed nucleotide codons are

underlined and changed nucleotide sequences and amino acid sequences are bold-faced.

Wild type and mutant recombinant vesicular stomatitis viruses (rVSV) were recovered from the cDNA plasmids by reverse genetics. The VSV reverse genetics employs the BHK21 cells expressing DNA dependant RNA polymerase of bacteriophage T7 (T7) and a plasmid which encodes full length genomic RNA of VSV (pVSV) and 3 plasmids expressing nucleocapsid protein (pN), phosphoprotein (pP), and VSV polymerase L protein (pL). The transcription of the full length genomic RNA and the messenger RNAs for N, P, and L proteins are under the control of T7 RNA polymerase. Internal ribosome entry site (IRES) at the upstream of each VSV N, P, and L gene enhances the translation of proteins. The plasmids are transfected into BHK-T7 cells with Lipofectamine™ 2000 in concentrations of 10 μg of pN, 10 μg of pP, μ5 g of pL, and 15 μg of pVSV. The culture media from the transfected cells were harvested when the cells showed about 50-70% of CPE.

FIG. 36 illustrates the construction of a cDNA plasmid for the full length genomic RNA of $rVSV_{Ind}$(GLM)-new.

First, AGG of M51R in the *M* gene of $prVSV_{Ind}$(GLM) was changed to CGA by megaprimer method. The primers, Ind(GLM) M (F) and Ind (GLM) M(M51R) was used to amplify the part of M gene with the additional nucleotide changes by polymerase chain reaction. The first PCR product was used as a megaprimer together with Ind(GLM) M (R) to amplify the whole M gene with the nucleotide changes, and the second PCR product was cloned into pBluescript II KS vector (Invitrogen) at the restriction site *Not I*. After confirmation of the nucleotide changes in the *M* gene clone with the CGA at the R51, the additional nucleotide change GCA for the Alanine was introduced by megaprimer PCR method using the primers Ind(GLM) M(F) and Ind(GLM) M(L111A), and Ind(GLM) M(R). The PCR product was cloned again into the pBluescript II KS vector at the *Not I* site. The M gene with the nucleotide changes of GAA(E)/CGA(R)/GCA(A) was cut from the pBluescript II KS vector with restriction enzymes, Pac I and Not I and cloned into the $prVSV_{Ind}$(GLM), which M gene was removed by cutting with Pac I and Not I.

Primers:

Ind(GLM) M (F); 5'-cgggcggccgcttaattaaactatgaaaaaaagtaacagat -3' [SEQ ID NO:11]

Ind (GLM) M(M51R); 5'-catgagtgtctcgctcgtcaac-3' [SEQ ID NO:12]

Ind (GLM) M(L111A); 5'-aagatcttggctttlgcaggttcttc-3' [SEQ ID NO:13]

Ind(GLM) M (R); 5'-cgggcggccgctagactagctcatttg-3' [SEQ ID NO:14]

FIG. 37 illustrates the construction of a cDNA plasmid for the full length genomic RNA of $rVSV_{NJ}$(GMM)-new

The nucleotides for the M48R and M51R in the M gene of $rVSV_{NJ}$ was changed by the megaprimer PCR method. The part of M gene with the nucleotide changes were amplified with the primers, NJ-M(F) and NJ-M(M48R+M51R), and the PCR product was used as a megaprimer with the primer NJ-G(R) to amplify the full length NJ M gene and G gene in order to use the restriction enzyme site, *Sac I*. The M and G PCR product was cut with Pac I and replaced the M and G gene of $prVSV_{NJ}$(GMM).

Primers:

NJ-M (F); 5'-tccccgcggttaattaagatgaacgatatgaaaaaaact-3' [SEQ ID NO:15]

NJ-M(M48R+M51R); 5'- tatcatataaatctcgatcctctcgtccgaagaagtca-3' [SEQ ID NO:16]

NJ-G (R); 5'-tccccgcggttaattaatttagcggaagtgagccat-3' [SEQ ID NO:17]

FIG. 38 shows that $rVSV_{Ind}$(GLM)-new (G21E/L111A/M51R) with additional nucleotide changes shows the same temperature sensitivity as original $rVSV_{Ind}$(GLM) (G21E/L111F/M51R).

The recovered viruses were purified 3 times by plaque picking and were amplified for a larger volume of stock viruses by infecting BHK21 cells with an MOI of 0.1 at 31°C. The inventors infected BHK21 cells with an MOI of 0.1 of $rVSV_{Ind}$(GLM) and $rVSV_{Ind}$(GLM)-New. The infected cells were incubated at permissive temperature (31°C) and semi-permissive temperature (37°C, body temperature) to determine the temperature sensitivity of the new M mutants in the assembly of virus particles. Culture media from the infected cells were collected at 20 hrs post-infection. The number of infectious viral particles in the culture media was determined by plaque assay with Vero E6 cells. The cells infected with the mutant viruses for the plaque

assay were incubated at 31°C. Both $rVSV_{Ind}$(GLM) and $rVSV_{Ind}$(GLM)-New showed the same temperature sensitivity at 37°C indicating that that the additional nucleotide changes in the M gene did not alter the temperature sensitivity of the $rVSV_{Ind}$(GLM). The mutation L111A shows the same temperature sensitivity as L111F indicating that the mutation L111A is not a silent mutation.

The inventors further demonstrated the stability of the new rVSV vector after 20 passages at 37C (about body temperature) and at 31C (the temperature for virus amplification) (data not shown). The new GLM mutant (G21E/L111A/M51R) with further nucleotide changes (codons gca for A and cga for R) did not change back to wild type sequences (reversion) after 20 passages in contrast to the old GLM mutant (G21E/L110F/M51R). The result demonstrated that the new GLM mutant is more stable and safer to use for vaccination in humans and other animals.

Table 1. Mouse Vaccination Groups and Regimen

Vaccination Groups	Prime		Boost		# of Mice
	Viruses	Titer (PFU)/50 µl	Viruses	Titer (PFU)/50 µl	
1	Ind (WT)-Gag	$5X10^6$	NJ (WT)-Gag	$5X10^6$	6
2	Ind (GLM)-Gag	$5X10^6$	NJ (GLM)-Gag	$5X10^6$	6
3	Ind (GLM)-Gag	$5X10^7$	NJ (GLM)-Gag	$5X10^7$	6
4	Ind (GLM)-Gag	$5X10^8$	NJ (GLM)-Gag	$5X10^8$	6
5	Ind (GLM)-Gag	$5X10^9$	NJ (GLM)-Gag	$5X10^9$	6
6	Ind (GLM)-Gag	$5X10^6$	NJ (GM)-Gag	$5X10^6$	6
7	Ind (GLM)-Gag	$5X10^7$	NJ (GM)-Gag	$5X10^7$	6
8	Ind (GLM)-Gag	$5X10^8$	NJ (GM)-Gag	$5X10^8$	6
9	Ind (GLM)-Gag	$5X10^9$	NJ (GM)-Gag	$5X10^9$	6
10	NJ (WT)-Gag	$5X10^6$	Ind (WT)-Gag	$5X10^6$	6
Total Mice					60

Table 2. Neucleotide Sequence Comparison between M Genes of VSV Indiana serotype, Wild Type (SEQ ID NO: 1) and a Mutant G21E/L111A/M51R (SEQ ID NO 2)

```
                1                                                   50
SEQ ID NO: 1: ATGAGTTCCT TAAAGAAGAT TCTCGGTCTG AAGGGGAAAG GTAAGAAATC
SEQ ID NO: 2: ATGAGTTCCT TAAAGAAGAT TCTCGGTCTG AAGGGGAAAG GTAAGAAATC

               51
      100
SEQ ID NO: 1: TAAGAAATTA GGGATCGCAC CACCCCCTTA TGAAGAGGAC ACTAACATGG
SEQ ID NO: 2: TAAGAAATTA GAAATCGCAC CACCCCCTTA TGAAGAGGAC ACTAACATGG

               101
150
SEQ ID NO: 1: AGTATGCTCC GAGCGCTCCA ATTGACAAAT CCTATTTTGG AGTTGACGAG
SEQ ID NO: 2: AGTATGCTCC GAGCGCTCCA ATTGACAAAT CCTATTTTGG AGTTGACGAG

               151
200
SEQ ID NO: 1: ATGGACACTC ATGATCCGCA TCAATTAAGA TATGAGAAAT TCTTCTTTAC
SEQ ID NO: 2: CGAGACACTC ATGATCCGCA TCAATTAAGA TATGAGAAAT TCTTCTTTAC

               201
250
SEQ ID NO: 1: AGTGAAAATG ACGGTTAGAT CTAATCGTCC GTTCAGAACA TACTCAGATG
SEQ ID NO: 2: AGTGAAAATG ACGGTTAGAT CTAATCGTCC GTTCAGAACA TACTCAGATG

               251
300
SEQ ID NO: 1: TGGCAGCCGC TGTATCCCAT TGGGATCACA TGTACATCGG AATGGCAGGG
SEQ ID NO: 2: TGGCAGCCGC TGTATCCCAT TGGGATCACA TGTACATCGG AATGGCAGGG

               301
350
SEQ ID NO: 1: AAACGTCCCT TCTACAAGAT CTTGGCTTTT TTGGGTTCTT CTAATCTAAA
SEQ ID NO: 2: AAACGTCCCT TCTACAAGAT CTTGGCTTTT GCAGGTTCTT CTAATCTAAA

               351
400
SEQ ID NO: 1: GGCCACTCCA GCGGTATTGG CAGATCAAGG TCAACCAGAG TATCACGCTC
SEQ ID NO: 2: GGCCACTCCA GCGGTATTGG CAGATCAAGG TCAACCAGAG TATCACGCTC

               401
450
SEQ ID NO: 1: ACTGTGAAGG CAGGGCTTAT TTGCCACACA GAATGGGGAA GACCCCTCCC
SEQ ID NO: 2: ACTGTGAAGG CAGGGCTTAT TTGCCACACA GAATGGGGAA GACCCCTCCC

               451
500
SEQ ID NO: 1: ATGCTCAATG TACCAGAGCA CTTCAGAAGA CCATTCAATA TAGGTCTTTA
```

```
SEQ ID NO: 2: ATGCTCAATG TACCAGAGCA CTTCAGAAGA CCATTCAATA TAGGTCTTTA

                501
550
SEQ ID NO: 1: CAAGGGAACG GTTGAGCTCA CAATGACCAT CTACGATGAT GAGTCACTGG
SEQ ID NO: 2: CAAGGGAACG GTTGAGCTCA CAATGACCAT CTACGATGAT GAGTCACTGG

                551
600
SEQ ID NO: 1: AAGCAGCTCC TATGATCTGG GATCATTTCA ATTCTTCCAA ATTTTCTGAT
SEQ ID NO: 2: AAGCAGCTCC TATGATCTGG GATCATTTCA ATTCTTCCAA ATTTTCTGAT

                601
650
SEQ ID NO: 1: TTCAGAGATA AGGCCTTAAT GTTTGGCCTG ATTGTCGAGA AAAAGGCATC
SEQ ID NO: 2: TTCAGAGATA AGGCCTTAAT GTTTGGCCTG ATTGTCGAGA AAAAGGCATC

                651
700
SEQ ID NO: 1: TGGAGCTTGG GTCCTGGATT CTGTCAGCCA CTTCAAATGA
SEQ ID NO: 2: TGGAGCTTGG GTCCTGGATT CTGTCAGCCA CTTCAAATGA
```

Table 3. Amino Acid Sequence Comparison between M Proteins of VSV Indiana serotype Wild Type (SEQ ID NO: 3) and a Mutant G21E/L111A/M51R (SEQ ID NO: 4)

```
                1                   21                            50
SEQ ID NO: 3: MSSLKKILGL KGKGKKSKKL GIAPPPYEED TNMEYAPSAP IDKSYFGVDE
SEQ ID NO: 4: MSSLKKILGL KGKGKKSKKL EIAPPPYEED TNMEYAPSAP IDKSYFGVDE

                51
100
SEQ ID NO: 3: MDTHDPHQLR YEKFFFTVKM TVRSNRPFRT YSDVAAAVSH WDHMYIGMAG
SEQ ID NO: 4: RDTHDPHQLR YEKFFFTVKM TVRSNRPFRT YSDVAAAVSH WDHMYIGMAG

                101       111
150
SEQ ID NO: 3: KRPFYKILAF LGSSNLKATP AVLADQGQPE YHAHCEGRAY LPHRMGKTPP
SEQ ID NO: 4: KRPFYKILAF AGSSNLKATP AVLADQGQPE YHAHCEGRAY LPHRMGKTPP

                151
200
SEQ ID NO: 3: MLNVPEHFRR PFNIGLYKGT VELTMTIYDD ESLEAAPMIW DHFNSSKFSD
SEQ ID NO: 4: MLNVPEHFRR PFNIGLYKGT VELTMTIYDD ESLEAAPMIW DHFNSSKFSD

                201                         229
250
SEQ ID NO: 3: FRDKALMFGL IVEKKASGAW VLDSVSHFK
SEQ ID NO: 4: FRDKALMFGL IVEKKASGAW VLDSVSHFK
```

Table 4. Nucleotide Sequence Comparison between M Genes of VSV New Jersey serotype Wild Type (SEQ ID NO: 5) and Mutants, G22E/M48R/M51R (SEQ ID NO: 6) and G22E/L110A/M48R/M51R (SEQ ID NO: 7)

```
                1                                                   50
SEQ ID NO: 5:   ATGAGTTCCT TCAAAAAGAT TCTGGGATTT TCTTCAAAAA GTCACAAGAA
SEQ ID NO: 6:   ATGAGTTCCT TCAAAAAGAT TCTGGGATTT TCTTCAAAAA GTCACAAGAA
ID NO: 7:       ATGAGTTCCT TCAAAAAGAT TCTGGGATTT TCTTCAAAAA GTCACAAGAA

               51                                                  100
SEQ ID NO: 5 :ATCAAAGAAA CTAGGCTTGC CACCTCCTTA TGAGGAATCA AGTCCTATGG
SEQ ID NO: 6 :ATCAAAGAAA CTAGAATTGC CACCTCCTTA TGAGGAATCA AGTCCTATGG
SEQ ID NO: 7 :ATCAAAGAAA CTAGAATTGC CACCTCCTTA TGAGGAATCA AGTCCTATGG

               101                                                 150
SEQ ID NO: 5 :AGATTCAACC ATCTGCCCCA TTATCAAATG ACTTCTTCGG AATGGAGGAT
SEQ ID NO: 6 :AGATTCAACC ATCTGCCCCA TTATCAAATG ACTTCTTCGG ACGAGAGGAT
SEQ ID NO: 7 :AGATTCAACC ATCTGCCCCA TTATCAAATG ACTTCTTCGG ACGAGAGGAT

               151                                                 200
SEQ ID NO: 5 :ATGGATTTAT ATGATAAGGA CTCCTTGAGA TATGAGAAGT TCCGCTTTAT
SEQ ID NO: 6 :CGAGATTTAT ATGATAAGGA CTCCTTGAGA TATGAGAAGT TCCGCTTTAT
SEQ ID NO: 7 :CGAGATTTAT ATGATAAGGA CTCCTTGAGA TATGAGAAGT TCCGCTTTAT

               201                                                 250
SEQ ID NO: 5 :GTTGAAGATG ACTGTTAGAG CTAACAAGCC CTTCAGATCG TATGATGATG
SEQ ID NO: 6 :GTTGAAGATG ACTGTTAGAG CTAACAAGCC CTTCAGATCG TATGATGATG
SEQ ID NO: 7 :GTTGAAGATG ACTGTTAGAG CTAACAAGCC CTTCAGATCG TATGATGATG

               251                                                 300
SEQ ID NO: 5 :TCACCGCAGC GGTATCACAA TGGGATAATT CATACATTGG AATGGTTGGA
SEQ ID NO: 6 :TCACCGCAGC GGTATCACAA TGGGATAATT CATACATTGG AATGGTTGGA
SEQ ID NO: 7 :TCACCGCAGC GGTATCACAA TGGGATAATT CATACATTGG AATGGTTGGA

               301                                                 350
SEQ ID NO: 5 :AAGCGTCCTT TCTACAAGAT AATTGCTCTG ATTGGCTCCA GTCATCTGCA
SEQ ID NO: 6 :AAGCGTCCTT TCTACAAGAT AATTGCTCTG ATTGGCTCCA GTCATCTGCA
SEQ ID NO: 7 :AAGCGTCCTT TCTACAAGAT AATTGCTGCA ATTGGCTCCA GTCATCTGCA

               351                                                 400
SEQ ID NO: 5 :AGCAACTCCA GCTGTGTTGG CAGACTTAAA TCAACCAGAG TATTATGCCA
SEQ ID NO: 6 :AGCAACTCCA GCTGTGTTGG CAGACTTAAA TCAACCAGAG TATTATGCCA
SEQ ID NO: 7 :AGCAACTCCA GCTGTGTTGG CAGACTTAAA TCAACCAGAG TATTATGCCA

               401                                                 450
SEQ ID NO: 5 :CACTAACAGG TCGTTGTTTT CTTCCTCACC GACTCGGATT GATCCCACCG
SEQ ID NO: 6 :CACTAACAGG TCGTTGTTTT CTTCCTCACC GACTCGGATT GATCCCACCG
SEQ ID NO: 7 :CACTAACAGG TCGTTGTTTT CTTCCTCACC GACTCGGATT GATCCCACCG
```

```
                451                                                 500
SEQ ID NO: 5 :ATGTTTAATG TGTCCGAAAC TTTCAGAAAA CCATTCAATA TTGGGATATA
SEQ ID NO: 6 :ATGTTTAATG TGTCCGAAAC TTTCAGAAAA CCATTCAATA TTGGGATATA
SEQ ID NO: 7 :ATGTTTAATG TGTCCGAAAC TTTCAGAAAA CCATTCAATA TTGGGATATA

                501                                                 550
SEQ ID NO: 5 :CAAAGGGACT CTCGACTTCA CCTTTACAGT TTCAGATGAT GAGTCTAATG
SEQ ID NO: 6 :CAAAGGGACT CTCGACTTCA CCTTTACAGT TTCAGATGAT GAGTCTAATG
SEQ ID NO: 7 :CAAAGGGACT CTCGACTTCA CCTTTACAGT TTCAGATGAT GAGTCTAATG

                551                                                 600
SEQ ID NO: 5 :AAAAAGTCCC TCATGTTTGG GAATACATGA ACCCAAAATA TCAATCTCAG
SEQ ID NO: 6 :AAAAAGTCCC TCATGTTTGG GAATACATGA ACCCAAAATA TCAATCTCAG
SEQ ID NO: 7 :AAAAAGTCCC TCATGTTTGG GAATACATGA ACCCAAAATA TCAATCTCAG

                601                                                 650
SEQ ID NO: 5 :ATCCAAAAAG AAGGGCTTAA ATTCGGATTG ATTTTAAGCA AGAAAGCAAC
SEQ ID NO: 6 :ATCCAAAAAG AAGGGCTTAA ATTCGGATTG ATTTTAAGCA AGAAAGCAAC
SEQ ID NO: 7 :ATCCAAAAAG AAGGGCTTAA ATTCGGATTG ATTTTAAGCA AGAAAGCAAC

                651                                                 700
SEQ ID NO: 5 :GGGAACTTGG GTGTTAGACC AATTGAGTCC GTTTAA
SEQ ID NO: 6 :GGGAACTTGG GTGTTAGACC AATTGAGTCC GTTTAA
SEQ ID NO: 7 :GGGAACTTGG GTGTTAGACC AATTGAGTCC GTTTAA
```

Table 5. Amino Acid Sequence Comparison between M Proteins of VSV New Jersey serotype Wild Type (SEQ ID NO: 8) and Mutants, G22E/M48R/M51R (SEQ ID NO: 9) and G22E/L110A/M48R/M51R (SEQ ID NO: 10)

```
                1                       22                        48 50
SEQ ID NO: 8 :MSSFKKILGF SSKSHKKSKK LGLPPPYEES SPMEIQPSAP LSNDFFGMED
SEQ ID NO: 9 :MSSFKKILGF SSKSHKKSKK LELPPPYEES SPMEIQPSAP LSNDFFGRED
SEQ ID NO: 10:MSSFKKILGF SSKSHKKSKK LELPPPYEES SPMEIQPSAP LSNDFFGRED

                51                                                  100
SEQ ID NO: 8 :MDLYDKDSLR YEKFRFMLKM TVRANKPFRS YDDVTAAVSQ WDNSYIGMVG
SEQ ID NO: 9 :RDLYDKDSLR YEKFRFMLKM TVRANKPFRS YDDVTAAVSQ WDNSYIGMVG
SEQ ID NO: 10:RDLYDKDSLR YEKFRFMLKM TVRANKPFRS YDDVTAAVSQ WDNSYIGMVG

                101     110                                         150
SEQ ID NO: 8 :KRPFYKIIAL IGSSHLQATP AVLADLNQPE YYATLTGRCF LPHRLGLIPP
SEQ ID NO: 9 :KRPFYKIIAL IGSSHLQATP AVLADLNQPE YYATLTGRCF LPHRLGLIPP
SEQ ID NO: 10:KRPFYKIIAA IGSSHLQATP AVLADLNQPE YYATLTGRCF LPHRLGLIPP

                151                                                 200
SEQ ID NO: 8 :MFNVSETFRK PFNIGIYKGT LDFTFTVSDD ESNEKVPHVW EYMNPKYQSQ
```

SEQ ID NO: 9 :MFNVSETFRK PFNIGIYKGT LDFTFTVSDD ESNEKVPHVW EYMNPKYQSQ
SEQ ID NO: 10:MFNVSETFRK PFNIGIYKGT LDFTFTVSDD ESNEKVPHVW EYMNPKYQSQ

201 250
SEQ ID NO: 8 :IQKEGLKFGL ILSKKATGTW VLDQLSPFK
SEQ ID NO: 9 :IQKEGLKFGL ILSKKATGTW VLDQLSPFK
SEQ ID NO: 10:IQKEGLKFGL ILSKKATGTW VLDQLSPFK

Table 6. Vaccination studies in mice for broad range CD8+ T cell responses and humoral immune responses against HIV-1 proteins

Vaccination Groups	rVSV w/ HIV-1 Proteins	
	Prime	**Boost**
G 1	$rVSV_{Ind}$(GLM)	$rVSV_{NJ}$(GM)
G 2	$rVSV_{Ind}$(GLM)-HIV-1 Gag-En	$rVSV_{NJ}$(GM)-HIV-1 Gag-En
G 3	$rVSV_{Ind}$(GLM)-HIV-1 Gp160mss	$rVSV_{NJ}$(GM)-HIV-1 Gp160mss
G 4	$rVSV_{Ind}$(GLM)-HIV-1 Pol	$rVSV_{NJ}$(GM)-HIV-1 Pol
G 5	$rVSV_{Ind}$(GLM)-HIV-1 Gag-En + $rVSV_{Ind}$(GLM)-Gp160mss + $rVSV_{Ind}$(GLM)-HIV-1 Pol	$rVSV_{NJ}$(GM)-HIV-1 Gag-En + $rVSV_{NJ}$(GM)-Gp160mss + $rVSV_{NJ}$(GM)-HIV1 Pol
G 6	$rVSV_{Ind}$(GLM)-HIV-1 Gag-A+ $rVSV_{Ind}$(GLM)-HIV-1 Gag-B+ $rVSV_{Ind}$(GLM)-HIV-1 Gag-C	$rVSV_{NJ}$(GM)-HIV-1 Gag-A+ $rVSV_{NJ}$(GM)-HIV-1 Gag-B+ $rVSV_{NJ}$(GM)-HIV-1 Gag-C

CLAIMS

What is claimed is:

1. A modified matrix (M) protein of a non-cytolytic and avirulent vesicular stomatitis virus (VSV), wherein the modified M protein comprises an amino acid sequence selected from: (i) SEQ ID NO: 3 including at least the following substitutions: G21E/L111A/M51R; and (ii) SEQ ID NO: 8 including at least the following substitutions: G22E/L110A/M48R/M51R.

2. The modified M protein of claim 1, wherein the modified M protein of (i) comprises an amino acid sequence SEQ ID NO:4 and the modified M protein of (ii) comprises the amino acid sequence SEQ ID NO:10.

3. The modified M protein of claim 1, wherein the E at position 21 in (i) and the E at position 22 in (ii) are encoded by a gaa codon, and wherein the R at position 51 in (i) and (ii) and the R at position 48 in (ii) is encoded by a cga codon.

4. The modified M protein of claim 1, wherein the A at position 111 in (i) and the A at position 110 in (ii) is encoded by a gca codon.

5. The modified M protein of claim 1, wherein the amino acid sequence of (i) is encoded by a gene comprising SEQ ID NO:2, and the amino acid sequence of (ii) is encoded by a gene comprising SEQ ID NO:7.

6. A nucleotide molecule that encodes for a modified matrix protein of a vesicular stomatitis virus, wherein the nucleotide molecule comprises a sequence selected from: SEQ ID NO:2, SEQ ID NO:6 and SEQ ID NO:7.

7. A non-cytolytic and avirulent recombinant vesicular stomatitis virus (rVSV), wherein said rVSV includes a nucleotide molecule that encodes for a modified matrix (M) protein, the nucleotide molecule comprising a sequence selected from SEQ ID NO:2, SEQ ID NO:6 and SEQ ID NO:7.

8. A non-cytolytic and avirulent recombinant vesicular stomatitis virus (rVSV), wherein said non-cytolytic and avirulent rVSV comprises a modified matrix (M) protein, wherein (i) the rVSV is of Indiana serotype ($rVSV_{Ind}$) and the modified M protein comprises SEQ ID NO: 3 including at least the following substitutions: G21E/L111A/M51R; or (ii) the rVSV is of New jersey serotype ($rVSV_{NJ}$) and the modified M protein comprises SEQ ID NO: 8 including at least the following substitutions: G22E/L110A/M48R/M51R.

Date Reçue/Date Received 2021-06-02

9. The non-cytolytic and avirulent rVSV of claim 8, wherein the E at position 21 in (i) and the E at position 22 in (ii) are encoded by a gaa codon, and wherein the R at position 51 in (i) and (ii) and the R at position 48 in (ii) is encoded by a cga codon.

10. The non-cytolytic and avirulent rVSV of claim 8, wherein the A at position 111 in (i) and the A at position 110 in (ii) are encoded by a gca codon.

11. The non-cytolytic and avirulent rVSV of claim 8, wherein said rVSV is the $rVSV_{Ind}$, and wherein the modified M protein comprises the amino acid sequence of SEQ ID NO: 4.

12. The non-cytolytic and avirulent rVSV of claim 11, wherein the modified M protein is encoded by a gene comprising a nucleotide sequence of SEQ ID NO: 2.

13. The non-cytolytic and avirulent rVSV according to any one of claims 11 to 12, wherein the $rVSV_{Ind}$ produces VSV_{Ind} particles at permissive temperatures of about 31°C.

14. The non-cytolytic and avirulent rVSV of claim 8, wherein said rVSV is the $rVSV_{NJ}$ and wherein the modified M protein comprises the amino acid sequence of SEQ ID NO: 10.

15. The rVSV of claim 14, wherein the modified M protein is encoded by a gene comprising a nucleotide sequence of SEQ ID NO: 7

16. The non-cytolytic and avirulent rVSV of claims 8 to 15, wherein the rVSV is a non-cytolytic and avirulent chimeric rVSV that expresses a protein of a foreign pathogen.

17. The non-cytolytic and avirulent rVSV of claim 16, wherein said foreign pathogen is a viral, fungal, bacterial or parasitic pathogen.

18. A vaccine, the vaccine including a modified matrix (M) protein of a vesicular stomatitis virus (VSV) wherein the modified M protein is encoded by a nucleotide molecule comprising a sequence selected from: SEQ ID NO:2, SEQ ID NO:6 and SEQ ID NO:7.

19. A vaccine, wherein the vaccine comprises an effective amount of one or more of the non-cytolytic and avirulent recombinant vesicular stomatitis viruses (rVSVs) of claims 7 to 18.

20. The vaccine of claim 19, wherein said vaccine induces a humoral, cellular and mucosal immune response.

21. The vaccine of claim 18 or 19, wherein said vaccine further includes an adjuvant.

22. A prime boost combination vaccine, wherein the prime boost combination vaccine comprises: (a) an effective amount of a vaccine comprising a non-cytolytic and avirulent recombinant vesicular stomatitis virus (rVSV) of one serotype having a first modified matrix (M)

Date Reçue/Date Received 2021-06-02

protein comprising the amino acid sequence of SEQ ID NO:4; and (b) an effective amount of a vaccine comprising a non-cytolytic and avirulent rVSV of another serotype having a second modified M protein comprising the amino acid sequence of SEQ ID NO:9 or SEQ ID NO:10.

23. The prime boost combination vaccine of claim 22, wherein SEQ ID NO:4 is encoded by a gene comprising SEQ ID NO:2.

24. The prime boost combination vaccine of claim 22, wherein SEQ ID NO:9 is encoded by a gene comprising SEQ ID NO:6, and SEQ ID NO:10 is encoded by a gene comprising SEQ ID NO:7.

25. The prime boost combination vaccine of claim 22, wherein (a) is a priming vaccine and (b) is a booster vaccine.

26. The prime boost combination vaccine of claim 22, wherein (b) is a priming vaccine and (a) is a booster vaccine.

27. The prime boost combination vaccine of claim 22, wherein the two attenuated rVSVs are a non-cytolytic and avirulent chimeric rVSVs that express a protein of a foreign pathogen, and wherein the two chimeric rVSVs induce an immune response to the protein.

28. The prime boost combination vaccine of claim 27, wherein the pathogen is a viral, a fungal, a bacterial or a parasitic pathogen.

29. The prime boost combination vaccine of claim 27, wherein the foreign pathogen is a lentivirus.

30. The prime boost combination vaccine of claim 29, wherein the lentivirus is a HIV and the protein is a HIV protein.

31. The prime boost combination vaccine of claim 30, wherein the rVSV of one serotype and the rVSV of the other serotype include a surface glycoprotein (G) gene and a RNA dependent RNA polymerase (L) gene, and wherein a gene for expressing the HIV protein is inserted in between the G gene and the L gene.

32. The prime boost combination vaccine of claim 31, wherein the HIV gene is selected from the group of HIV genes consisting of env, gag and pol.

33. The prime boost combination vaccine of claim 30, wherein the foreign pathogen is HCV and the epitope is a HCV protein.

Date Reçue/Date Received 2021-06-02

34. The prime boost combination vaccine of claim 33, wherein the rVSV of one serotype and the rVSV of the other serotype include a surface glycoprotein (G) gene and a RNA dependent RNA polymerase (L) gene, and wherein a gene for expressing the HCV protein is inserted in between the G gene and the L gene.

35. The prime boost combination vaccine of claim 34, wherein the HCV protein is a structural or a non-structural HCV protein.

36. The prime boost combination vaccine of any one of claims 27 to 35, wherein each one of the two vaccines comprises a mixture of the attenuated chimeric rVSVs, and wherein at least two of the attenuated chimeric rVSVs in the mixture have a different protein of the pathogen.

37. The prime boost combination vaccine of any one of claims 22 to 36, wherein each one of the two vaccines induces humoral, cellular and mucosal immune responses.

38. The prime boost combination vaccine of any one of claims 22 to 37, wherein the serotype of vaccine (a) is Indiana and the serotype of vaccine (b) is New Jersey.

39. The prime boost combination vaccine of any one of claims 22 to 38, wherein each one of vaccine (a) and vaccine (b) further comprises an adjuvant.

40. A kit comprising: (a) at least one dose of an effective amount of a vaccine comprising a recombinant vesicular stomatitis virus Indiana serotype ($rVSV_{Ind}$) having a modified matrix (M) protein comprising the amino acid sequence of SEQ ID NO:4, and (b) at least one dose of an effective amount of a vaccine comprising a recombinant vesicular stomatitis virus New Jersey serotype ($rVSV_{NJ}$) having a modified M protein comprising the amino acid sequence of SEQ ID NO:9 or the amino acid sequence of SEQ ID NO:10.

41. The kit of claim 40, wherein (a) and (b) are formulated in a pharmaceutically acceptable carrier.

42. The kit of claim 40, wherein SEQ ID NO:4 is encoded by a gene comprising SEQ ID NO:2.

43. The kit of claim 40, wherein SEQ ID NO:9 is encoded by a gene comprising SEQ ID NO:6 and SEQ ID NO:10 is encoded by a gene comprising SEQ ID NO:7.

44. An isolated peptide comprising an amino acid sequence selected from the group of amino acid sequences listed as SEQ ID NOs: 4 and 10.

45. An isolated nucleotide molecule comprising a nucleotide sequence selected from the group SEQ ID NO:2, SEQ ID NO:6 and SEQ ID NO:7.

Date Reçue/Date Received 2021-06-02

46. A use of the vaccine of claim 18 or 19 to induce an immune response in a subject.

47. A use of the prime boost combination vaccine of claim 22 to induce an immune response in a subject.

Date Reçue/Date Received 2021-06-02

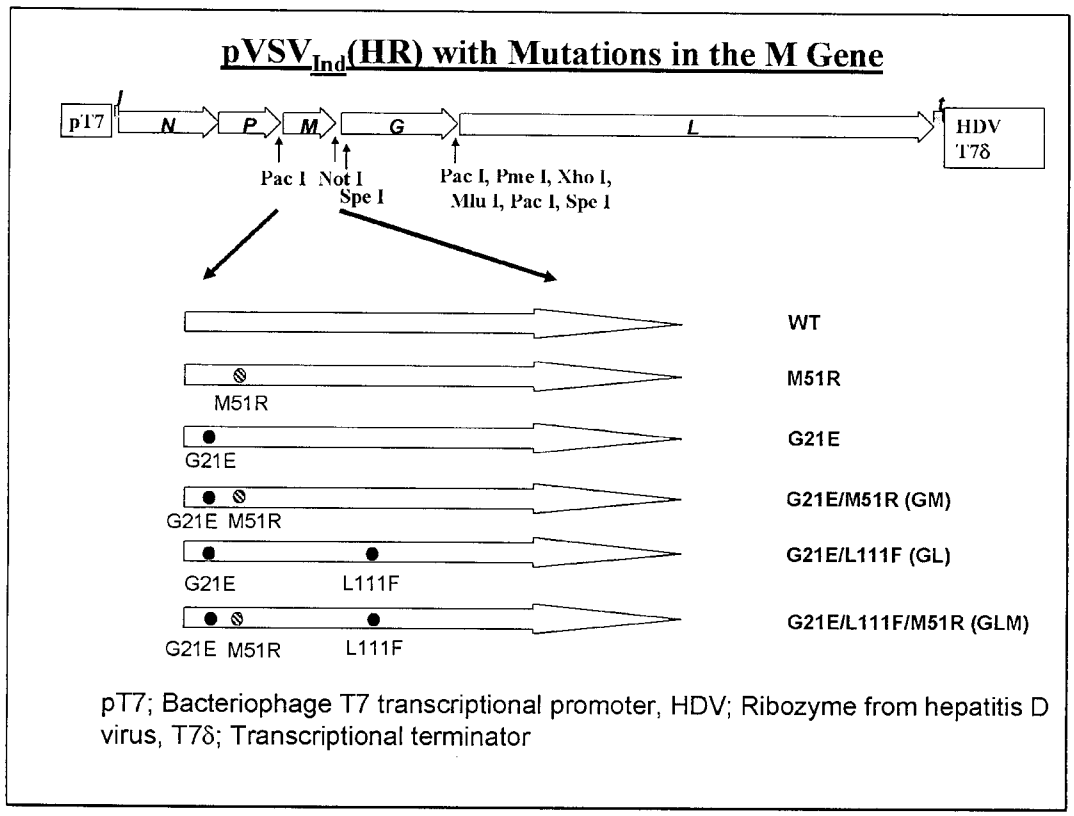

pVSV$_{Ind}$(HR) with Mutations in the M Gene
pT7
N
P
M
G
L
HDV
Tδ
Pac I
Not I
Spe I
Pac I, Pme I, Xho I,
Mlu I, Pac I, Spe I
WT
M51R
M51R
G21E
G21E
G21E M51R
G21E/M51R (GM)
G21E
L111F
G21E/L111F (GL)
G21E M51R
L111F
G21E/L111F/M51R (GLM)
pT7; Bacteriophage T7 transcriptional promoter, HDV; Ribozyme from hepatitis D virus, Tδ; Transcriptional terminator

FIGURE 1

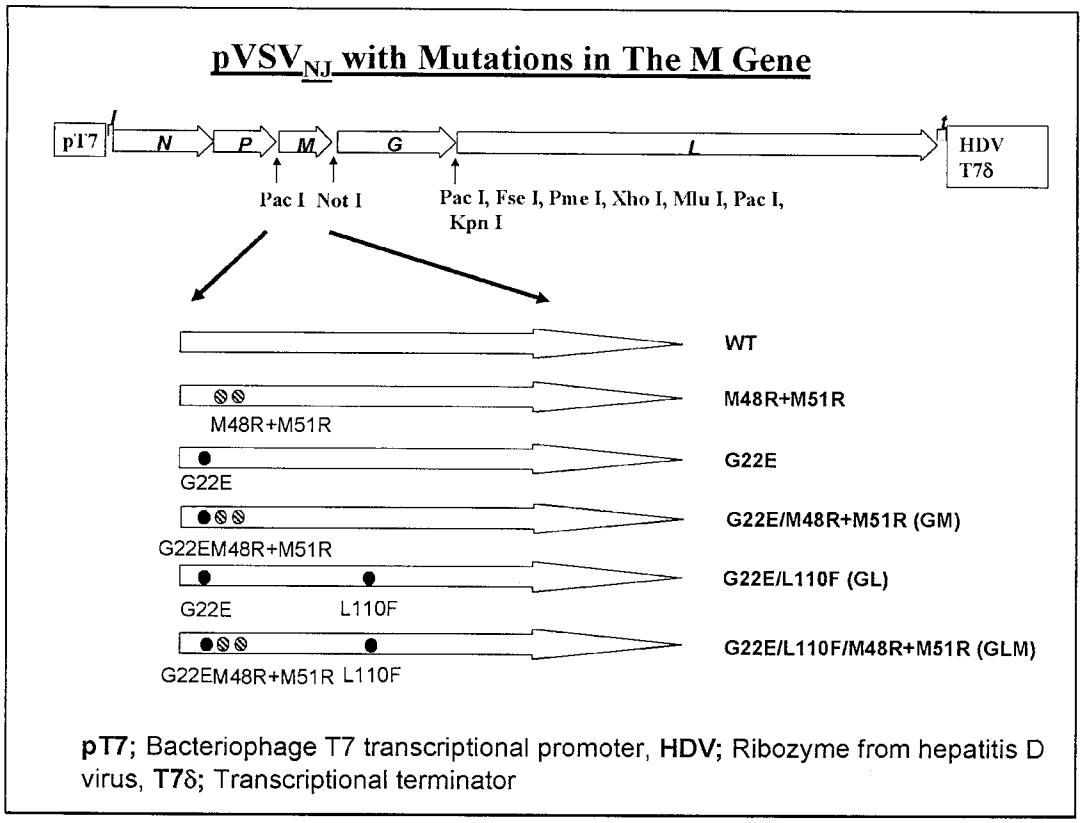
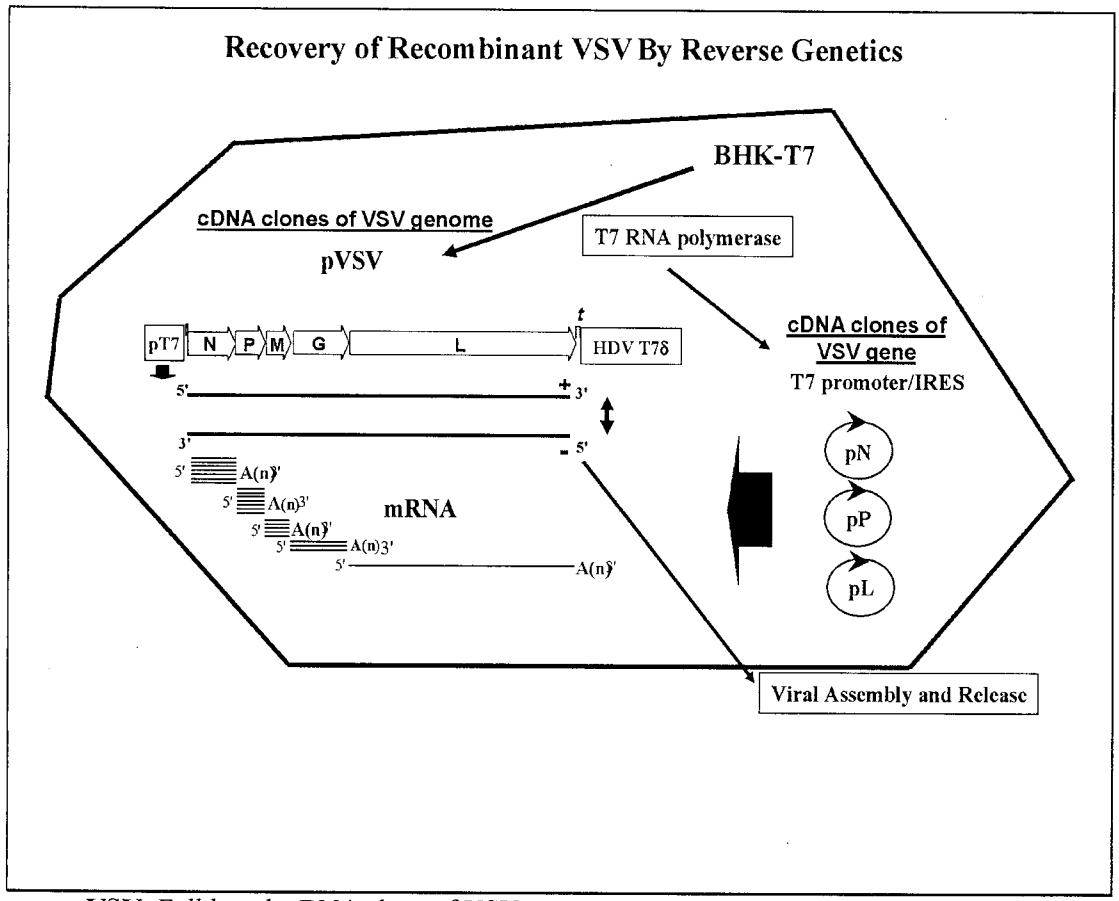

pVSV$_{NJ}$ with Mutations in The M Gene
pT7
N
P
M
G
L
HDV
T7δ
Pac I Not I
Pac I, Fse I, Pme I, Xho I, Mlu I, Pac I, Kpn I
WT
M48R+M51R
M48R+M51R
G22E
G22E
G22E/M48R+M51R (GM)
G22EM48R+M51R
G22E/L110F (GL)
G22E
L110F
G22E/L110F/M48R+M51R (GLM)
G22EM48R+M51R L110F
pT7; Bacteriophage T7 transcriptional promoter, HDV; Ribozyme from hepatitis D virus, T7δ; Transcriptional terminator

FIGURE 2



pVSV; Full length cDNA clone of VSV genome, **BHK-T7;** BHK21 cells constitutive expressing T7 DNA dependent RNA polymerase,

pN; A plasmid expressing VSV nucleocapsid protein, **pP;** A plasmid expressing VSV phosphoprotein,

pL; A plasmid expressing VSV large protein, **IRES;** Internal ribosomal entry site

FIGURE 3

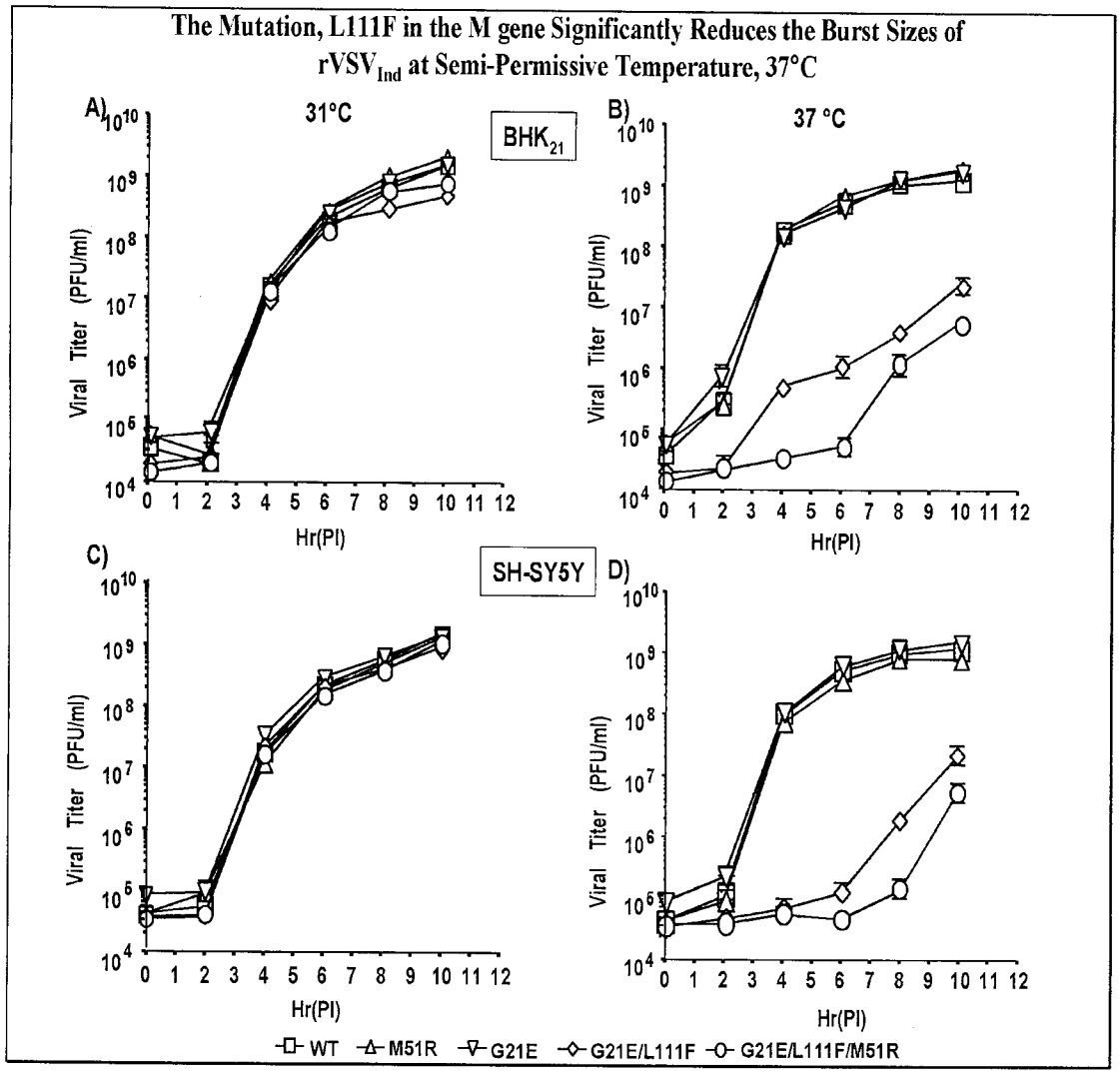
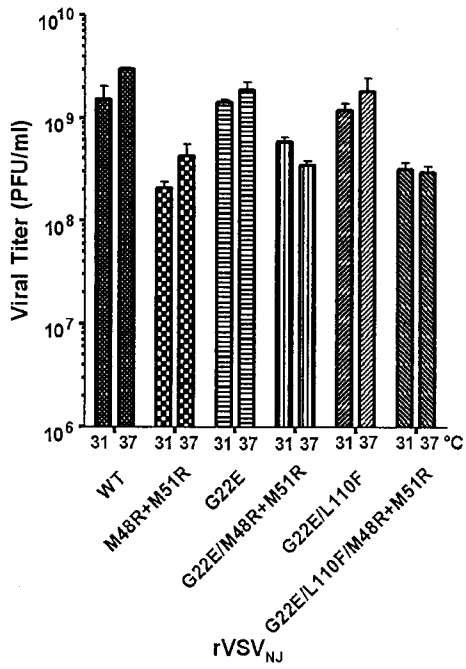

The Mutation, L111F in the M gene Significantly Reduces the Burst Sizes of rVSV$_{Ind}$ at Semi-Permissive Temperature, 37°C
A)
31°C
BHK$_{21}$
B)
37 °C
C)
SH-SY5Y
D)
Viral Titer (PFU/ml)
Hr(PI)
WT
M51R
G21E
G21E/L111F
G21E/L111F/M51R

FIGURE 4

Mutations in the M Gene of $rVSV_{NJ}$, G22E and L110F Do Not Reduce the Burst Size of the $rVSV_{NJ}$ at 37 °C



$rVSV_{NJ}$	Burst Size (PFU/ml)	
	31°C	37°C
WT	$1.53X10^9$	$3.03X10^9$
M48R+M51R	$2.06X10^8$	$4.23X10^8$
G22E	$1.43X10^9$	$1.9X10^9$
G22E/M48R+M51R	$5.78X10^8$	$3.48X10^8$
G22E/L110F	$1.20X10^9$	$1.85X10^9$
G22E/L110F/M48R+ M51R	$3.15X10^8$	$2.98X10^8$

FIGURE 5

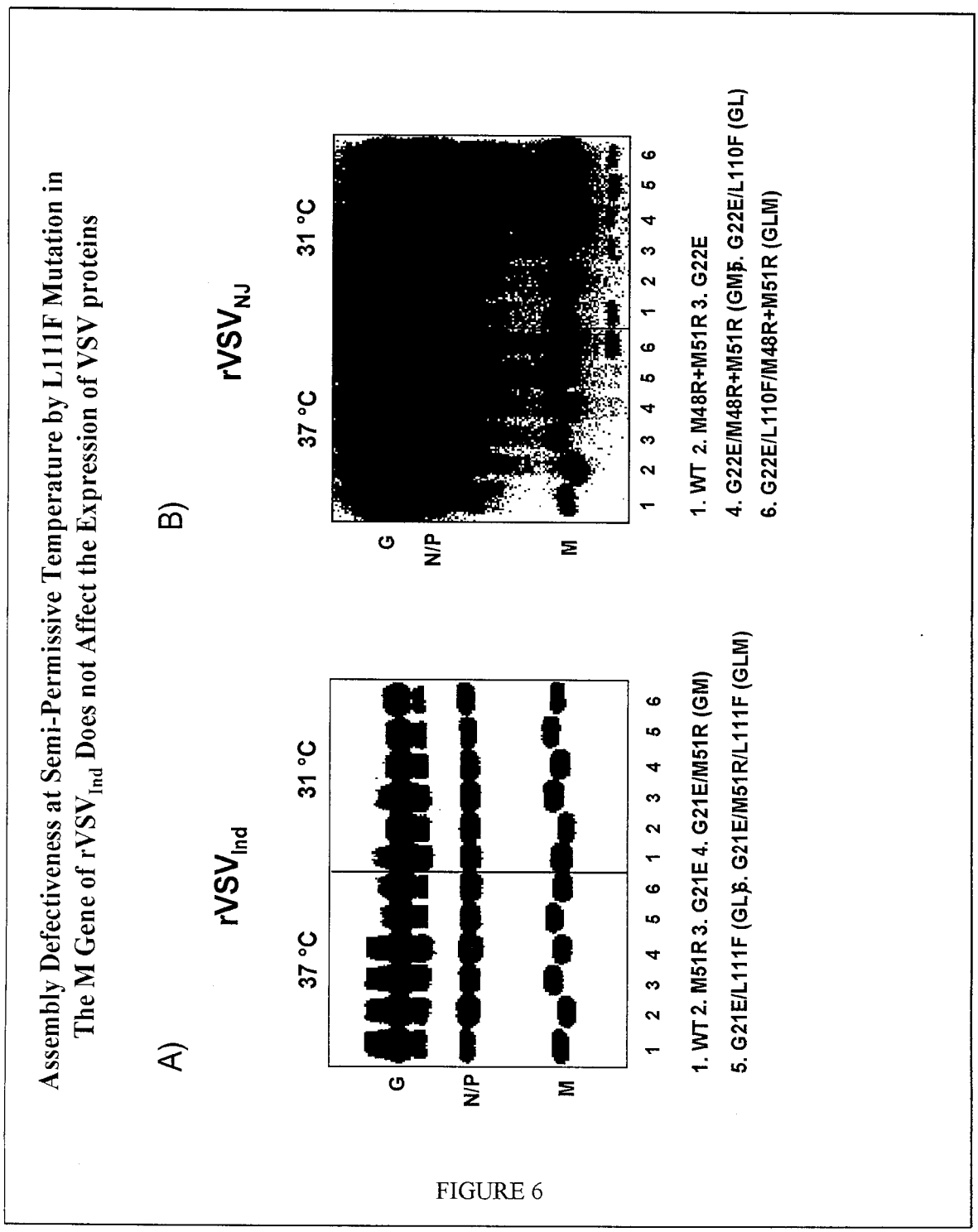

Assembly Defectiveness at Semi-Permissive Temperature by L111F Mutation in The M Gene of rVSV$_{Ind}$ Does not Affect the Expression of VSV proteins
A)
rVSV$_{Ind}$
37 °C
31 °C
G
N/P
M
1 2 3 4 5 6 1 2 3 4 5 6
1. WT 2. M51R 3. G21E 4. G21E/M51R (GM)
5. G21E/L111F (GLβ. G21E/M51R/L111F (GLM)
B)
rVSV$_{NJ}$
37 °C
31 °C
G
N/P
M
1 2 3 4 5 6 1 2 3 4 5 6
1. WT 2. M48R+M51R 3. G22E
4. G22E/M48R+M51R (GMβ. G22E/L110F (GL)
6. G22E/L110F/M48R+M51R (GLM)

FIGURE 6

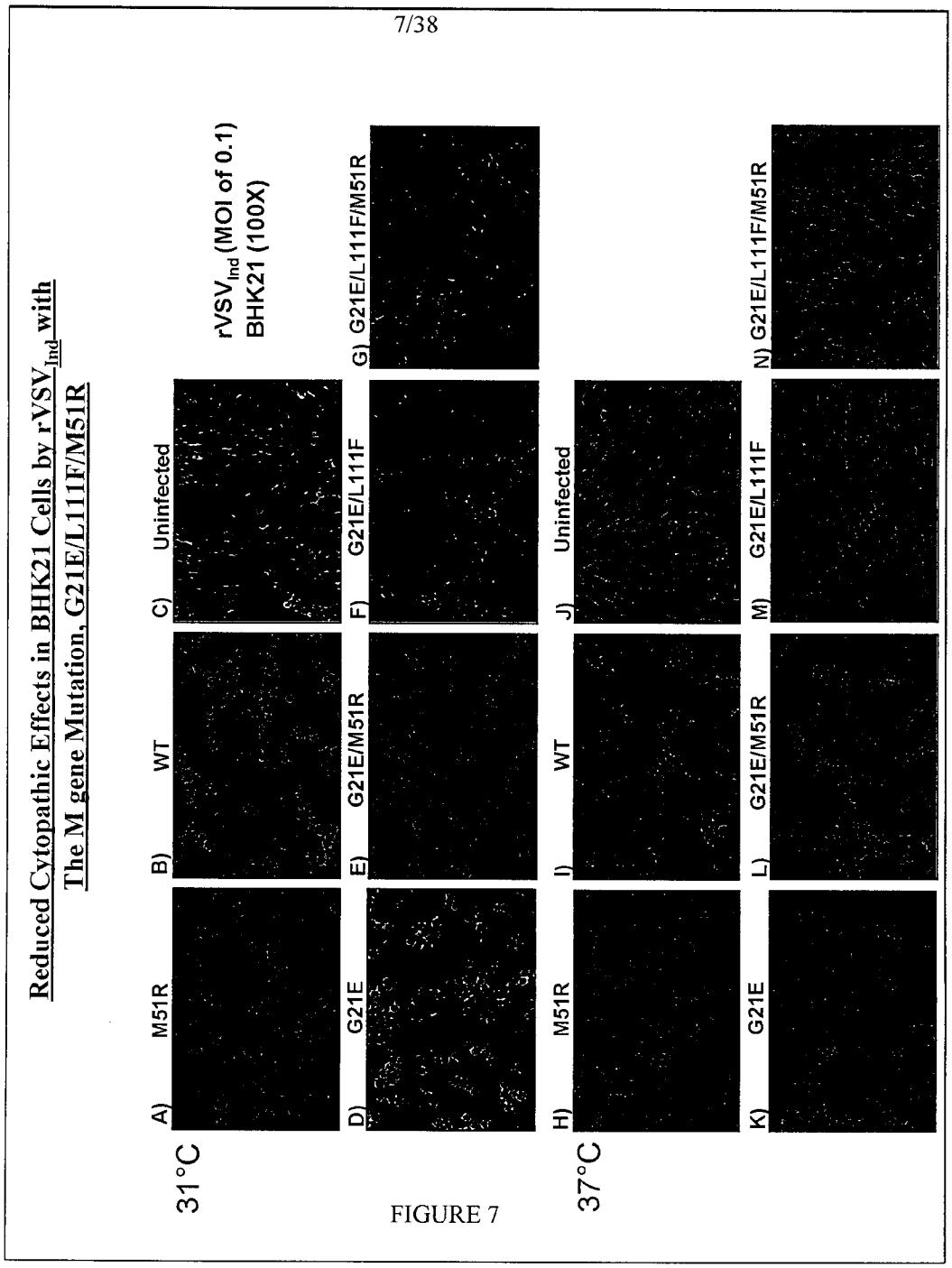

Reduced Cytopathic Effects in BHK21 Cells by rVSV$_{Ind}$ with The M gene Mutation, G21E/L111F/M51R
rVSV$_{Ind}$ (MOI of 0.1) BHK21 (100X)
31°C
37°C
A) M51R
B) WT
C) Uninfected
D) G21E
E) G21E/M51R
F) G21E/L111F
G) G21E/L111F/M51R
H) M51R
I) WT
J) Uninfected
K) G21E
L) G21E/M51R
M) G21E/L111F
N) G21E/L111F/M51R

FIGURE 7

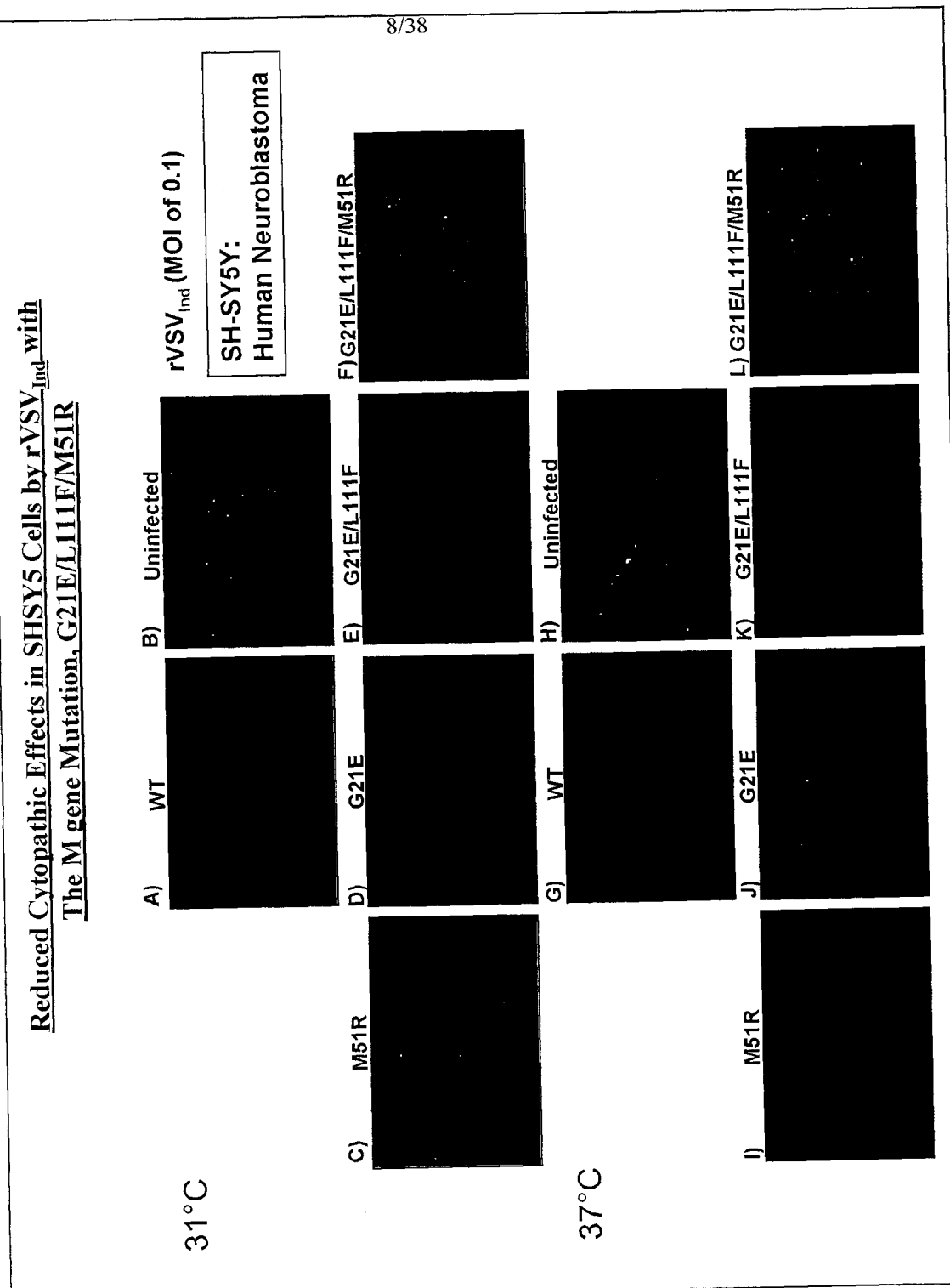

Reduced Cytopathic Effects in SHSY5 Cells by rVSV$_{Ind}$ with The M gene Mutation, G21E/L111F/M51R
rVSV$_{Ind}$ (MOI of 0.1)
SH-SY5Y: Human Neuroblastoma
A) WT
B) Uninfected
C) M51R
D) G21E
E) G21E/L111F
F) G21E/L111F/M51R
G) WT
H) Uninfected
I) M51R
J) G21E
K) G21E/L111F
L) G21E/L111F/M51R
31°C
37°C

FIGURE 8

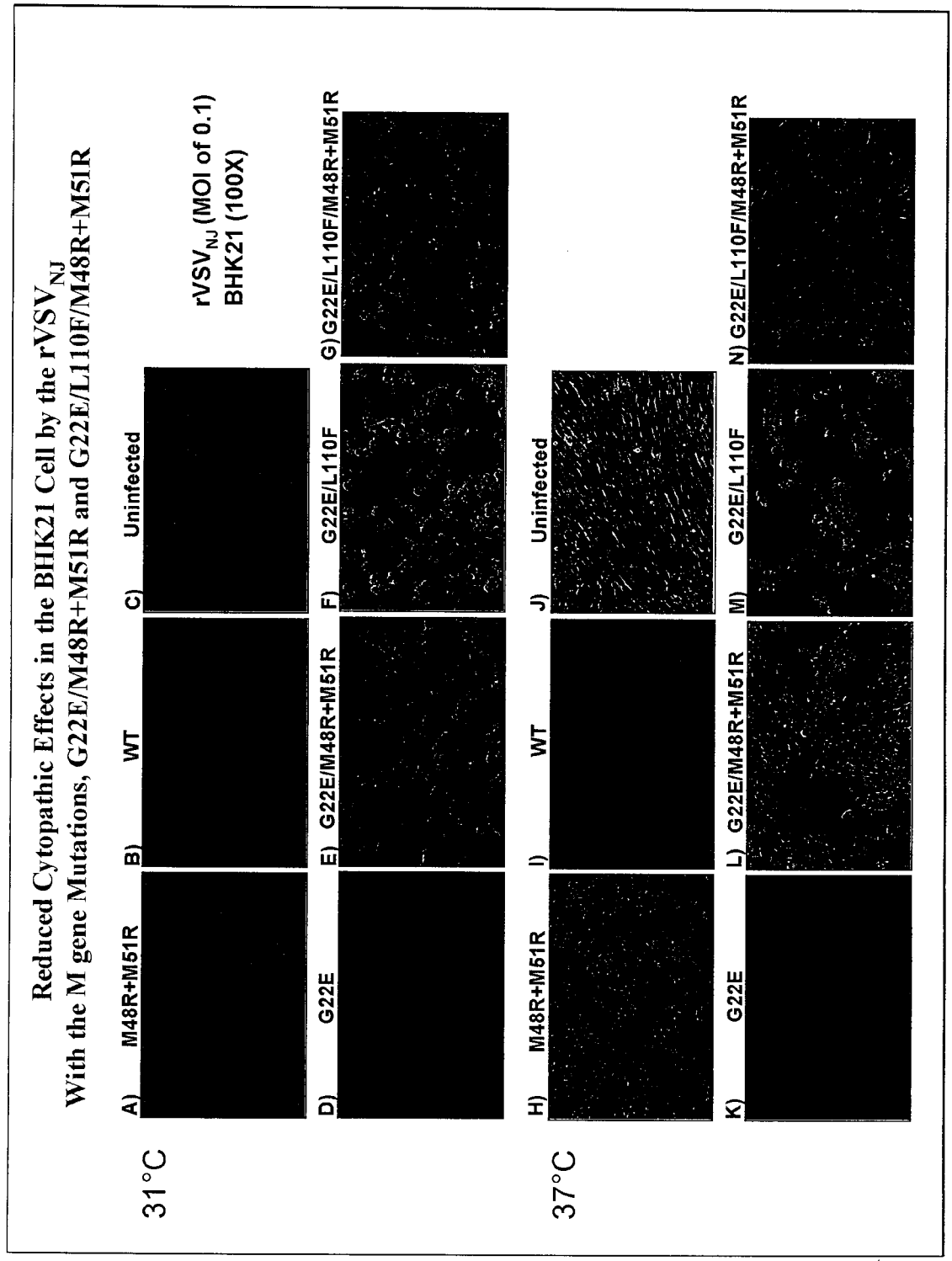

Reduced Cytopathic Effects in the BHK21 Cell by the rVSV$_{NJ}$
With the M gene Mutations, G22E/M48R+M51R and G22E/L110F/M48R+M51R
rVSV$_{NJ}$ (MOI of 0.1)
BHK21 (100X)
31°C
37°C
A) M48R+M51R
B) WT
C) Uninfected
D) G22E
E) G22E/M48R+M51R
F) G22E/L110F
G) G22E/L110F/M48R+M51R
H) M48R+M61R
I) WT
J) Uninfected
K) G22E
L) G22E/M48R+M51R
M) G22E/L110F
N) G22E/L110F/M48R+M51R

FIGURE 9

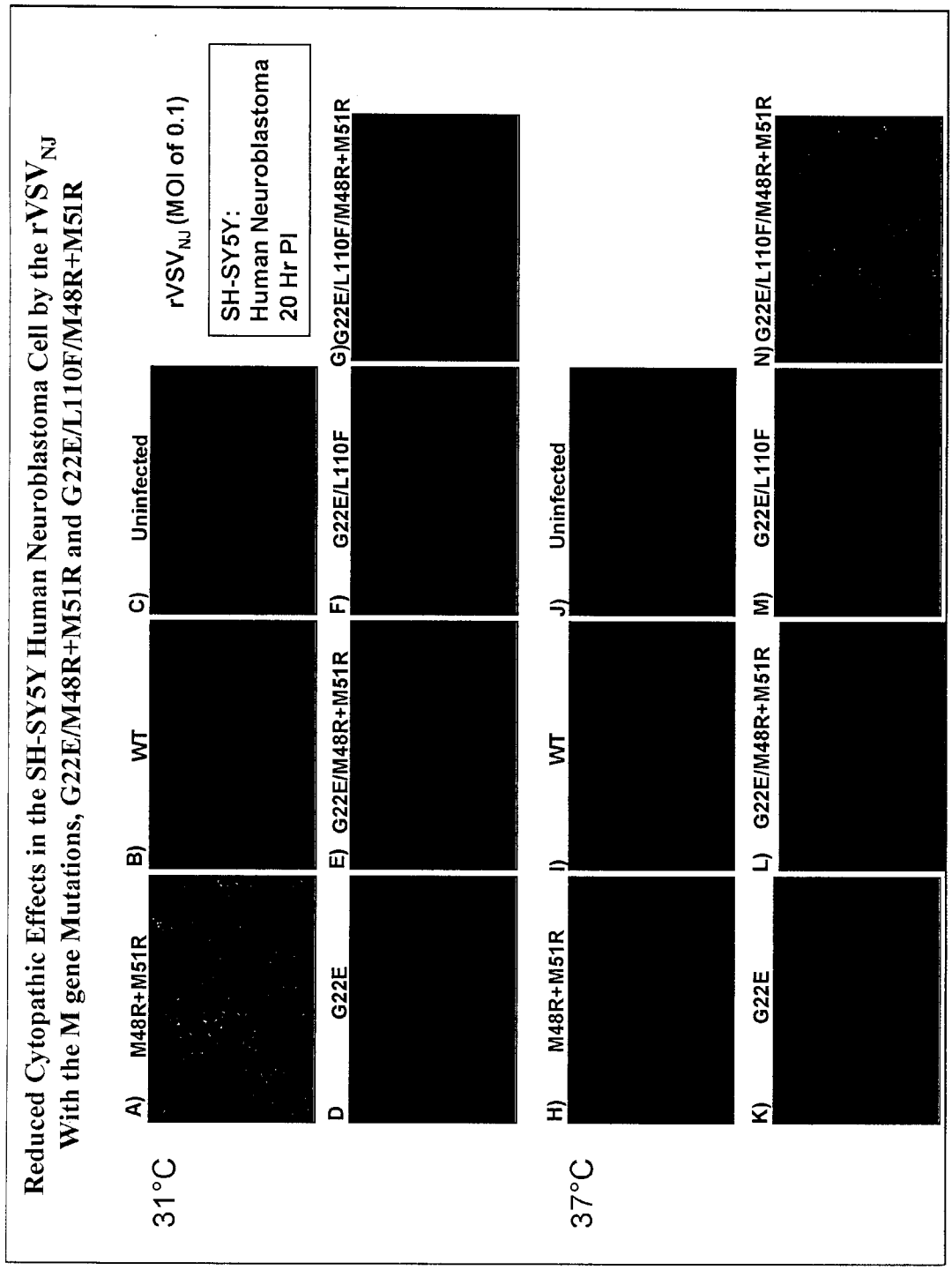

Reduced Cytopathic Effects in the SH-SY5Y Human Neuroblastoma Cell by the rVSV$_{NJ}$ With the M gene Mutations, G22E/M48R+M51R and G22E/L110F/M48R+M51R
rVSV$_{NJ}$ (MOI of 0.1)
SH-SY5Y: Human Neuroblastoma 20 Hr PI
31°C
37°C
A) M48R+M51R
B) WT
C) Uninfected
D G22E
E) G22E/M48R+M51R
F) G22E/L110F
G) G22E/L110F/M48R+M51R
H) M48R+M51R
I) WT
J) Uninfected
K) G22E
L) G22E/M48R+M51R
M) G22E/L110F
N) G22E/L110F/M48R+M51R

FIGURE 10

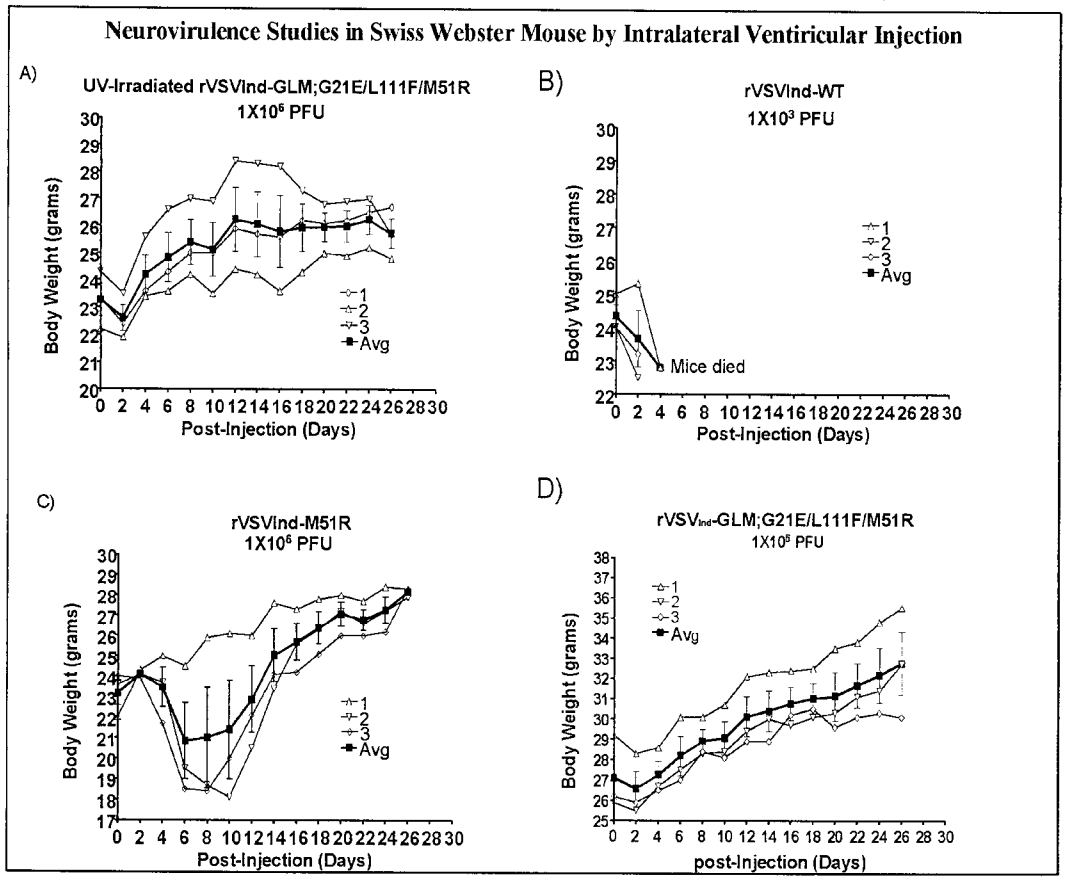

Neurovirulence Studies in Swiss Webster Mouse by Intralateral Ventiricular Injection
A)
UV-Irradiated rVSVInd-GLM;G21E/L111F/M51R
1X10⁶ PFU
Body Weight (grams)
Post-Injection (Days)
1
2
3
Avg
B)
rVSVInd-WT
1X10³ PFU
Mice died
C)
rVSVInd-M51R
1X10⁶ PFU
D)
rVSVInd-GLM;G21E/L111F/M51R
1X10⁵ PFU
post-Injection (Days)

FIGURE 11

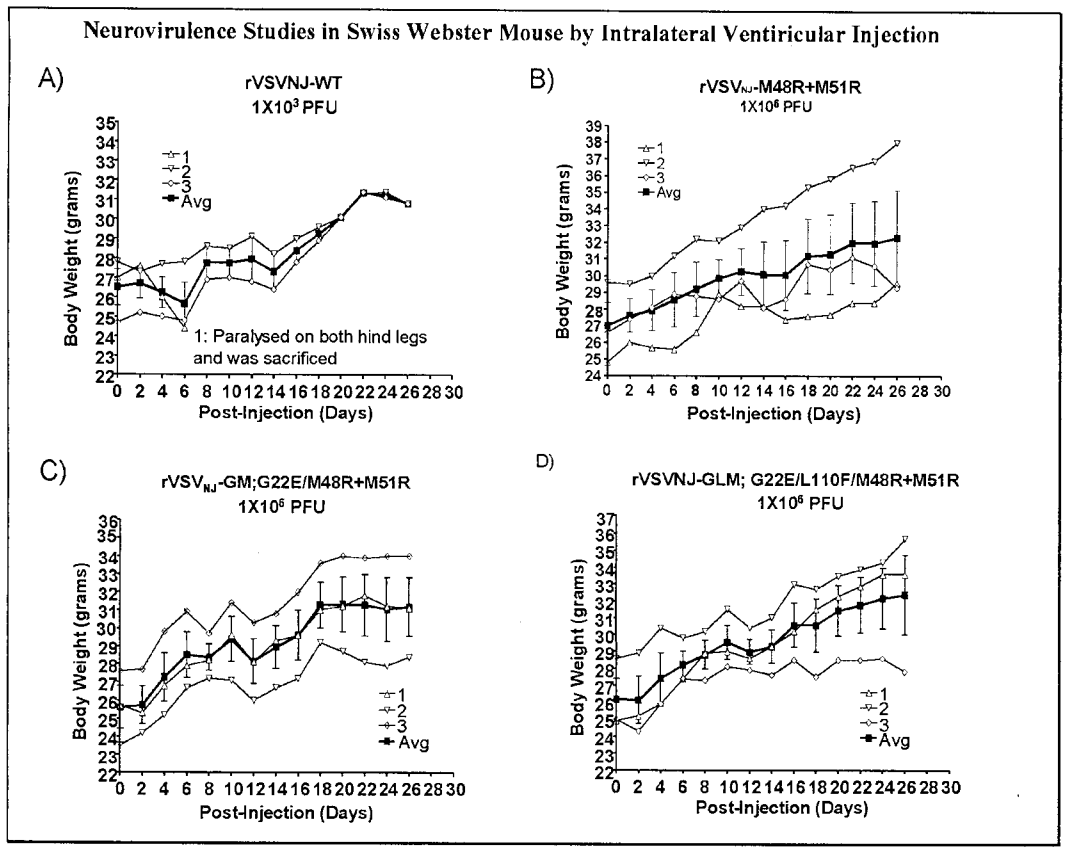

Neurovirulence Studies in Swiss Webster Mouse by Intralateral Ventiricular Injection
A)
rVSVNJ-WT
1X10³ PFU
Body Weight (grams)
Post-Injection (Days)
1
2
3
Avg
1: Paralysed on both hind legs and was sacrificed
B)
rVSVNJ-M48R+M51R
1X10⁶ PFU
Body Weight (grams)
Post-Injection (Days)
C)
rVSVNJ-GM;G22E/M48R+M51R
1X10⁶ PFU
Body Weight (grams)
Post-Injection (Days)
D)
rVSVNJ-GLM; G22E/L110F/M48R+M51R
1X10⁶ PFU
Body Weight (grams)
Post-Injection (Days)

FIGURE 12

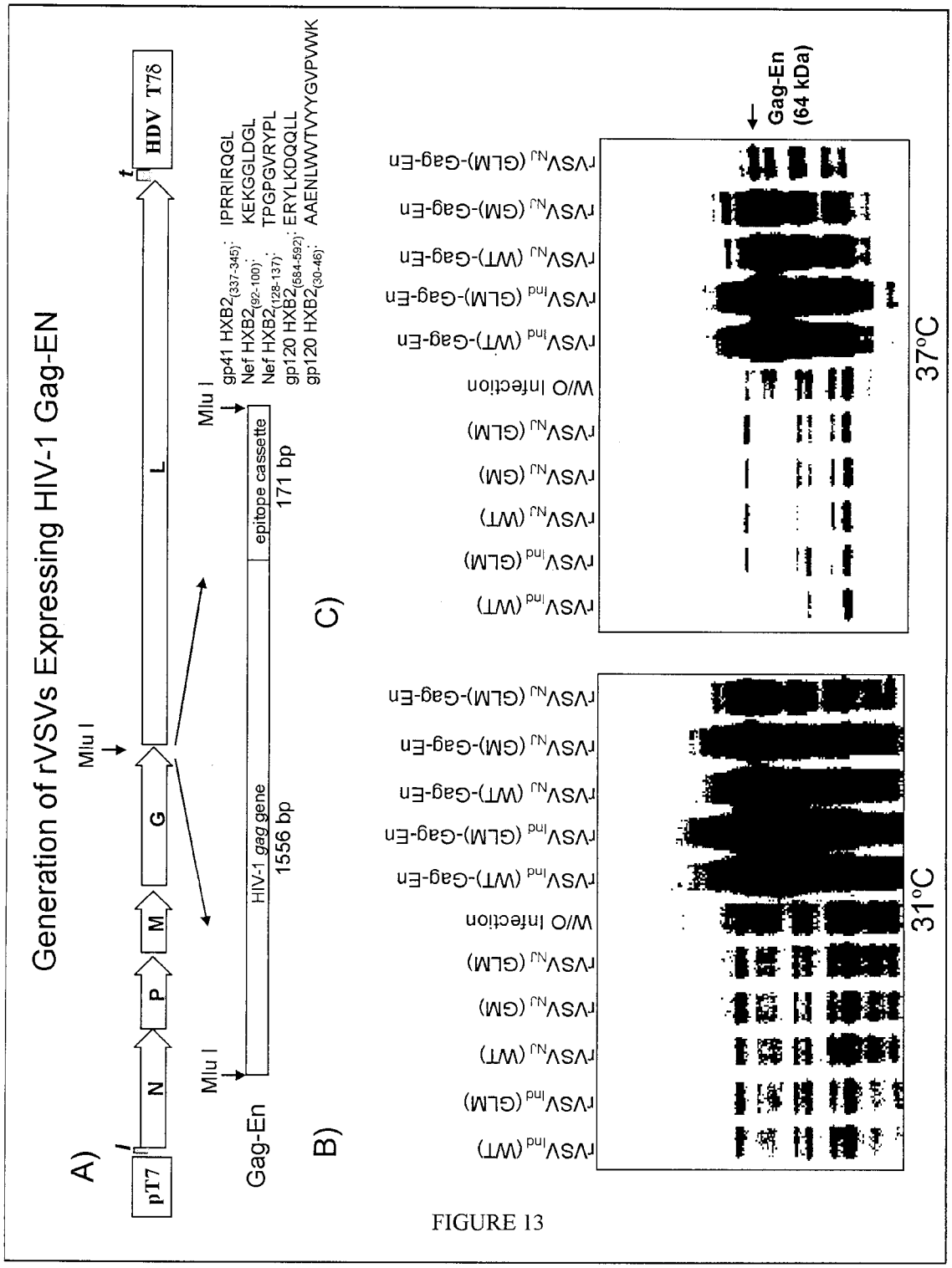
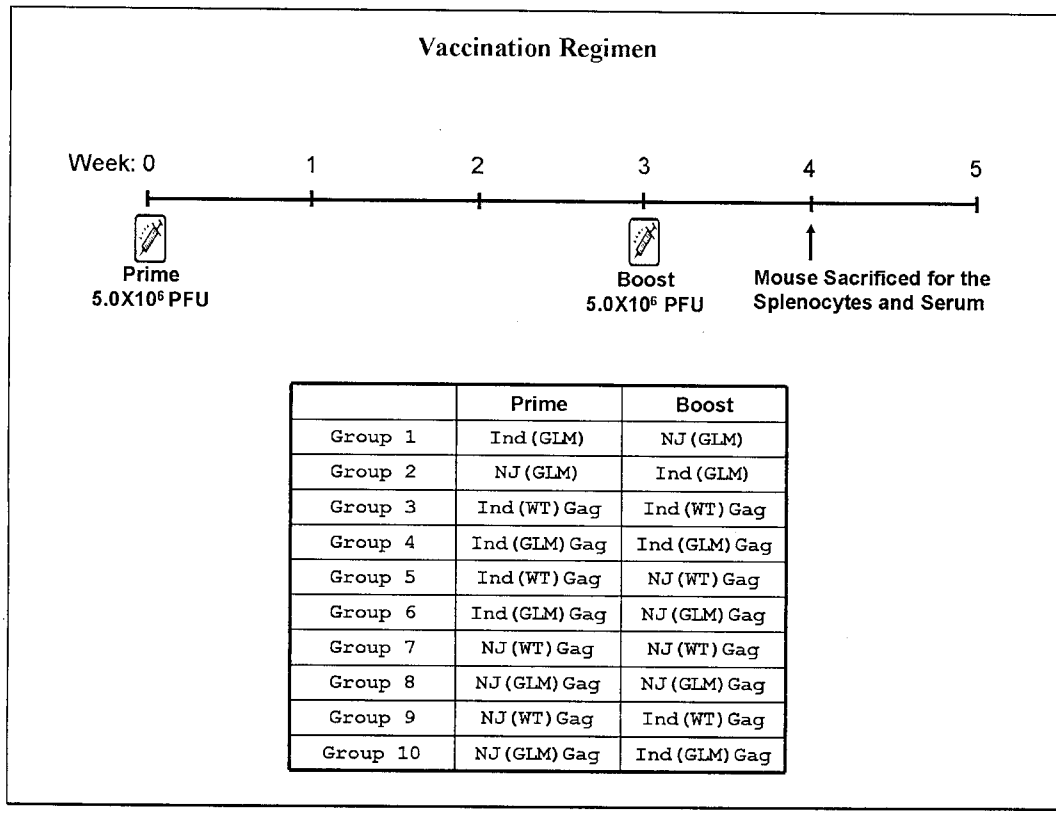

Generation of rVSVs Expressing HIV-1 Gag-EN
A)
pT7
N
P
M
G
L
HDV Tδ
Mlu I
Mlu I
Gag-En
HIV-1 gag gene
1556 bp
epitope cassette
171 bp
gp41 HXB2(337-345): IPRRIRQGL
Nef HXB2(92-100): KEKGGLDGL
Nef HXB2(128-137): TPGPGVRYPL
gp120 HXB2(684-592): ERYLKDQQLL
gp120 HXB2(30-46): AAENLWVTVYYGVPVWK
B)
rVSVInd (WT)
rVSVInd (GLM)
rVSVNJ (WT)
rVSVNJ (GM)
rVSVNJ (GLM)
W/O Infection
rVSVInd (WT)-Gag-En
rVSVInd (GLM)-Gag-En
rVSVNJ (WT)-Gag-En
rVSVNJ (GM)-Gag-En
rVSVNJ (GLM)-Gag-En
31°C
Gag-En (64 kDa)
37°C

FIGURE 13



	Prime	Boost
Group 1	Ind (GLM)	NJ (GLM)
Group 2	NJ (GLM)	Ind (GLM)
Group 3	Ind (WT) Gag	Ind (WT) Gag
Group 4	Ind (GLM) Gag	Ind (GLM) Gag
Group 5	Ind (WT) Gag	NJ (WT) Gag
Group 6	Ind (GLM) Gag	NJ (GLM) Gag
Group 7	NJ (WT) Gag	NJ (WT) Gag
Group 8	NJ (GLM) Gag	NJ (GLM) Gag
Group 9	NJ (WT) Gag	Ind (WT) Gag
Group 10	NJ (GLM) Gag	Ind (GLM) Gag

FIGURE 14

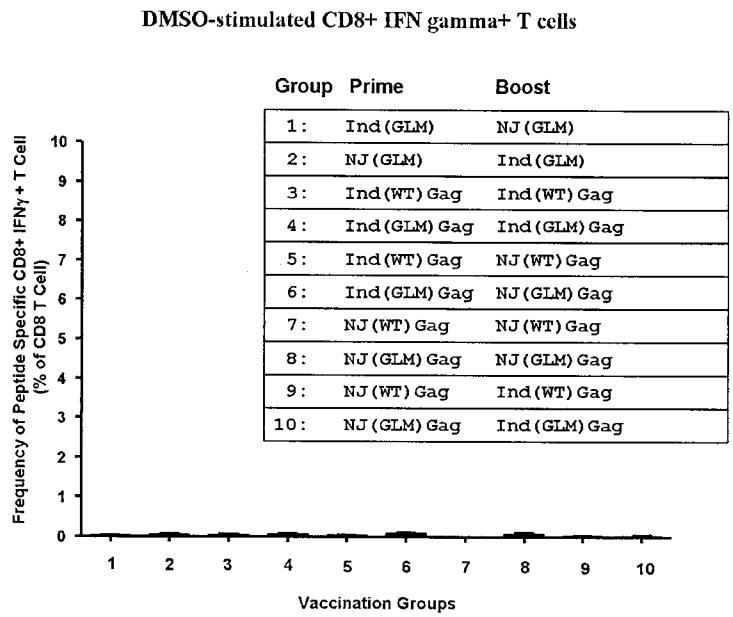
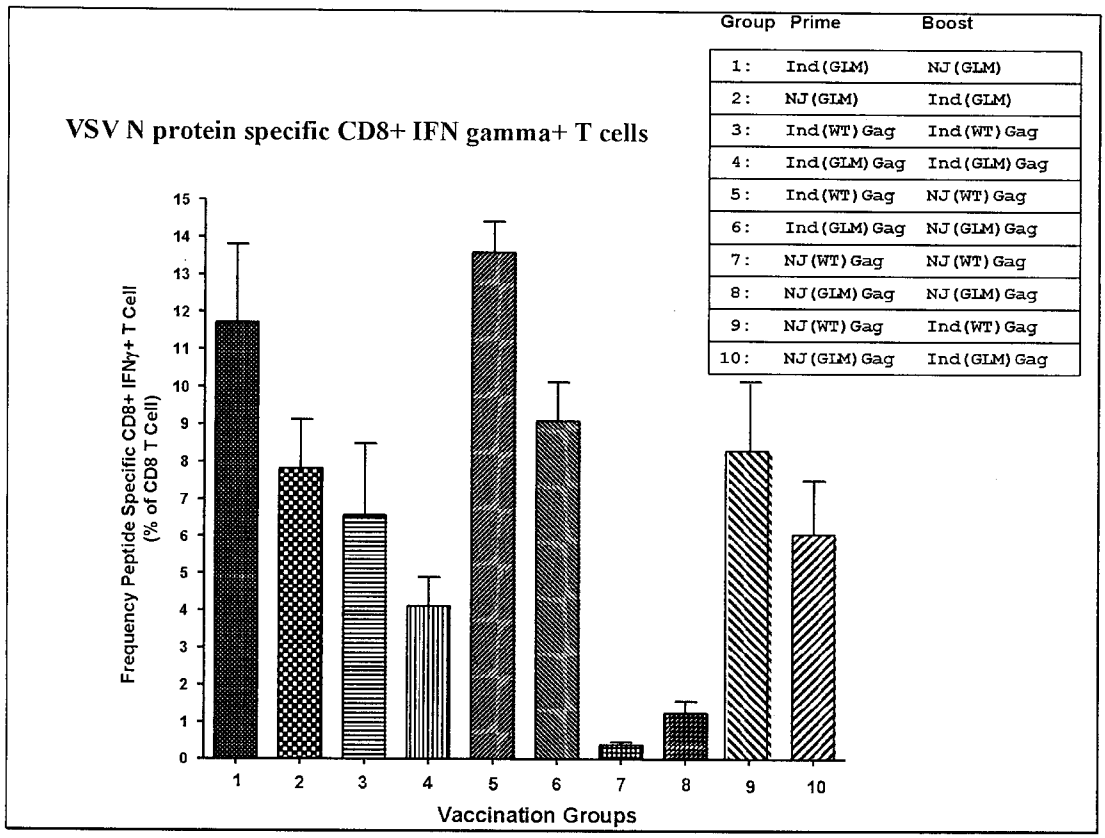

DMSO-stimulated CD8+ IFN gamma+ T cells
Group Prime Boost
1: Ind(GLM) NJ(GLM)
2: NJ(GLM) Ind(GLM)
3: Ind(WT) Gag Ind(WT) Gag
4: Ind(GLM) Gag Ind(GLM) Gag
5: Ind(WT) Gag NJ(WT) Gag
6: Ind(GLM) Gag NJ(GLM) Gag
7: NJ(WT) Gag NJ(WT) Gag
8: NJ(GLM) Gag NJ(GLM) Gag
9: NJ(WT) Gag Ind(WT) Gag
10: NJ(GLM) Gag Ind(GLM) Gag
Frequency of Peptide Specific CD8+ IFNγ + T Cell (% of CD8 T Cell)
0
1
2
3
4
5
6
7
8
9
10
1 2 3 4 5 6 7 8 9 10
Vaccination Groups

FIGURE 15



Group	Prime	Boost
1:	Ind(GLM)	NJ(GLM)
2:	NJ(GLM)	Ind(GLM)
3:	Ind(WT)Gag	Ind(WT)Gag
4:	Ind(GLM)Gag	Ind(GLM)Gag
5:	Ind(WT)Gag	NJ(WT)Gag
6:	Ind(GLM)Gag	NJ(GLM)Gag
7:	NJ(WT)Gag	NJ(WT)Gag
8:	NJ(GLM)Gag	NJ(GLM)Gag
9:	NJ(WT)Gag	Ind(WT)Gag
10:	NJ(GLM)Gag	Ind(GLM)Gag

FIGURE 16

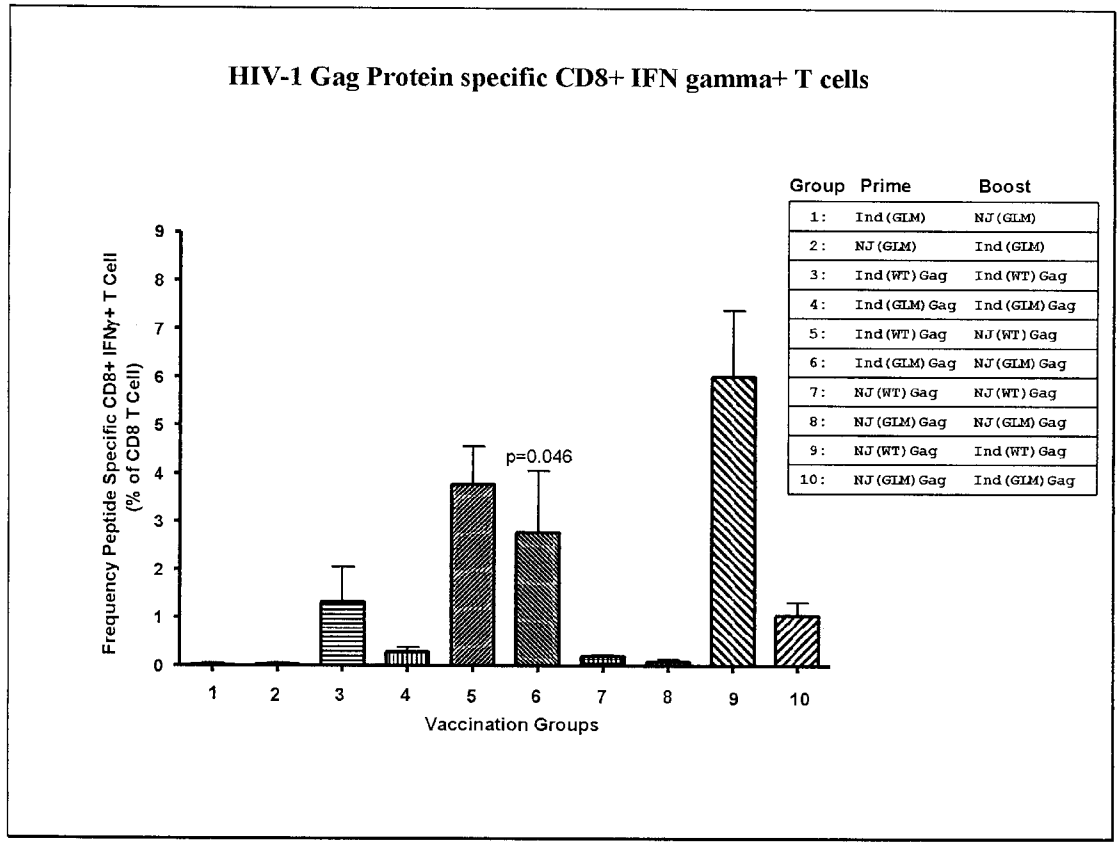

HIV-1 Gag Protein specific CD8+ IFN gamma+ T cells
Frequency Peptide Specific CD8+ IFNγ+ T Cell (% of CD8 T Cell)
0
1
2
3
4
5
6
7
8
9
p=0.046
1
2
3
4
5
6
7
8
9
10
Vaccination Groups
Group Prime Boost
1: Ind(GLM) NJ(GLM)
2: NJ(GLM) Ind(GLM)
3: Ind(WT)Gag Ind(WT)Gag
4: Ind(GLM)Gag Ind(GLM)Gag
5: Ind(WT)Gag NJ(WT)Gag
6: Ind(GLM)Gag NJ(GLM)Gag
7: NJ(WT)Gag NJ(WT)Gag
8: NJ(GLM)Gag NJ(GLM)Gag
9: NJ(WT)Gag Ind(WT)Gag
10: NJ(GLM)Gag Ind(GLM)Gag

FIGURE 17

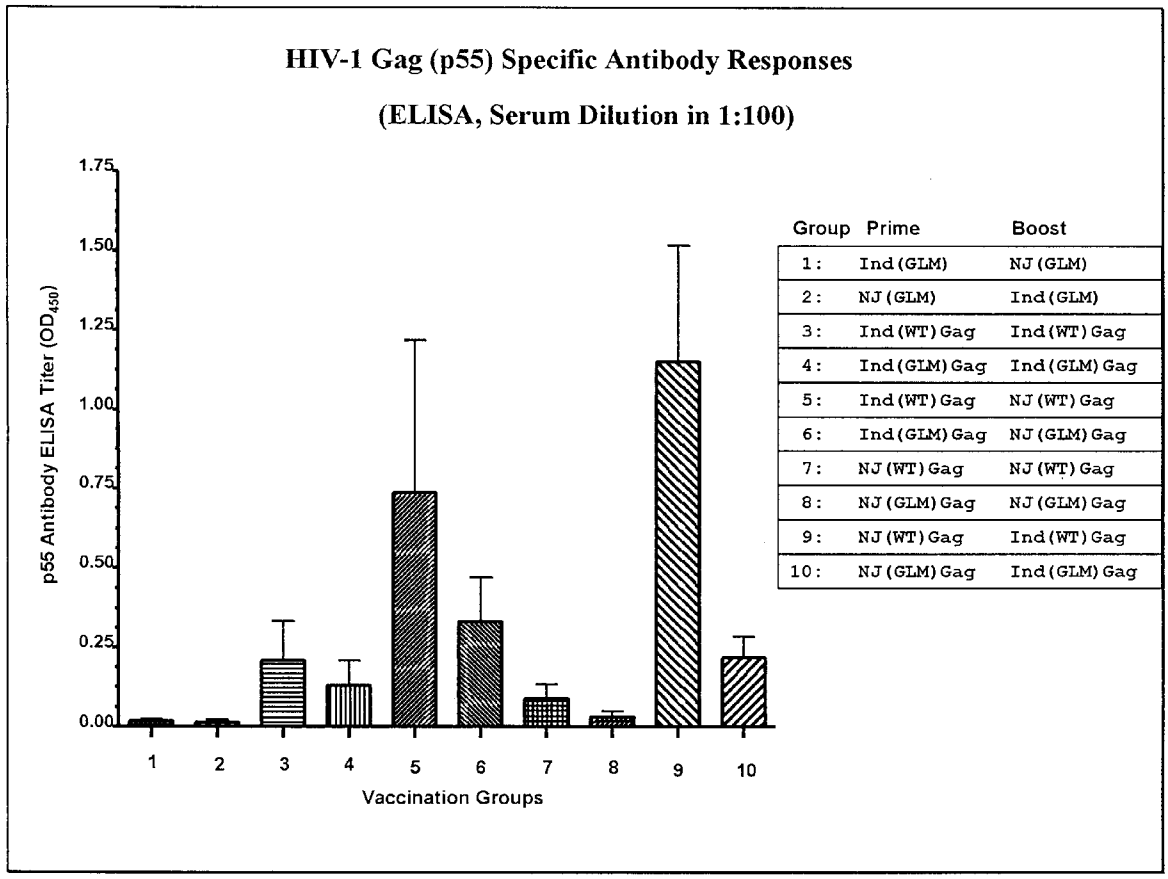

HIV-1 Gag (p55) Specific Antibody Responses
(ELISA, Serum Dilution in 1:100)
p55 Antibody ELISA Titer (OD_{450})
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1 2 3 4 5 6 7 8 9 10
Vaccination Groups
Group Prime Boost
1: Ind(GLM) NJ(GLM)
2: NJ(GLM) Ind(GLM)
3: Ind(WT)Gag Ind(WT)Gag
4: Ind(GLM)Gag Ind(GLM)Gag
5: Ind(WT)Gag NJ(WT)Gag
6: Ind(GLM)Gag NJ(GLM)Gag
7: NJ(WT)Gag NJ(WT)Gag
8: NJ(GLM)Gag NJ(GLM)Gag
9: NJ(WT)Gag Ind(WT)Gag
10: NJ(GLM)Gag Ind(GLM)Gag

FIGURE 18

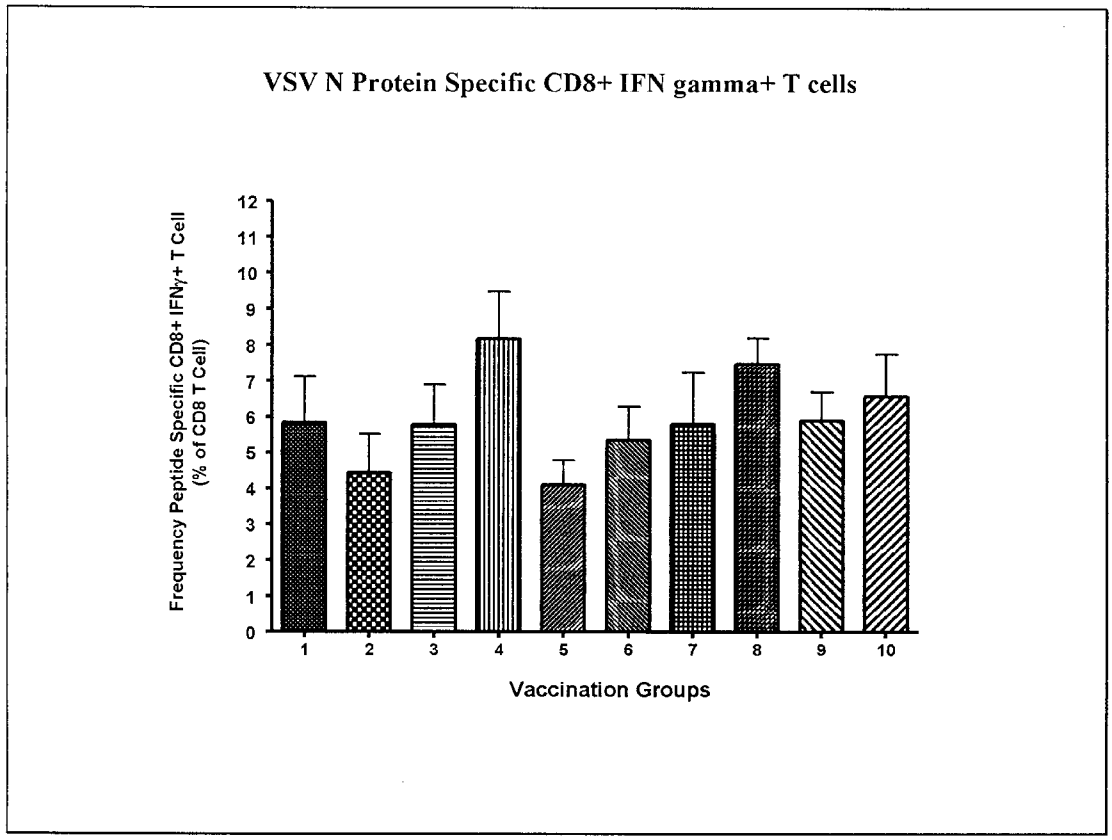

VSV N Protein Specific CD8+ IFN gamma+ T cells
Frequency Peptide Specific CD8+ IFNγ+ T Cell (% of CD8 T Cell)
0
1
2
3
4
5
6
7
8
9
10
11
12
1
2
3
4
5
6
7
8
9
10
Vaccination Groups

FIGURE 19

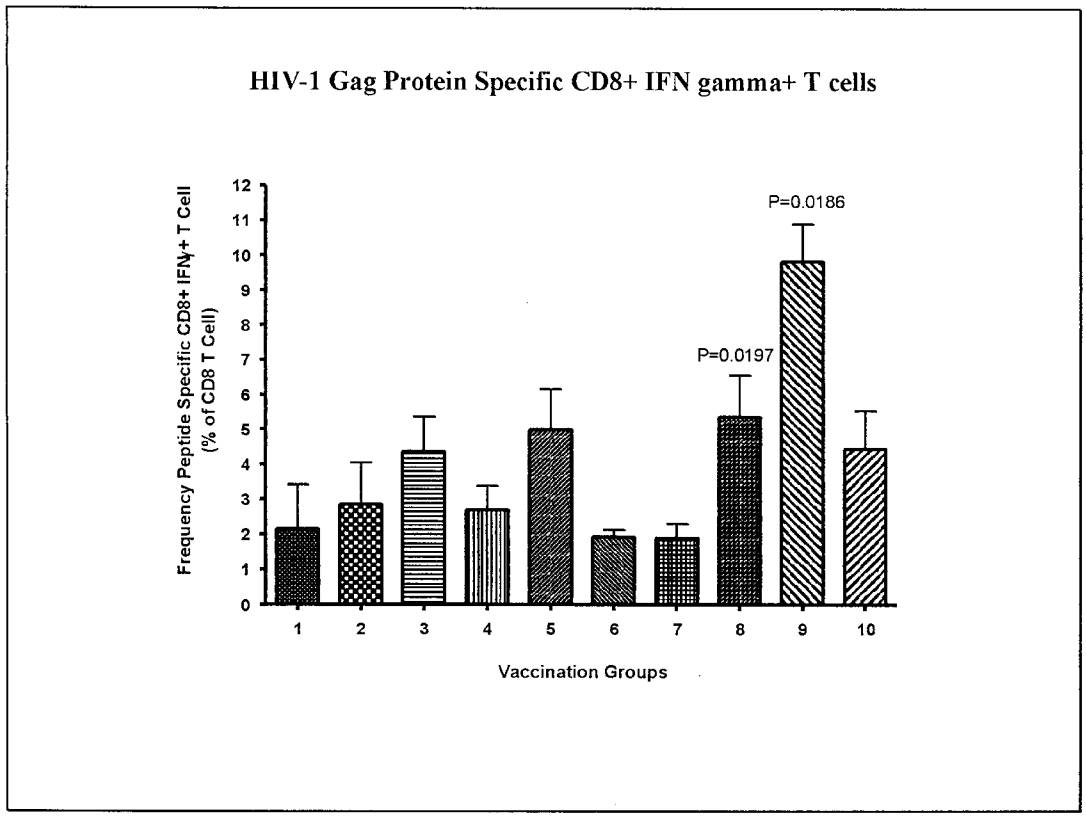

HIV-1 Gag Protein Specific CD8+ IFN gamma+ T cells
Frequency Peptide Specific CD8+ IFNγ+ T Cell (% of CD8 T Cell)
0
1
2
3
4
5
6
7
8
9
10
11
12
P=0.0197
P=0.0186
1
2
3
4
5
6
7
8
9
10
Vaccination Groups

FIGURE 20

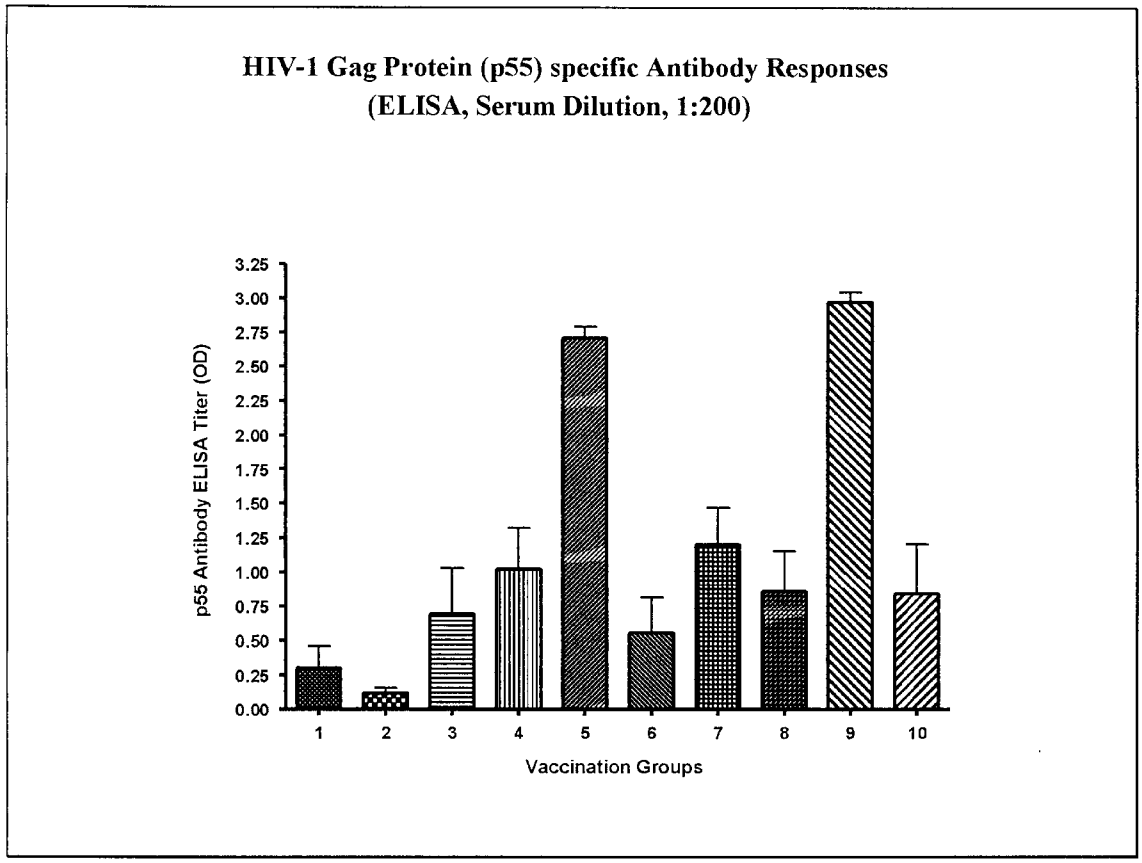

HIV-1 Gag Protein (p55) specific Antibody Responses
(ELISA, Serum Dilution, 1:200)
p55 Antibody ELISA Titer (OD)
3.25
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1
2
3
4
5
6
7
8
9
10
Vaccination Groups

FIGURE 21

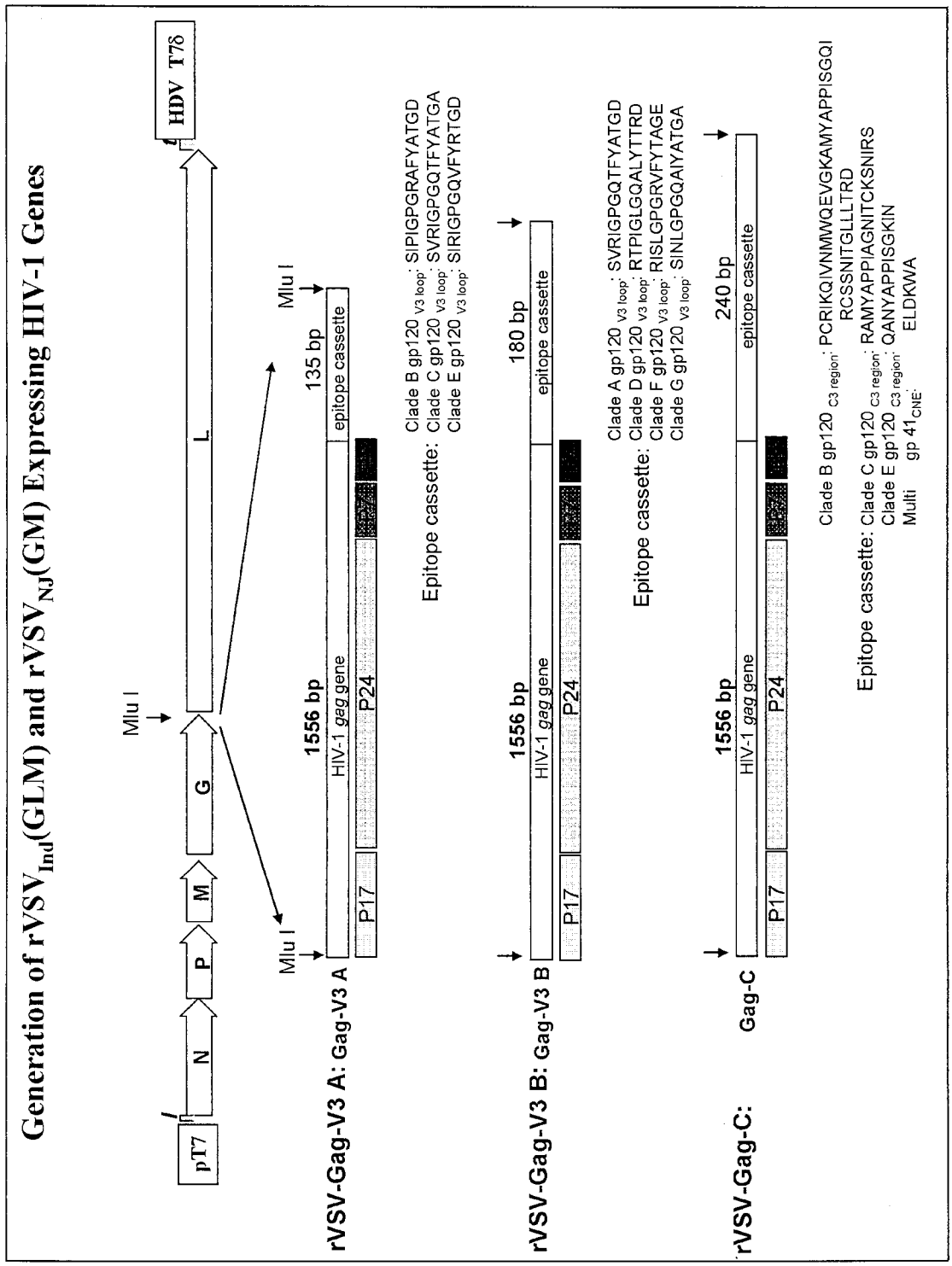

Generation of rVSV$_{Ind}$(GLM) and rVSV$_{NJ}$(GM) Expressing HIV-1 Genes
pT7
N
P
M
G
L
HDV Tδ
Mlu I
Mlu I
Mlu I
rVSV-Gag-V3 A: Gag-V3 A
1556 bp
HIV-1 gag gene
P17
P24
135 bp
epitope cassette
Epitope cassette:
Clade B gp120 V3 loop: SIPIGPGRAFYATGD
Clade C gp120 V3 loop: SVRIGPGQTFYATGA
Clade E gp120 V3 loop: SIRIGPGQVFYRTGD
rVSV-Gag-V3 B: Gag-V3 B
1556 bp
HIV-1 gag gene
P17
P24
180 bp
epitope cassette
Epitope cassette:
Clade A gp120 V3 loop: SVRIGPGQTFYATGD
Clade D gp120 V3 loop: RTPIGLGQALYTTRD
Clade F gp120 V3 loop: RISLGPGRVFYTAGE
Clade G gp120 V3 loop: SINLGPGQAIYATGA
rVSV-Gag-C:
Gag-C
1556 bp
HIV-1 gag gene
P17
P24
240 bp
epitope cassette
Epitope cassette:
Clade B gp120 C3 region: PCRIKQIVNMWQEVGKAMYAPPISGQI
RCSSNITGLLLTRD
Clade C gp120 C3 region: RAMYAPPIAGNITCKSNIRS
Clade E gp120 C3 region: QANYAPPISGKIN
Multi gp 41CNE: ELDKWA

FIGURE 22

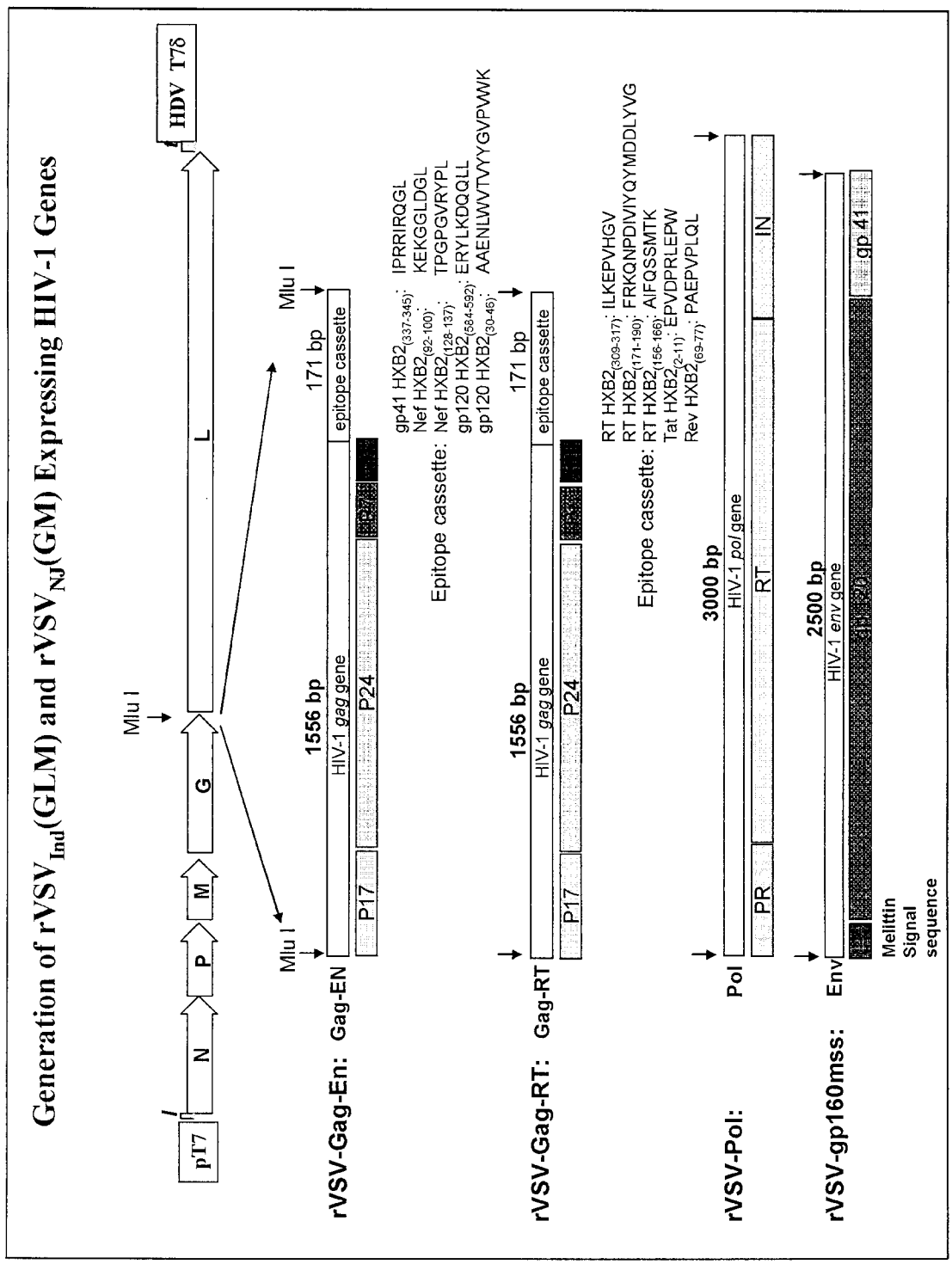

Generation of rVSV$_{Ind}$(GLM) and rVSV$_{NJ}$(GM) Expressing HIV-1 Genes
pT7
N
P
M
G
L
HDV Tδ
Mlu I
Mlu I
Mlu I
rVSV-Gag-En: Gag-EN
1556 bp
HIV-1 gag gene
P17
P24
171 bp
epitope cassette
Epitope cassette: Nef HXB2(128-137): TPGPGVRYPL
gp120 HXB2(584-592): ERYLKDQQLL
gp120 HXB2(30-46): AAENLWVTVYYGVPVWK
gp41 HXB2(337-345): IPRRIRQGL
Nef HXB2(92-100): KEKGGLDGL
rVSV-Gag-RT: Gag-RT
1556 bp
HIV-1 gag gene
P17
P24
171 bp
epitope cassette
Epitope cassette: RT HXB2(309-317): ILKEPVHGV
RT HXB2(171-190): FRKQNPDIVIYQYMDDLYVG
RT HXB2(156-166): AIFQSSMTK
Tat HXB2(2-11): EPVDPRLEPW
Rev HXB2(69-77): PAEPVPLQL
rVSV-Pol:
Pol
3000 bp
HIV-1 pol gene
PR
RT
IN
rVSV-gp160mss:
Env
2500 bp
HIV-1 env gene
gp41
Melittin
Signal
sequence

FIGURE 23

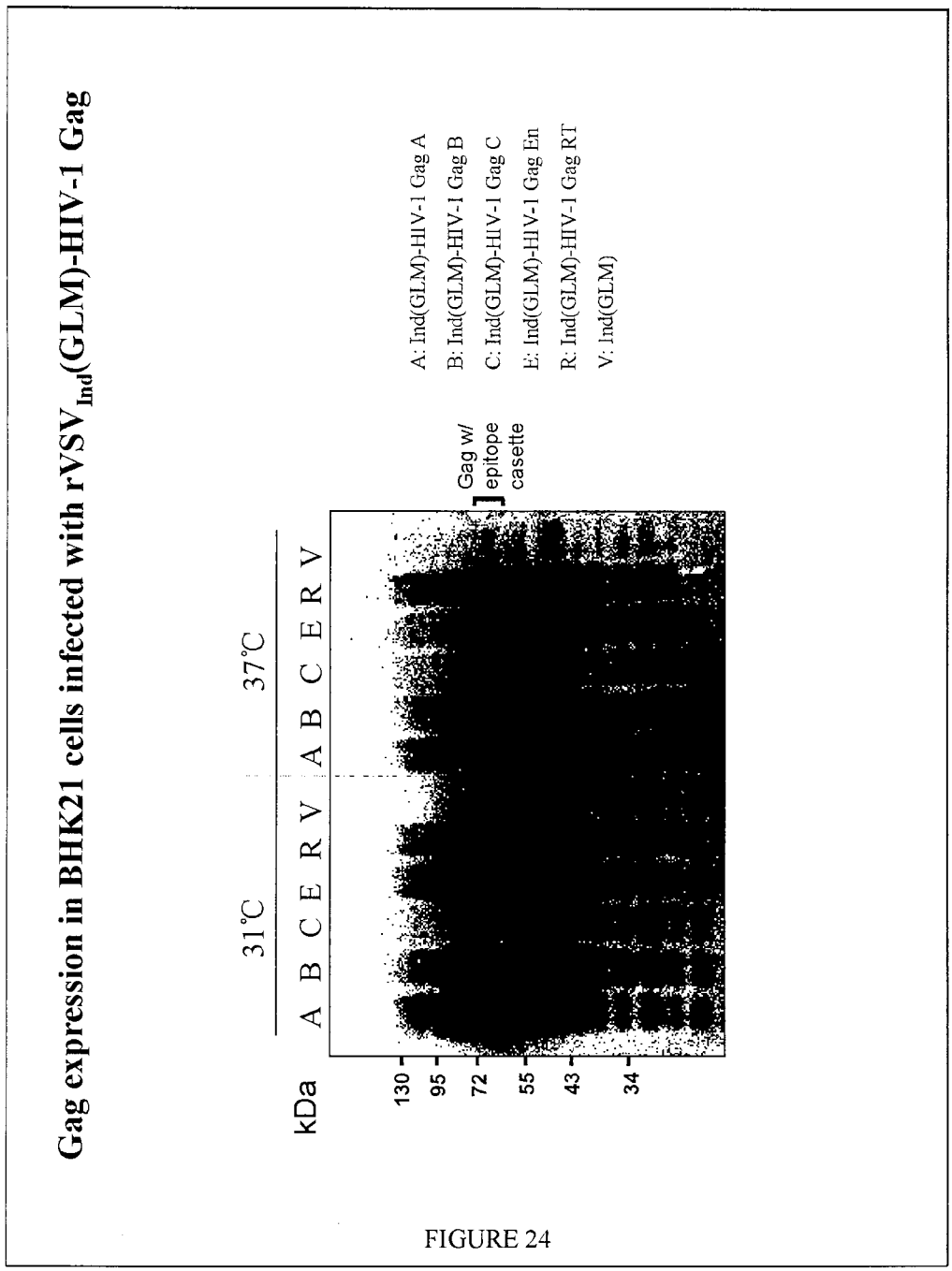

Gag expression in BHK21 cells infected with rVSV Ind(GLM)-HIV-1 Gag
31°C
37°C
A B C E R V A B C E R V
kDa
130
95
72
55
43
34
Gag w/ epitope casette
A: Ind(GLM)-HIV-1 Gag A
B: Ind(GLM)-HIV-1 Gag B
C: Ind(GLM)-HIV-1 Gag C
E: Ind(GLM)-HIV-1 Gag En
R: Ind(GLM)-HIV-1 Gag RT
V: Ind(GLM)

FIGURE 24

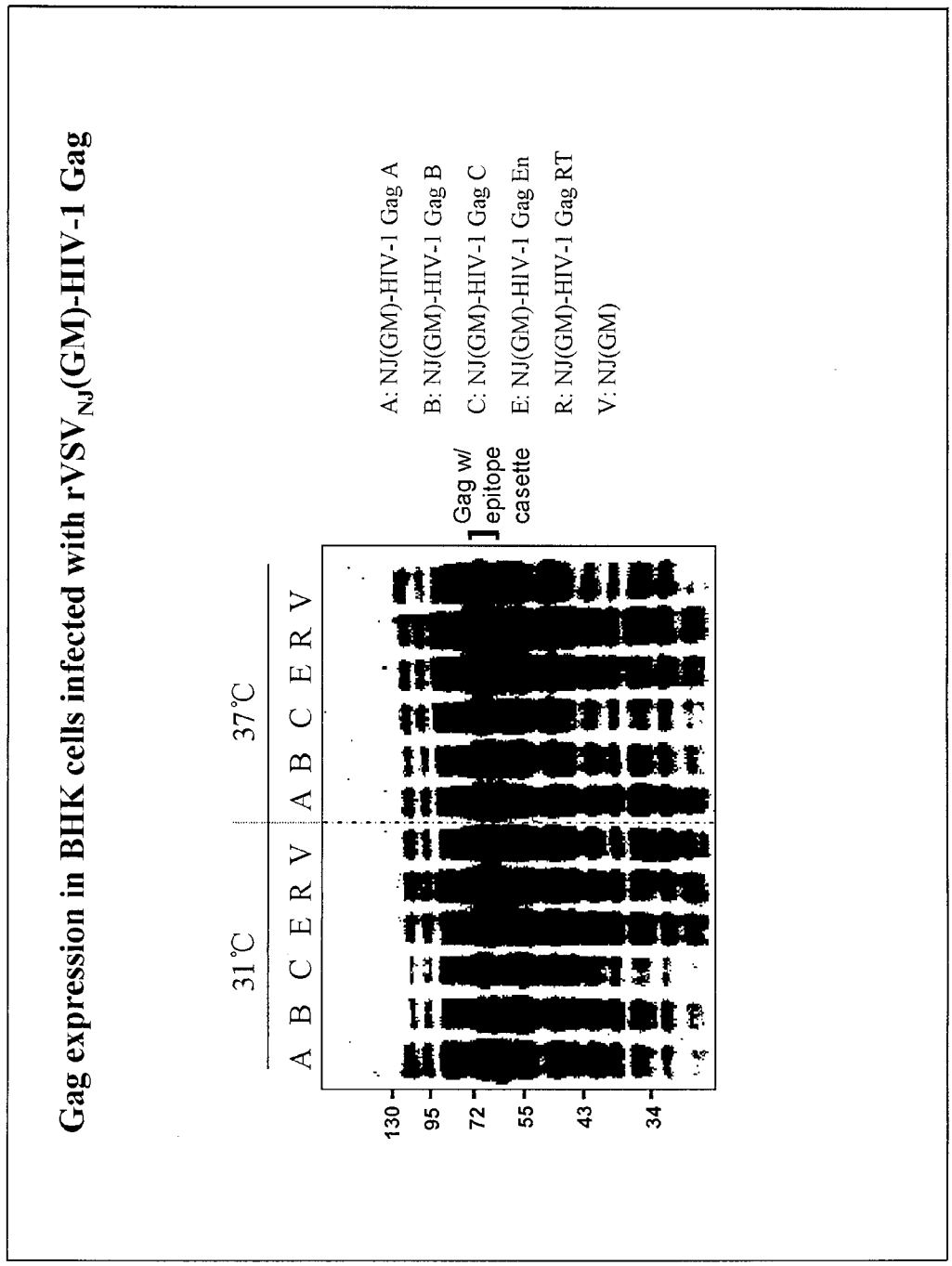

Gag expression in BHK cells infected with rVSV$_{NJ}$(GM)-HIV-1 Gag
31°C
37°C
A B C E R V A B C E R V
Gag w/ epitope casette
A: NJ(GM)-HIV-1 Gag A
B: NJ(GM)-HIV-1 Gag B
C: NJ(GM)-HIV-1 Gag C
E: NJ(GM)-HIV-1 Gag En
R: NJ(GM)-HIV-1 Gag RT
V: NJ(GM)
130
95
72
55
43
34

FIGURE 25

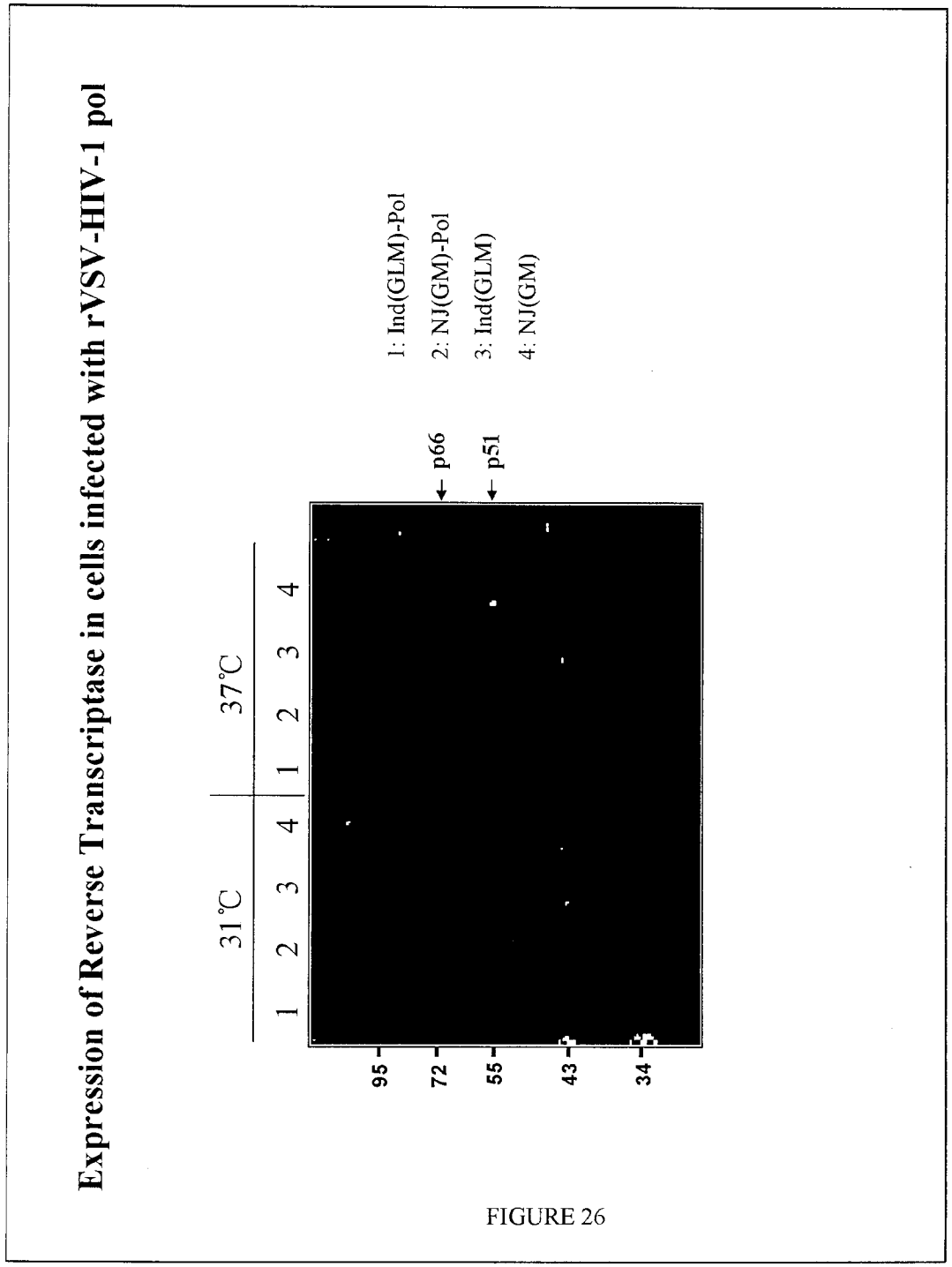

Expression of Reverse Transcriptase in cells infected with rVSV-HIV-1 pol
1: Ind(GLM)-Pol
2: NJ(GM)-Pol
3: Ind(GLM)
4: NJ(GM)
p66
p51
31°C
37°C
1 2 3 4 1 2 3 4
95
72
55
43
34

FIGURE 26

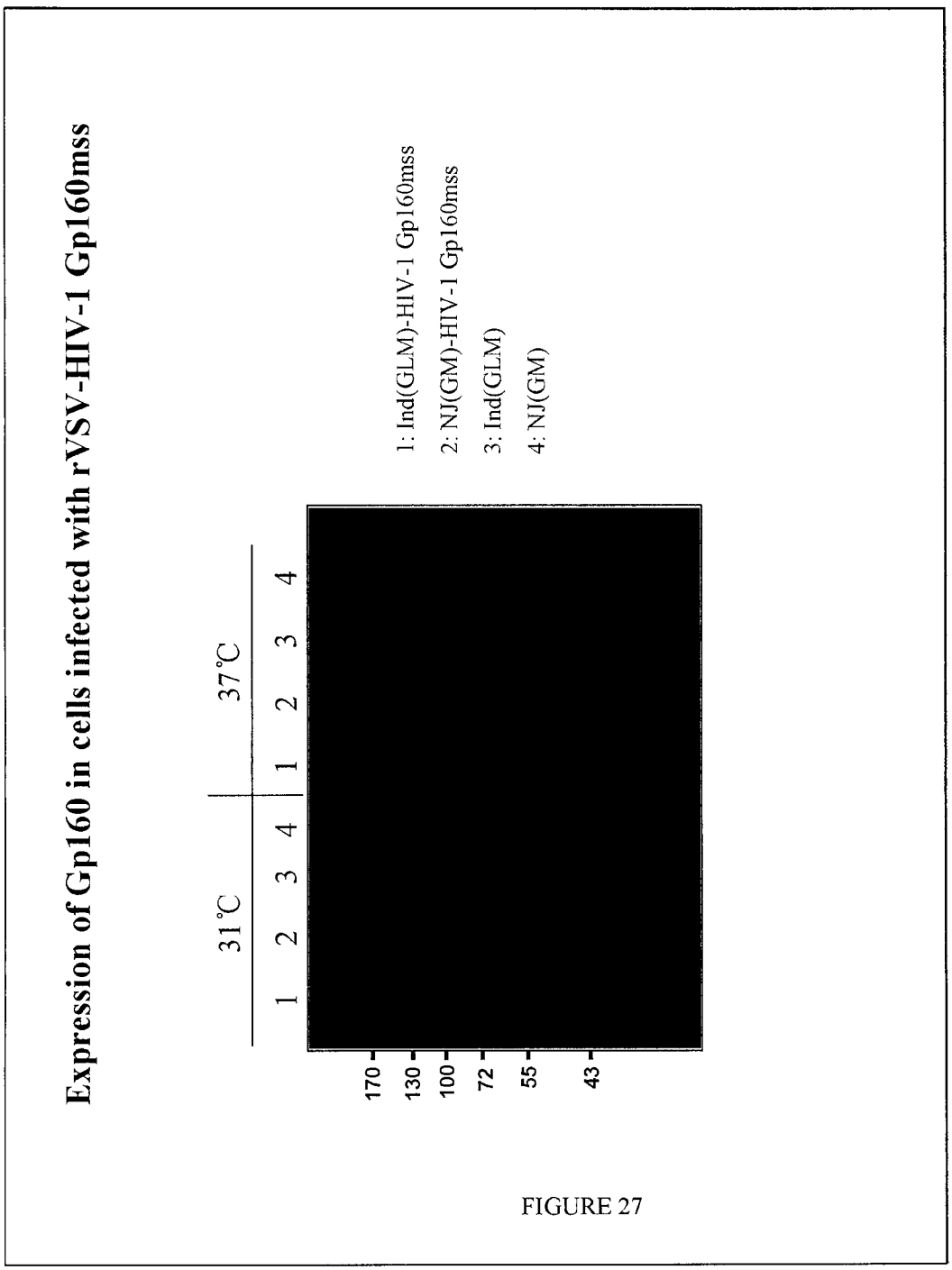

Expression of Gp160 in cells infected with rVSV-HIV-1 Gp160mss
31℃
37℃
1 2 3 4 1 2 3 4
1: Ind(GLM)-HIV-1 Gp160mss
2: NJ(GM)-HIV-1 Gp160mss
3: Ind(GLM)
4: NJ(GM)
170
130
100
72
55
43

FIGURE 27

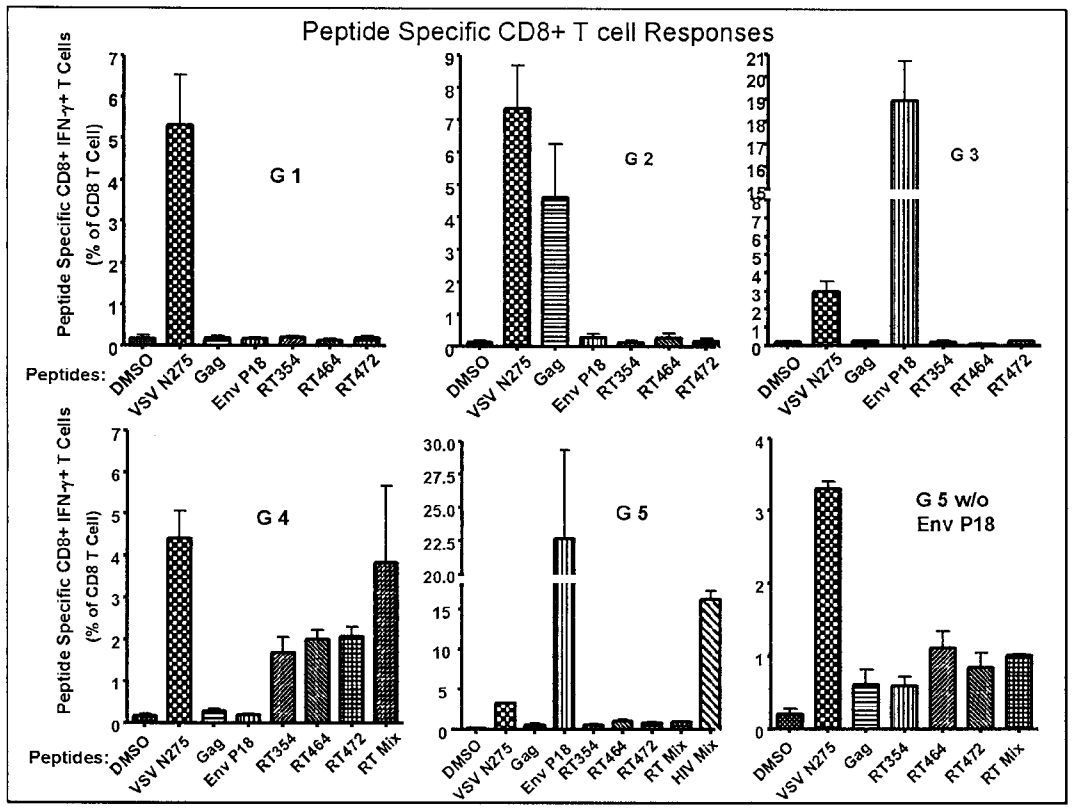

Peptide Specific CD8+ T cell Responses
Peptide Specific CD8+ IFN-γ+ T Cells (% of CD8 T Cell)
G 1
G 2
G 3
G 4
G 5
G 5 w/o Env P18
Peptides:
DMSO
VSV N275
Gag
Env P18
RT354
RT464
RT472
RT Mix
HIV Mix

FIGURE 28

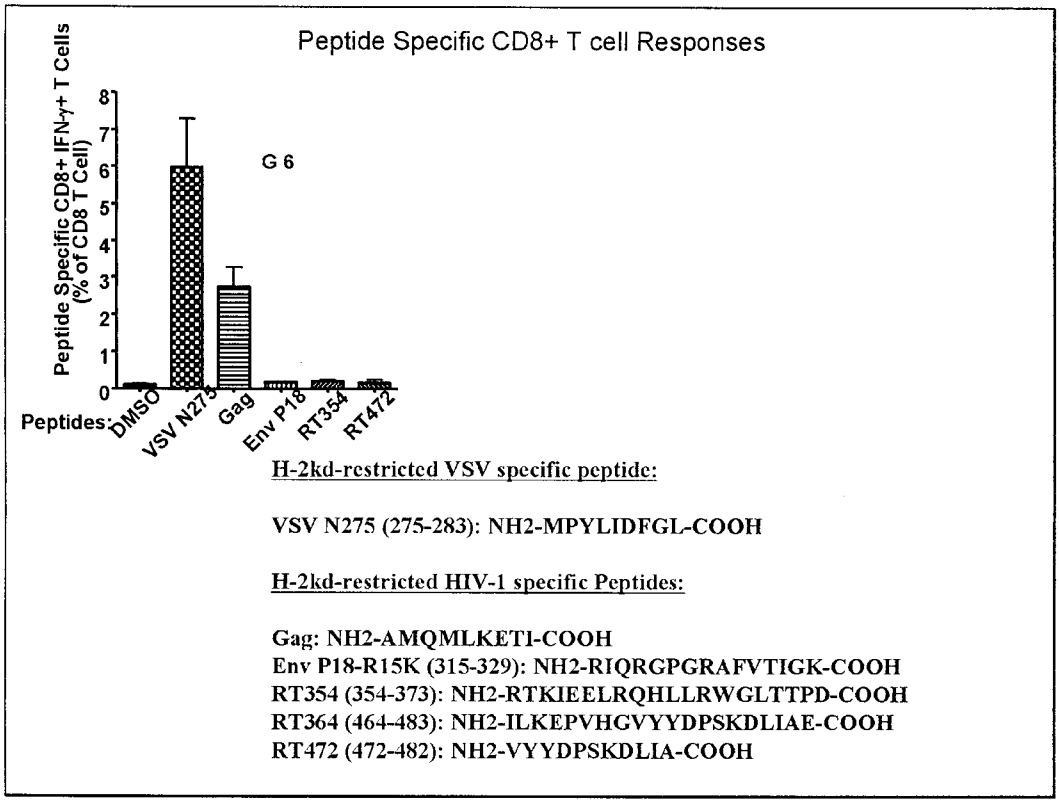

Peptide Specific CD8+ T cell Responses
Peptide Specific CD8+ IFN-γ+ T Cells (% of CD8 T Cell)
G 6
Peptides:
DMSO
VSV N275
Gag
Env P18
RT354
RT472
H-2kd-restricted VSV specific peptide:
VSV N275 (275-283): NH2-MPYLIDFGL-COOH
H-2kd-restricted HIV-1 specific Peptides:
Gag: NH2-AMQMLKETI-COOH
Env P18-R15K (315-329): NH2-RIQRGPGRAFVTIGK-COOH
RT354 (354-373): NH2-RTKIEELRQHLLRWGLTTPD-COOH
RT364 (464-483): NH2-ILKEPVHGVYYDPSKDLIAE-COOH
RT472 (472-482): NH2-VYYDPSKDLIA-COOH

FIGURE 29

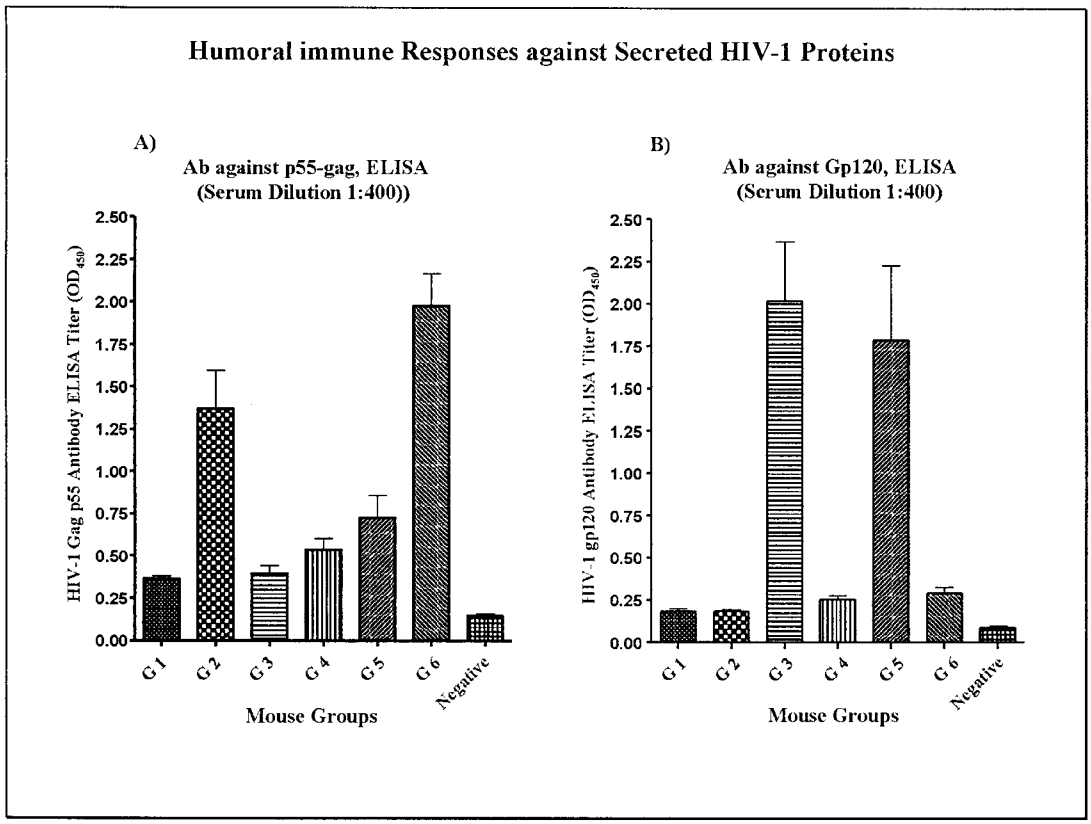

Humoral immune Responses against Secreted HIV-1 Proteins
A)
Ab against p55-gag, ELISA
(Serum Dilution 1:400))
HIV-1 Gag p55 Antibody ELISA Titer (OD450)
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
G1
G2
G3
G4
G5
G6
Negative
Mouse Groups
B)
Ab against Gp120, ELISA
(Serum Dilution 1:400)
HIV-1 gp120 Antibody ELISA Titer (OD450)
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
G1
G2
G3
G4
G5
G6
Negative
Mouse Groups

FIGURE 30

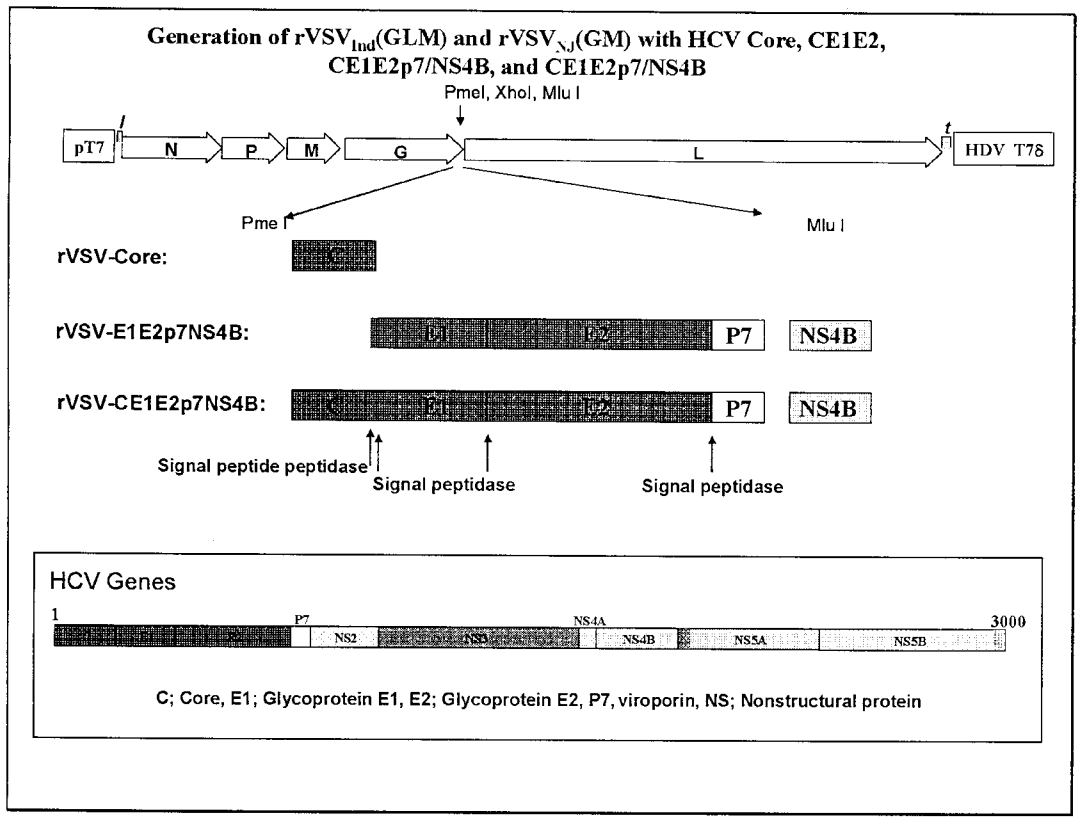

Generation of rVSV$_{Ind}$(GLM) and rVSV$_{NJ}$(GM) with HCV Core, CE1E2, CE1E2p7/NS4B, and CE1E2p7/NS4B
PmeI, XhoI, MluI
pT7
N
P
M
G
L
HDV T7δ
Pme I
Mlu I
rVSV-Core:
C
rVSV-E1E2p7NS4B:
E1
E2
P7
NS4B
rVSV-CE1E2p7NS4B:
C
E1
E2
P7
NS4B
Signal peptide peptidase
Signal peptidase
Signal peptidase
HCV Genes
1
P7
NS4A
3000
C
NS2
NS3
NS4B
NS5A
NS5B
C; Core, E1; Glycoprotein E1, E2; Glycoprotein E2, P7, viroporin, NS; Nonstructural protein

FIGURE 31

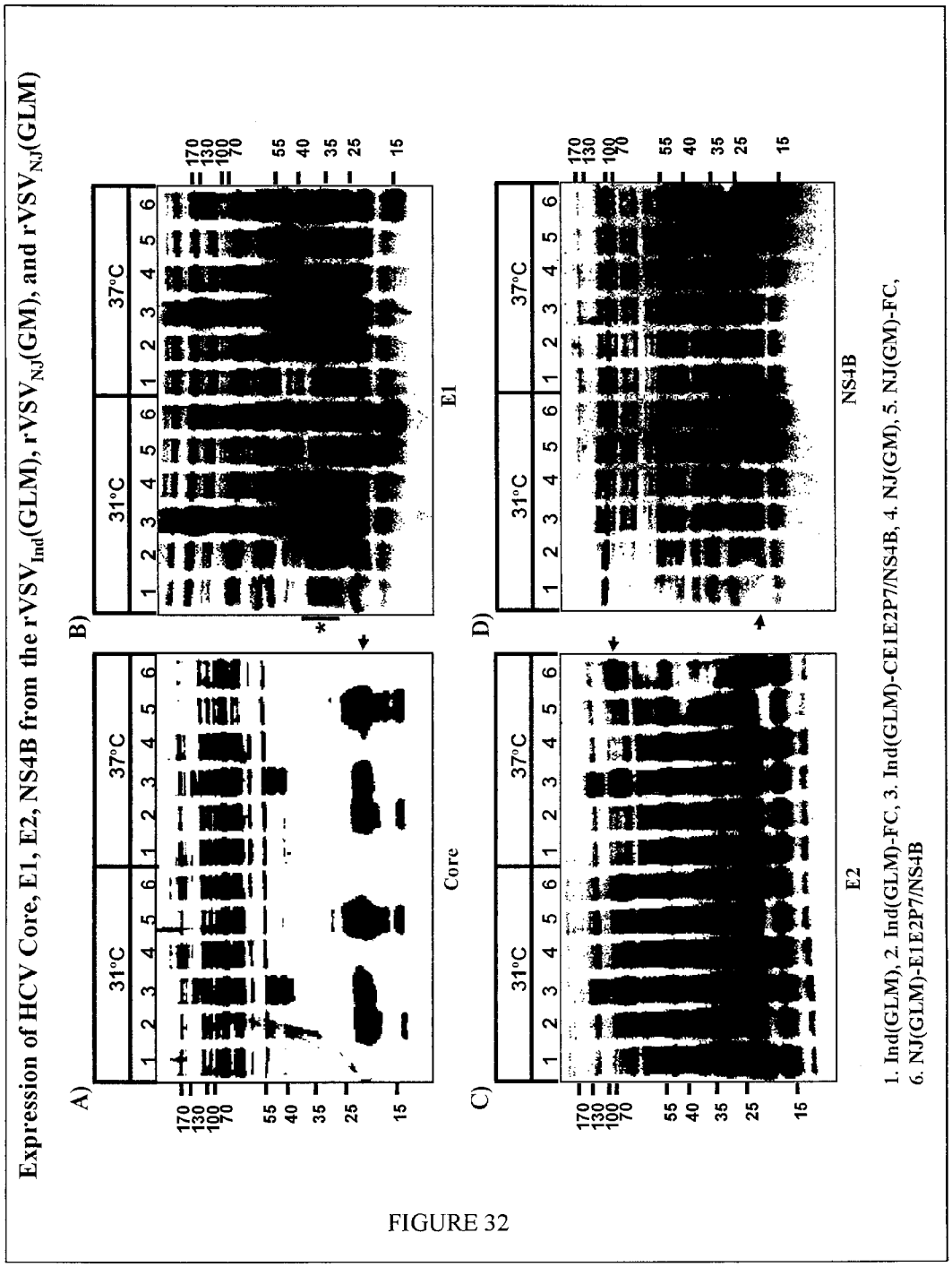

Expression of HCV Core, E1, E2, NS4B from the rVSV$_{Ind}$(GLM), rVSV$_{NJ}$(GM), and rVSV$_{NJ}$(GLM)
A)
B)
C)
D)
31°C
37°C
1 2 3 4 5 6
170
130
100
70
55
40
35
25
15
Core
E1
E2
NS4B
*
1. Ind(GLM), 2. Ind(GLM)-FC, 3. Ind(GLM)-CE1E2P7/NS4B, 4. NJ(GM), 5. NJ(GM)-FC,
6. NJ(GLM)-E1E2P7/NS4B

FIGURE 32

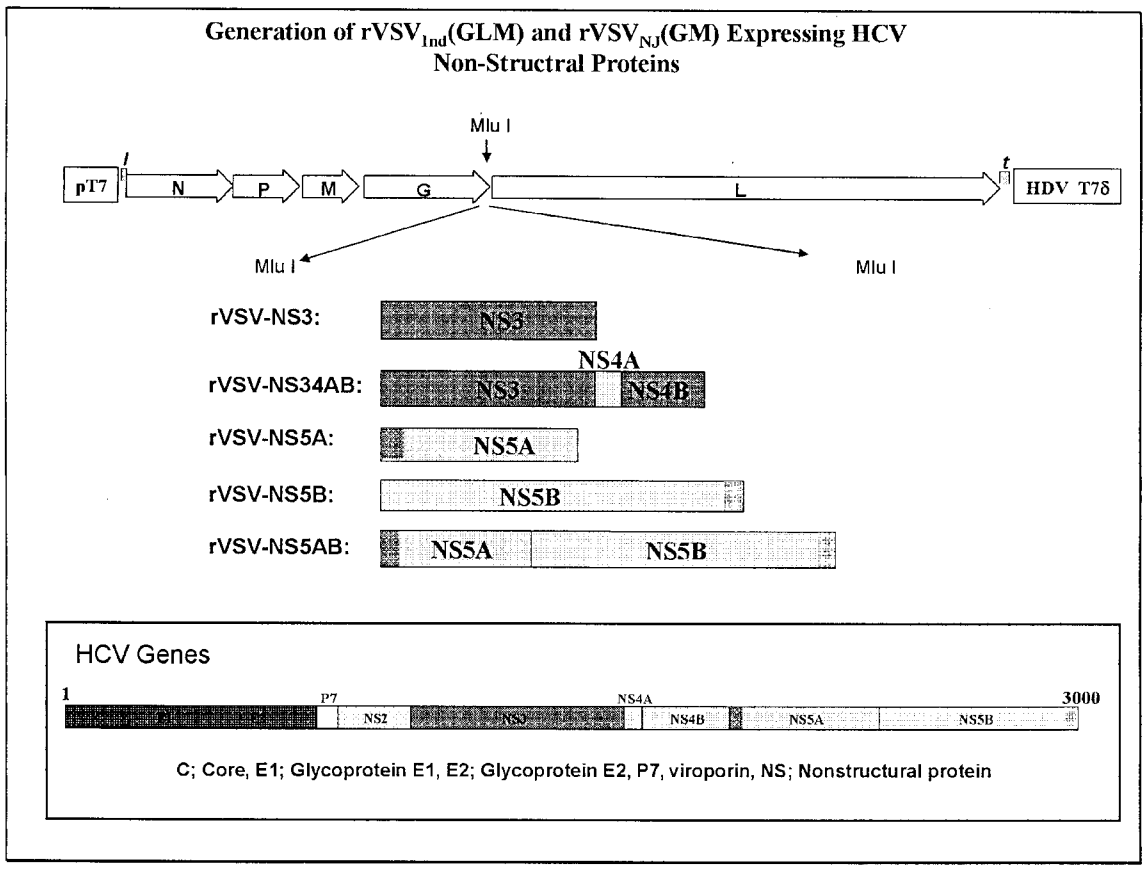

Generation of rVSV$_{Ind}$(GLM) and rVSV$_{NJ}$(GM) Expressing HCV Non-Structral Proteins
Mlu I
pT7
N
P
M
G
L
HDV T7δ
Mlu I
Mlu I
rVSV-NS3:
NS3
NS4A
rVSV-NS34AB:
NS3
NS4B
rVSV-NS5A:
NS5A
rVSV-NS5B:
NS5B
rVSV-NS5AB:
NS5A
NS5B
HCV Genes
1
P7
NS4A
3000
NS2
NS4B
NS5A
NS5B
C; Core, E1; Glycoprotein E1, E2; Glycoprotein E2, P7, viroporin, NS; Nonstructural protein

FIGURE 33

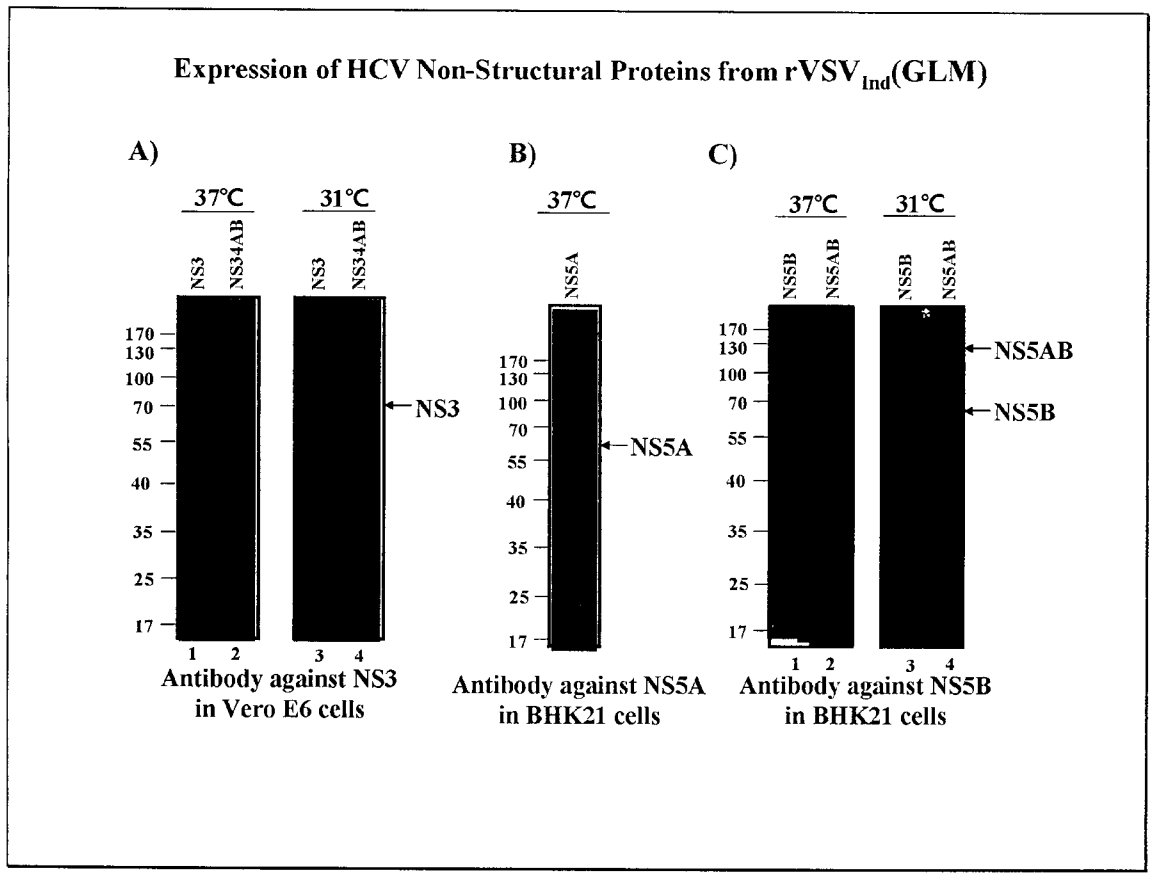

Expression of HCV Non-Structural Proteins from rVSV$_{Ind}$(GLM)
A)
B)
C)
37°C
31°C
NS3
NS34AB
NS5A
NS5B
NS5AB
170
130
100
70
55
40
35
25
17
NS3
NS5A
NS5AB
NS5B
1 2 3 4
Antibody against NS3 in Vero E6 cells
Antibody against NS5A in BHK21 cells
Antibody against NS5B in BHK21 cells

FIGURE 34

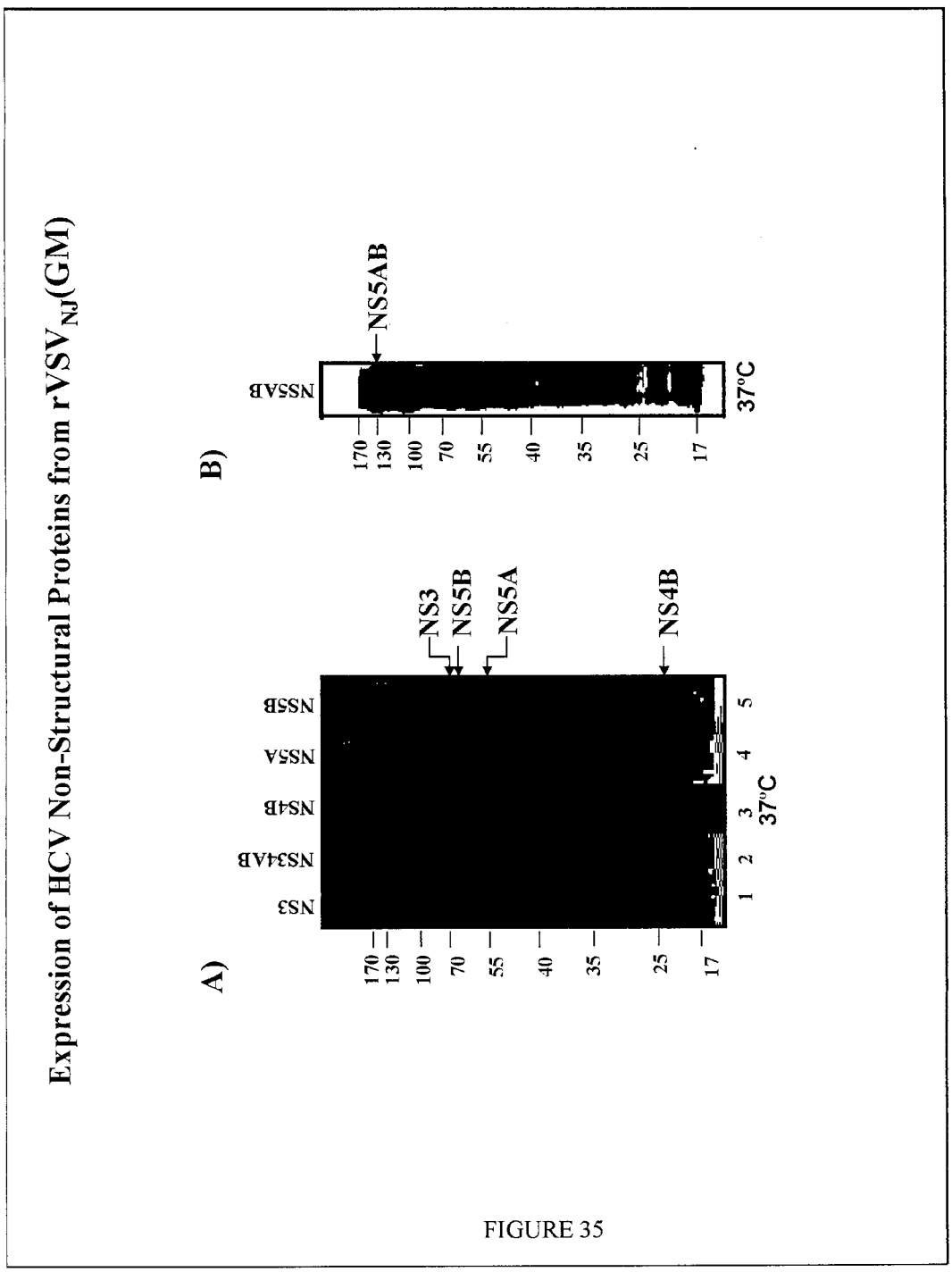

Expression of HCV Non-Structural Proteins from rVSV$_{NJ}$(GM)
A)
NS3
NS34AB
NS4B
NS5A
NS5B
NS3
NS5A
NS4B
NS5B
1 2 3 4 5
37°C
170
130
100
70
55
40
35
25
17
B)
NS5AB
NS5AB
37°C
170
130
100
70
55
40
35
25
17

FIGURE 35

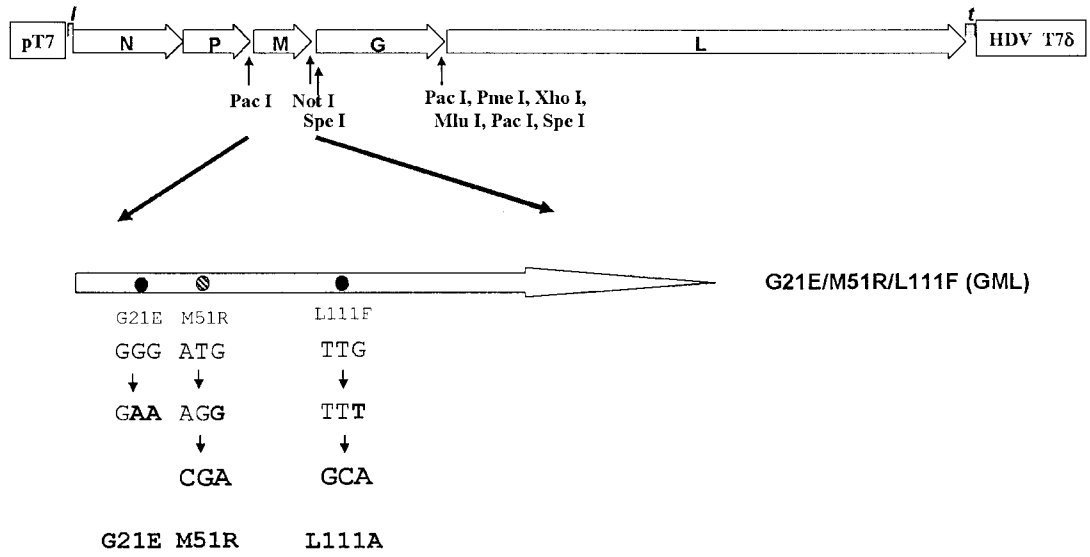

Construction of pVSV$_{Ind}$(GLM)-New
pT7
N
P
M
G
L
HDV T7δ
Pac I
Not I
Spe I
Pac I, Pme I, Xho I,
Mlu I, Pac I, Spe I
G21E/M51R/L111F (GML)
G21E M51R L111F
GGG ATG TTG
GAA AGG TTT
CGA GCA
G21E M51R L111A

FIGURE 36

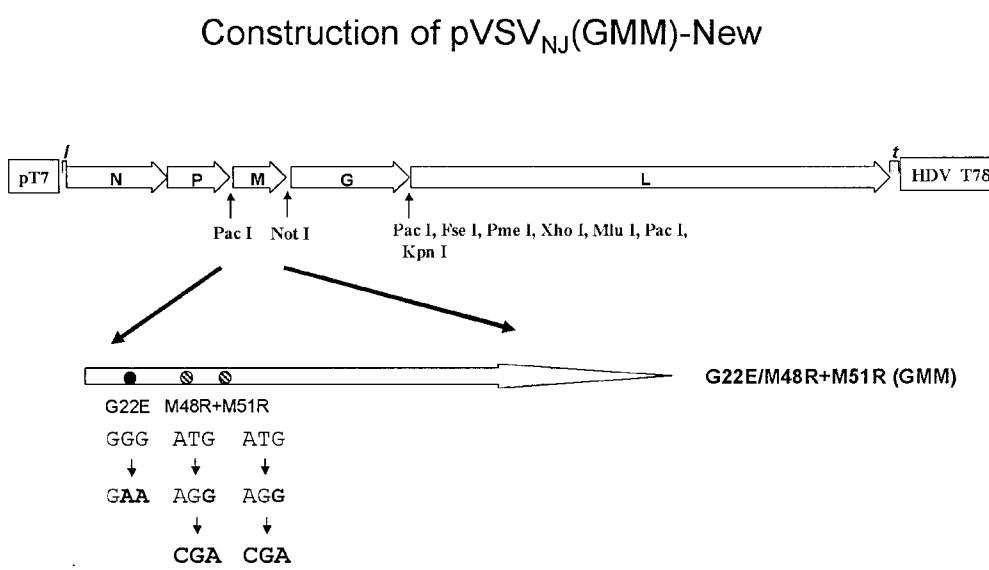

Construction of pVSV$_{NJ}$(GMM)-New
pT7
N
P
M
G
L
HDV T7t
Pac I
Not I
Pac I, Fse I, Pme I, Xho I, Mlu I, Pac I, Kpn I
G22E/M48R+M51R (GMM)
G22E M48R+M51R
GGG ATG ATG
GAA AGG AGG
CGA CGA

FIGURE 37

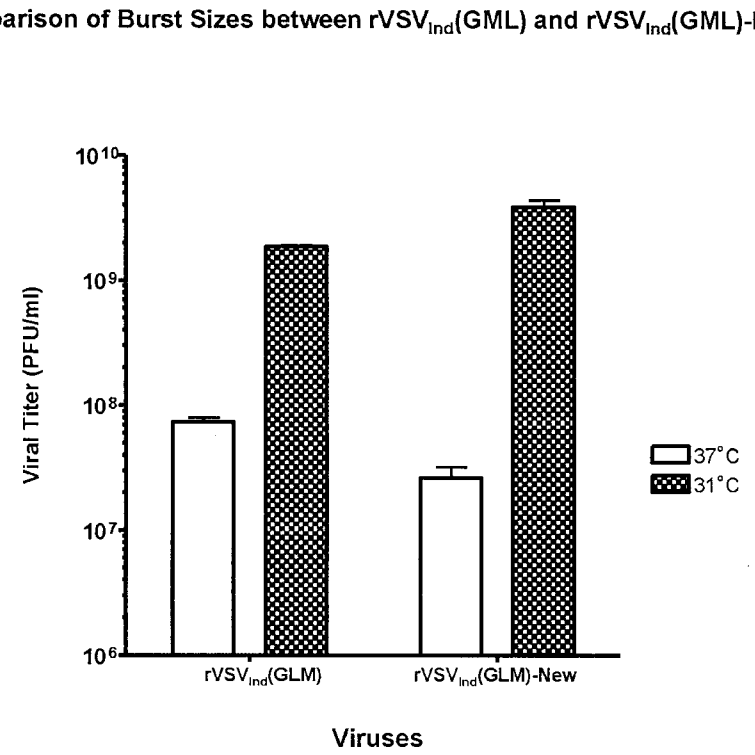

Comparison of Burst Sizes between rVSV$_{Ind}$(GML) and rVSV$_{Ind}$(GML)-New
Viral Titer (PFU/ml)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
rVSV$_{Ind}$(GLM)
rVSV$_{Ind}$(GLM)-New
Viruses
37°C
31°C

FIGURE 38

Construction of $pVSV_{Ind}$(GLM)-New

