NON-INTEGRATING RETROVIRAL VECTORS WITH PERSISTENT PAYLOAD EXPRESSION FOR APPLICATION TO GENE THERAPY AND VACCINOLOGY

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ABSTRACT

Stable integration of retroviral vectors into the host cell genome can achieve prolonged transgene expression, but poses the risk of insertional genotoxicity. To mitigate this risk, we have developed non-integrating retroviral vectors primarily by introducing single amino acid mutations into the integrase (IN) gene. However, relying on any individual mutation risks reversion to integration-competence, and previous non-integrating vectors have shown low expression levels, limiting their usefulness. Here we report the development of novel non-integrating gammaretroviral vector configurations and their utility for prodrug activator ('suicide') gene therapy and as a vaccine platform.

Non-integrating vectors derived from murine leukemia virus were generated by mutating 3 crucial residues of the IN catalytic core domain (D125A, D184A, E220A) individually or in combination, resulting in 7 different configurations of IN-defective retrovector (IDRV). In addition, vectors with complete deletion of integrase (dINT) from *gagpol* were also generated. Both IDRV and dINT vectors were produced at physical titers similar to those of control vectors with wildtype (WT) *gagpol*.

To evaluate their utility for suicide gene therapy, IDRV and dINT vectors were used to deliver an enhanced HSV-TK (HSV-eTK) transgene payload to A375 cells, and vector copy numbers quantitated by ddPCR of whole cell DNA. Measurable vector copies were detected at D1 post-transduction, but decreased to near or below limit of detection by D10, indicating gradual clearance due to lack of integration. Consistent with ddPCR results, flow cytometry showed high HSV-eTK protein expression at early time points in IDRV or dINT-transduced cells, followed by a steady decrease. In contrast, WT vector transduced cells maintained stable expression. Cytotoxicity assays after exposure of transduced cells to the prodrug ganciclovir, which is converted by HSV-eTK to a toxic nucleotide analog, showed similar cell-killing activity by IDRV and dINT as compared to WT for up to 7 days post-transduction. Cytotoxicity was decreased yet still detectable even at 14 days, consistent with flow cytometric data of expression levels of transgene over time.

Non-integrating vectors were also evaluated for potential utility as a vaccine platform. A conserved region of SARS-CoV-2 BA.2 strain envelope was inserted as a transgene into IDRV for prime-boost vaccination in BALB/C mice. ELISA data demonstrated antigen-specificity of antibodies generated by IDRV. Generation of neutralizing antibodies (NAb) against different strains was confirmed by *in vitro* pseudovirus neutralization assays, with 100% neutralization of a variant BA.2, 80% protection against BA.1, and no NAb activity against BA.2.75.

Taken together, our results indicate the utility of multiply IN-mutated IDRV and dINT retroviral vectors for both gene therapy and immunotherapy applications.

BACKGROUND

- Our current Phase I clinical trial utilizes a non-replicating vector derived from Moloney murine leukemia virus (MLV) for delivery of transgenes that integrate into the host cell genome (via integrase) during breakdown of the nuclear envelope during cell division [1]. Once integrated, the vector payload is constitutively expressed. For other uses, vectors lacking integrase that are non-integrating and can deliver a payload with a reduced risk of insertional mutagenesis are being explored in non-integrating lentivectors [3,4]. We describe the development of two non-integrating retrovectors that express the transgene for days.

Figure 1. GenVivo's MLV vector platform. To inactivate the integrase, the triad of residues D125, D184, E220, that form the Mg^{2+} binding motif within the catalytic core domain, was targeted and mutated to alanine (A) by single base-point mutations while integrase deleted has the integrase gene fully removed (Figures 2-3).

GenVivo's MLV Vector Platform

Wildtype *gagpol* (WT)	Integrase-Defective *gagpol* Retrovirus (IDRV) (IDRV1 (D184A) IDRV2 (D125A/D184A))	Deleted Integrase (dINT) *gagpol* Retrovirus
Integrates payload Potential for host genome mutagenesis	Dysfunctional Integrase present No Integration detected	No Integrase protein present No integration

Figure 2. Schematic representation of MLV gagpol polyprotein structural elements. GAG: Matrix Protein (p15), 130aa. RNA-binding phosphoprotein (p12), 84aa. Capsid protein (p30), 263aa. Nucleocapsid protein (p10), 56aa. **POL:** Protease, 125aa. Reverse Transcriptase/ribonuclease H, 671aa. Integrase, 408aa. Figure adapted from [5].

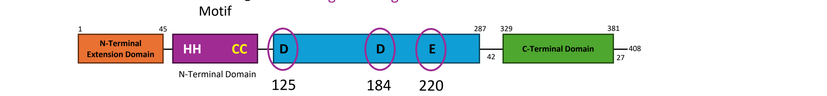
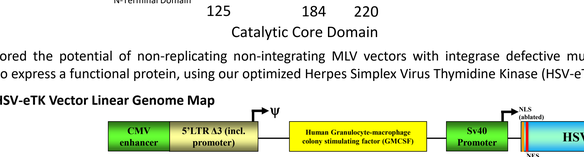


Figure 3. Schematic representation of MLV integrase protein, highlighting the highly conserved triad D125, D184, and E220 forming the Mg^{2+} binding motif in the catalytic core domain, and the HH-CC motif involved in Zinc binding motif. Figure adapted from [6].



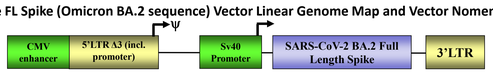
- We explored the potential of non-replicating non-integrating MLV vectors with integrase defective mutants and integrase deleted vectors to express a functional protein, using our optimized Herpes Simplex Virus Thymidine Kinase (HSV-eTK) gene construct (Figure 4).

Figure 4. HSV-eTK Vector Linear Genome Map

- MLV vectors that are both non-replicating and non-integrating have potential for applications in immunotherapy and vaccines with improved safety profile compared to technologies based on integrating vectors. A proof-of-concept *in vivo* study utilizing integrase-defective mutants with antigen SARS-CoV-2 BA.2 Omicron gene construct (Figure 5) was designed to test the ability of the GenVivo vaccine platform to produce an encoded functional gene as well as an antigen for immune responses.

Figure 5. SARS-CoV-2 vaccine FL Spike (Omicron BA.2 sequence) Vector Linear Genome Map and Vector Nomenclature

Vector Map



Vector Nomenclature

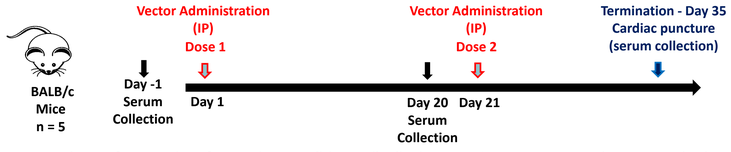
GEN4034: VSVg env/gagpol IDRV1 (D184A)/S:SARS-CoV-2 BA.2

GEN1044: MLV env/gagpol IDRV2 (D125A/D184A)/S:SARS-CoV-2 BA.2

METHODS

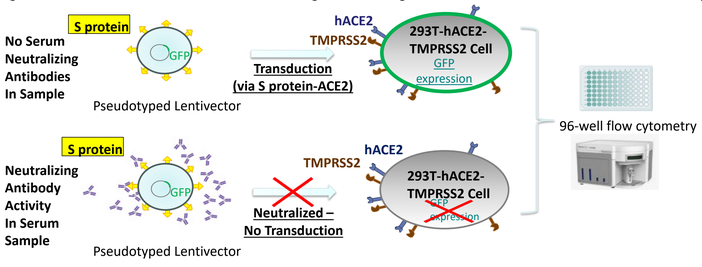
- **Vectors**. Non-replicating vectors were generated by transient triple transfection of 293T cells with plasmids bearing genes for: wild-type, integrase-defective IDRV1 (D184A), IDRV2 (D125A/D184A), or integrase-deleted (dINT) *gagpol;* MLV amphotropic envelope, or Vesicular Stomatitis Virus Glycoprotein (VSVg) pseudotyped envelope; and a payload plasmid. For the experiments shown here, the payload was an enhanced HSV-TK (HSV-eTK) or the S protein from SARS-CoV-2 BA.2. Titers were measured by RT-qPCR after isolation of the vector RNA.
- **Expression analysis.** Human melanoma cells, A375, were transduced with vectors. Payload protein expression was examined over time post-transduction by flow cytometry or Western blotting. Payload HSV-eTK protein activity was analyzed by harvesting the cells at given time points and seeding for cell kill assays in the presence of 20 μM GCV for 3-4 days. NTD are non-transduced cells.
- **Payload gene Psi copy and Vector Copy Number (VCN) analysis**. DNA was extracted from transduced cells using QIAcube (Qiagen) at different time points and tested for Psi signal and VCN copies by ddPCR.
- **Animals.** Immunocompetent BALB/C mice (Jackson Laboratory) were acclimated at Crown Bioscience (San Diego, CA) to eight to ten weeks of age. On Day 1 and Day 21, animals were injected Intraperitoneally (IP) at 20mL/kg, 2 times at 4 hours apart. Serum was collected by submandibular venipuncture before dosing on Day -1 and Day 20. Animals were anesthetized and euthanized at Day 35, and terminal blood was collected via cardiac puncture.

Figure 6. Experimental Timeline



- **Lentivector-Based Assay for Detection of S-Protein Neutralizing Antibodies in Mouse Serum.** Lentivectors bearing a payload encoding for a green fluorescent protein (GFP) were pseudotyped with the S protein of SARS-CoV-2 variants (BA.1, BA.2). 293T target cells expressing human Angiotensin-converting enzyme 2 (hACE2), to which the lentivector binds, were incubated with the lentivector in the presence of serum from mice immunized with the two retrovectors GEN4034 and GEN1044 expressing the S protein of SARS-CoV-2 BA.2 with mutated integrase genes. Cells were incubated for 3 days with mice serum and pseudotyped SARS-CoV-2 lentivector, then GFP expression was measured by FACS. The assay control is the pseudotyped lentivector alone with target cells as the baseline GFP % positive value. The percent neutralization antibody was determined by normalizing the GFP expression to the lentivector-only control for both 5-week post-vaccination and preimmunized mice serum.

Figure 7. Lentivector-Based Detection of Neutralizing Antibodies Against SARS-CoV-2 S-Protein in Mouse Serum Samples



RESULTS

In vitro, non-integrating gagpol vectors maintain HSV-eTK expression and functionality comparable to wild-type gagpol without integration in transduced cells

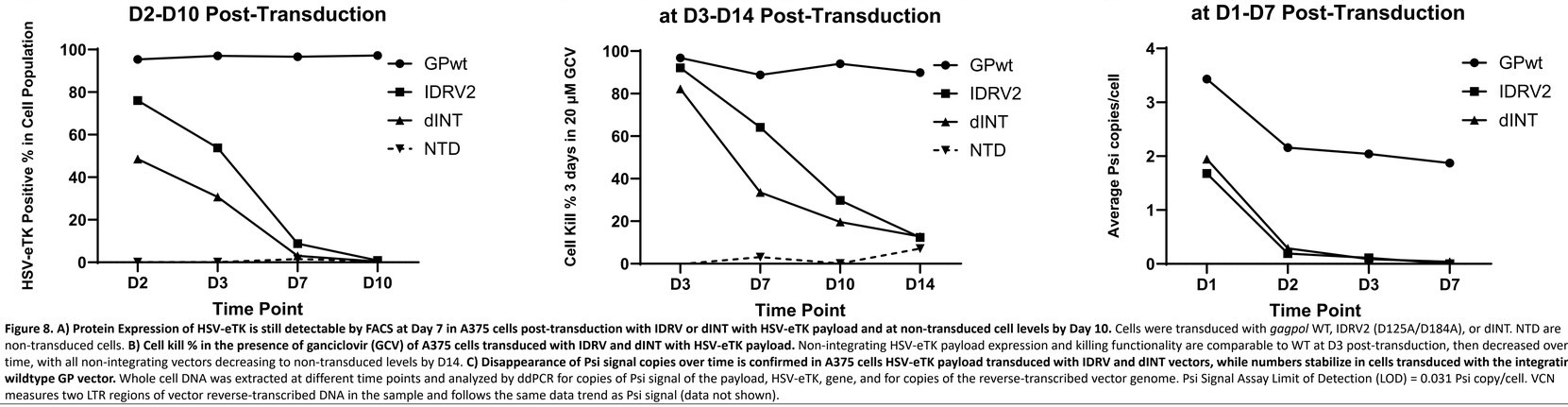


Figure 8. A) Protein Expression of HSV-eTK is still detectable by FACS at Day 7 in A375 cells post-transduction with IDRV or dINT with HSV-eTK payload and at non-transduced cell levels by Day 10. Cells were transduced with *gagpol* WT, IDRV2 (D125A/D184A), or dINT. NTD are non-transduced cells. **B) Cell kill % in the presence of ganciclovir (GCV) of A375 cells transduced with IDRV and dINT with HSV-eTK payload.** Non-integrating HSV-eTK payload expression and killing functionality are comparable to WT at D3 post-transduction, then decreased over time, with all non-integrating vectors decreasing to non-transduced levels by D14. **C) Disappearance of Psi signal copies over time is confirmed in A375 cells HSV-eTK payload transduced with IDRV and dINT vectors, while numbers stabilize in cells transduced with the integrating wildtype GP vector.** Whole cell DNA was extracted at different time points and analyzed by ddPCR for copies of Psi signal of the payload, HSV-eTK, gene, and for copies of the reverse-transcribed vector genome. Psi Signal Assay Limit of Detection (LOD) = 0.031 Psi copy/cell. VCN measures two LTR regions of vector reverse-transcribed DNA in the sample and follows the same data trend as Psi signal (data not shown).

In vivo, non-integrating gagpol vector injection produces measurable antibodies

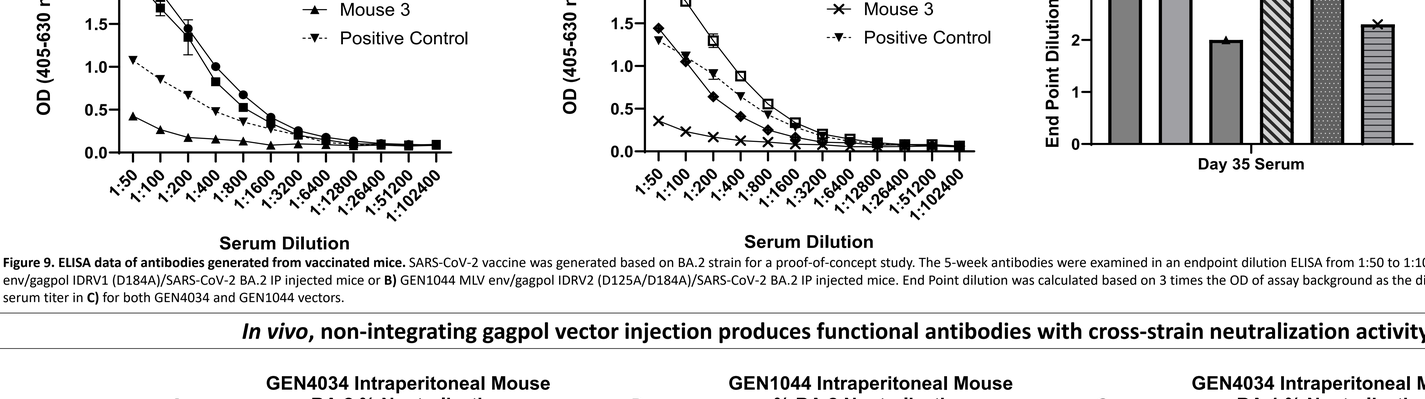


Figure 9. ELISA data of antibodies generated from vaccinated mice. SARS-CoV-2 vaccine was generated based on BA.2 strain for a proof-of-concept study. The 5-week antibodies were examined in an endpoint dilution ELISA from 1:50 to 1:102,400 dilution in **A)** GEN4034 VSVg env/gagpol IDRV1 (D184A)/SARS-CoV-2 BA.2 IP injected mice or **B)** GEN1044 MLV env/gagpol IDRV2 (D125A/D184A)/SARS-CoV-2 BA.2 IP injected mice. End Point dilution was calculated based on 3 times the OD of assay background as the dilution to calculate the antibody binding serum titer in **C)** for both GEN4034 and GEN1044 vectors.

In vivo, non-integrating gagpol vector injection produces functional antibodies with cross-strain neutralization activity

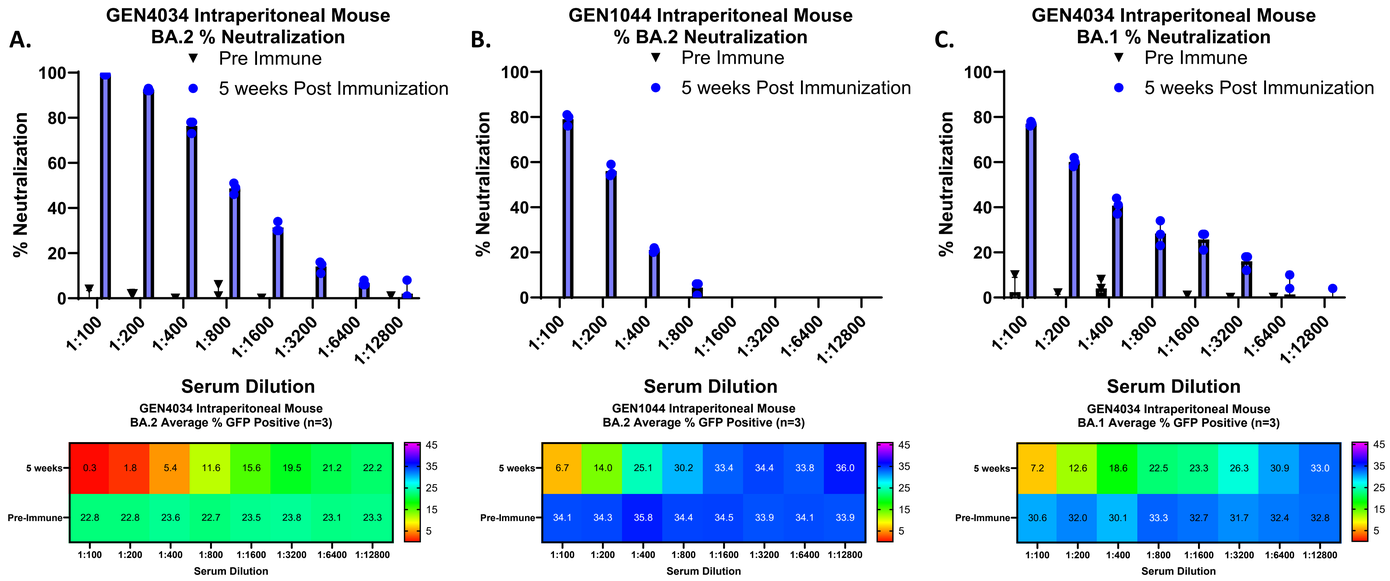


Figure 10. Representative data of serum neutralization activity against SARS-CoV-2 strain BA.1 and BA.2 with antibodies generated from vaccinated mice. 96-well FACS data was acquired at various dilutions of the serum tested, with the corresponding bar graph data generated after normalization by lentivector only control for both 5-week post-vaccination and preimmunized mice serum. **A)** GEN4034 IP injected mouse showed 100% inhibition at serum dilution 1:100 against BA.2. **B)** GEN1044 IP injected with almost 80% inhibition at serum dilution 1:100 against BA.2. **C)** GEN4034 VSVg env/gagpol IDRV1 (D184A)/SARS-CoV-2 BA.2 IP injected mouse showed 80% inhibition at serum dilution 1:100 against BA.1.

CONCLUSIONS

- Expression of transgene as a functional protein is demonstrated to occur and then persist for around 7 days in cells transduced with integrase-deleted vectors encoding HSV-eTK. Cell killing activity in cells transduced with HSV-eTK-encoding integrase-deleted (dINT) vectors is comparable at early time points with cells transduced with either an integrase-defective IDRV2 (D125A/D184A) or with wild-type gagpol vector. While payload expression remains steady in cells transduced with the integrating wildtype vectors, expression disappeared over time in cells transduced with dINT or IDRV2 vectors. The absence of payload gene integration was confirmed by ddPCR analysis.
- The ability of payload to be expressed and then to persist for days from a dINT vector without integrating into the cellular genome provides a gene delivery modality that improves on the persistence of mRNA while avoiding concerns about extended expression or integration of integrating vectors. Investigations are ongoing to assess the dINT vector in an *in vivo* model, with applications for both protein expression of therapeutic proteins and for immune responses against antigens for immunotherapy and prophylaxis of cancer and infectious diseases.
- Immunized animals were able to generate neutralizing antibodies in vivo against SARS-CoV-2 using vectors that both encoded the S protein of SARS-CoV-2, strain BA.2) in vectors that had mutations in the integrase gene, such that the vectors are non-integrating (GEN4034 which has a single amino acid mutation in integrase, (D184A), and GEN1044, which has two mutations, (D125A/D184A). The cross-strain SARS-CoV-2 virus protection demonstrates the versatility of the platform for generating vaccines for emerging pathogens.
- MLV vectors that are both non-replicating and non-integrating represent a novel and attractive tool that has potential advantages for certain applications compared to other gene delivery systems (DNA, mRNA, viral) for transient protein expression and use in gene therapy and vaccinology.

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