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Development and use of SIV-based Integrase defective lentiviral vector for immunization

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Abstract

Integrase (IN) defective lentiviral vectors have a high safety profile and might prove useful as immunizing agents especially against HIV-1. However, IN defective SIV-based vectors must be developed in order to test their potential in the non human primate models (NHP) of AIDS. To this aim we tested a novel SIV-based IN defective lentiviral vector for its ability to induce sustained immune responses in mice. BALB/c mice were immunized once intramuscularly with a SIV-based IN defective lentiviral vector expressing the model antigen enhanced green fluorescence protein (eGFP). Immune responses were evaluated 90 days after the injection and compared with those elicited with the IN competent counterpart. The IN defective vector was able to efficiently elicit specific and long-lasting polyfunctional immune responses as evaluated by enzyme-linked immunospot (ELISPOT) assays for interferon- γ (IFN- γ) in spleens, bone marrow (BM) and draining lymph nodes, and by intracellular staining (ICS) for IFN- γ , Interleukin-2 (IL-2) and tumor necrosis factor (TNF- α) in both splenocytes and BM cells without integration of the vector into the host genome. This is the first demonstration that an IN defective SIV-based lentiviral vector provides effective immunization, thus paving the way for the construction of IN defective vectors expressing SIV antigen(s) and test their efficacy against a SIV virus challenge in the NHP model of AIDS.

Keywords

HIV-1; SIV; integrase; episomal DNA; lentiviral vectors; vaccine

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1. Introduction

Non-replicating HIV-based lentiviral vectors have been shown to efficiently elicit strong and long-lasting cell-mediated immune responses in many settings [1]. In particular, lentiviral vectors expressing simian immunodeficiency virus (SIV) and human immunodeficiency virus-1 (HIV-1) antigens or polyepitopes have been shown to induce sustained immunization over a prolonged period of time *in vivo* in mouse models following a single inoculum [2-4]. However, there are safety concerns related to the use of integrating vectors, including the risk of insertional mutagenesis following viral transduction [5-6]. New approaches based on the use of Integrase (IN) defective lentiviral vectors have recently attracted much attention since they strongly minimize the risk of insertional mutagenesis and vector mobilization. The interest in IN defective vectors for delivery of antigens is based on recent studies demonstrating that unintegrated extrachromosomal forms of viral DNA (E-DNA) derived from IN defective lentiviral vectors are transcriptionally active and persistent *in vivo* [7-13]. E-DNA deriving from circularization of reverse transcribed viral DNA is naturally produced during lentiviral infections, and contains either a single copy or a tandem double copy of the long terminal repeat (LTR) promoter region [14]. In particular, HIV-derived IN defective lentiviral vectors produce transcriptionally active E-DNA in the absence of integrated provirus and can be adapted to mediate stable transduction *in vitro* and *in vivo* [15-16]. In this setting, we recently devised and engineered safer IN defective HIV-based lentiviral vectors in order to take advantage of the presentation and prolonged expression of viral antigens from E-DNA in the context of a non replicating whole virus. Importantly, we and others recently provided evidence that a single administration of an IN defective HIV-based lentiviral vector expressing the envelope protein of HIV-1 elicits a robust and sustained immune response in mice in the absence of vector integration [9,12,13]. However, HIV-1 and the derived HIV-based vectors do not efficiently transduce primary simian cells due to the presence of an innate restriction mechanism [17]. This complicates the evaluation of HIV-vector-based vaccine strategies in the non human primate (NHP) model of AIDS, and has led to the need to design and test SIV-based IN defective lentiviral vectors. A recent report showed that an IN mutant of wild type SIV behaves similarly to the HIV counterpart, including production of E-DNA and expression of viral protein from unintegrated templates in primary macrophages [18]. These encouraging observations prompted us to develop and evaluate SIV-based IN defective lentiviral vectors for gene delivery in an *in vivo* model of immunization. Results show that a single intramuscular administration is sufficient to provide sustained polyfunctional immune response in mice. These data support the development of SIV-based IN defective lentiviral vectors as a vaccine delivery system in NHP model of AIDS or other infectious diseases.

2. Materials and Methods

2.1. Vectors construction

A schematic depiction of the vectors used in this study is provided in Figure 1a. Briefly, the parental SIV-based self-inactivating lentiviral vector GAE-EFS-GFP [19] was obtained from Dr. F. L. Cosset and Dr. D. Nègre (INSERM, Paris, France). A ClaI/XhoI fragment of DNA containing the CMV promoter, the eGFP coding sequence and the WPRE sequence [9,20], was cloned in the ClaI/XhoI restricted GAE-EFS-GFP plasmid in place of the EF1 α promoter and eGFP sequence to produce the GAE-GFPW self-inactivating lentiviral vector. Control plasmid GAE-EmptyW was obtained by removal of the eGFP intervening sequence. Plasmid GAE-GFP-IRES-Neo was obtained by removal of the WPRE sequence and substitution with the IRES-Neo fragment of DNA from pFB-Neo (Stratagene, La Jolla, CA, USA). The IN competent packaging plasmid pAd-SIV3+, kindly provided by Dr. F. L. Cosset and Dr. D. Nègre (INSERM, Paris, France), produces all SIV viral proteins with the exception of Env [19]. For construction of the IN defective packaging plasmid pAd-D64V, the IN competent

pAd-SIV3+ was engineered to incorporate an aminoacid mutation in the IN catalytic triad (D64V) known to abolish the IN activity in the HIV-1 background [10,21,22]. Plasmid pMD.G [23], obtained from Dr. D. Trono (EPFL, Lausanne, Switzerland), produces the vesicular stomatitis virus envelope glycoprotein G (VSV.G).

2.2. Production of recombinant vectors

The human epithelium kidney 293T cell line was maintained in Dulbecco's Modified Eagles medium (Euroclone, Life Sciences Division, Pero, Milan, Italy) supplemented with 10 % fetal calf serum (Euroclone, Life Sciences Division, Pero, Milan, Italy) and 100 units/ml of penicillin-streptomycin-glutamine (Gibco Invitrogen, Paisley, UK). For production of recombinant lentiviral vectors, cells were transiently transfected using the Calcium Phosphate-based Profection Mammalian Transfection System (Promega Corporation, Madison WI, USA) as previously described [9]. To produce the control GAE-EmptyW recombinant vector, a fourth plasmid, pEGFP-C3 (Clontech, Palo Alto, CA, USA), was added during transfection in order to rule out the possibility of non specific immune responses from pseudo-transduction of eGFP-containing, but not expressing, lentiviral particles. Vector containing supernatants were concentrated by ultracentrifugation (Beckman Coulter, Inc., Fullerton, CA, USA) for 2 h at 27000 RPM on a 20% sucrose gradient (Sigma Chemical Co. St. Louis, MO, USA). Finally, the viral pellets were resuspended in 1X PBS and stored at -80 °C for further analyses. Viral titres were normalised by the RT [24] and p24 ELISA assays (Innotest, Innogenetics, Belgium).

2.3. Macrophage preparation and infection

Primary macrophages were obtained from murine bone marrow (BM) cells taken from tibiae by syringe insertion into one end of the bone and flushing with RPMI medium. Cells were seeded 100 µl on 96-well plates (Nunc, Roskilde, Denmark) at 1×10^7 cells per ml and were maintained in complete RPMI 1640 medium (Gibco, Invitrogen, Paisley, UK) containing 10% fetal calf serum (Euroclone, Life Sciences Division, Pero, Milan, Italy), 100 units/ml of penicillin-streptomycin-glutamine (Gibco Invitrogen, Paisley, UK), non-essential aminoacids 1X (Gibco, Invitrogen, Paisley, UK), sodium pyruvate 1 mM (Euroclone, Life Sciences Division, Pero, Milan, Italy), hepes buffer solution 25 mM (Euroclone, Life Sciences Division, Pero, Milan, Italy) 50 µM 2-Mercaptoethanol (Sigma Chemical Co. St. Louis, MO, USA). Murine GM-CSF (50 ng/ml Peprotech EC, London, UK) was added to the complete medium. The medium was changed after each three days. Confluent 10-day-old macrophages were transduced with GAE-GFPW/IN- and GAE-GFPW/IN+, observed with the fluorescence microscope every day and photographed at the indicated time points.

2.4. Residual integration activity of the IN defective vector

Human HeLa and murine colic adenocarcinoma C26 cells, were seeded at 5×10^4 per well in 6-well plates. Next day cells were transduced with serial ten-fold dilutions of IN competent GAE-GFP-IRES-Neo/IN+ and IN defective GAE-GFP-IRES-Neo/IN- vectors (range 1×10^5 to 1×10^1 RT counts for the IN competent vector and 1×10^6 to 1×10^2 RT counts for the IN defective vector). The medium was removed 24 h later and replaced with medium supplemented with 800 µg of Geneticin (Gibco Invitrogen, Paisley, UK) every 3 days. Cells were grown for two weeks, and developed clones were fixed with methanol and stained with Giemsa. Clones on each well were counted and expressed as number of colonies/ 10^6 RT counts.

2.5. Mice immunization

Six to eight weeks old BALB/c female mice (purchased from Harlan Italy, S. Pietro al Natisone, Italy), were used in these experiments. Mice were housed and fed in separate cages, according to the experimental group, in accordance with the European Union guidelines and Italian legislation. All studies were reviewed and approved by the internal institutional review

committee. Mice from all groups were injected once intramuscularly (right and left thigh) with 0.2 ml of viral preparation for a total amount of 1.3×10^7 RT units of each vector formulated in PBS. Four mice were injected for each group. Naïve, non immunized mice, were kept for parallel analysis. On day 30 mice were bled orbitally under metaphane-induced anesthesia to collect whole blood in K-EDTA anticoagulant. Leukocytes were obtained after Ammonium Chloride Potassium (ACK) lysis and then used in the IFN- γ ELISPOT assay. On day 90 mice were euthanized and the muscle injection sites were removed for DNA analysis while spleens, BM and draining lymph nodes were taken under sterile conditions for immunological analysis. Single cell suspensions was prepared by mechanical disruption and passage through cell strainers (BD Pharmingen, San Diego, CA) and transferred on tubes containing complete RPMI 1640 medium and used in the assays.

2.6. IFN- γ ELISPOT assay

The IFN- γ ELISPOT assay was performed using reagents from Mabtech (Mabtech AB Gamla Värmdöv, Sweden) as described [2,9]. Spot forming cells (SFC) were counted with an ELISPOT reader (AID, Amplimedical Bioline, Turin, Italy) and expressed as SCF/ 10^6 cells. A 9mer containing the H-2^d restricted GFP-9mer peptide (HYLSTQSAL) [25] (UFPeptides s.r.l., Ferrara, Italy) was used to stimulate GFP-specific CD8 T cells, medium alone and the unrelated H-2^d restricted HIV-1 gp120 V3 loop epitope peptide (JR-9mer, IGPGRAPHYT) (UFPeptides s.r.l., Ferrara, Italy) [26] were used as negative controls while Concanavalin A (SIGMA, Chemical Co. St. Louis, MO, USA) (5 μ g/ml) was used as a positive control. Samples (specific and unrelated peptides treated wells) were subtracted of the values obtained in the Medium-treated wells (background) and were scored positive when a minimum of 50 spots per 10^6 cells and a fold of 2 or higher compared to the unrelated peptide was observed.

2.7. Intracellular staining for cytokines (ICS)

Spleen and bone marrow-derived cells from each immunization group were pooled and cultured in the presence of GFP-specific (5 μ g/ml) or JR-unrelated (5 μ g/ml) peptides for 6 h in the presence of anti-mouse CD28 mAb (BD Pharmingen, San Diego CA, clone 37.51) at 2 μ g/ml. PMA (50 ng/ml) (Sigma Chemicals, Co., St Louis, MO, USA) in combination with Ionomycin (2 μ g/ml) (Sigma Chemicals, Co., St Louis, MO, USA) were used as positive control. One hour after stimulation, 10 μ g/ml of Brefeldin A (Sigma Chemicals Co., St Louis, MO, USA) was added to the cultures to inhibit cytokine secretion. Cells were stained on the membrane with direct fluorochrome conjugates PE-Cy5 anti-mouse CD8 (clone 53-6.7) (BD, Pharmingen, San Diego CA, USA) or the isotype-matched mAb (BD Pharmingen, San Diego, CA, USA), washed, and fixed with 4% paraformaldehyde (Sigma Chemicals, Co., St Louis, MO, USA), permeabilized in PBS-0.5% saponin (Sigma Chemicals, Co., St Louis, MO, USA) and stained with APC-labeled anti-mouse IFN- γ mAb (clone XMG1.2) and PE-labeled anti-mouse TNF- α (clone MP6-XT22) or PE-labeled anti-mouse IL-2 (clone JES6-5H4) or their isotype-matched controls (BD Pharmingen, San Diego CA, USA). Samples were washed and analyzed by flow cytometer (FACSanto, BD Biosciences).

2.8. DNA Isolation and PCR

Genomic DNA from the site of injection was extracted using the SV Total RNA Isolation System protocol, modified for DNA preparation (Promega Corporation, Madison WI, USA) [27]. All samples supported the amplification of the mouse Glyceraldehyde 3-Phosphate Dehydrogenase gene (GAPDH Control Amplimer Set, Clonthech, Mountain View, CA). Detection of the lentiviral vector DNA sequence was performed using 500 ng of DNA and a couple of primers spanning the CMV region of the vector (CMVfor: 5'-ACGCCAATAGGGACTTTCCATTGAC-3'; CMVas: 5'-ACGCCCATTGATGTACTGCCAAA-3'). Analysis of integrated vector sequence was

performed using a B2-PCR assay as described [9], with minor modifications. Briefly, in a first set of amplifications, primer B2AS (5'-ATATGTAAGTACACTGTAGC-3') was used together with the CMVfor primer. After the first amplification, a nested PCR was performed using two different internal primers in the eGFP sequence of the vector genome (GFPfor1: 5'-CTGACCTACGGCGTGCAGTGCTTCA-3'; GFPprev2: 5'-TGTGCCCCAGGATGTTGCCG-3'). A nested PCR approach was performed to detect the 2-LTR form of lentiviral vector E-DNA using outer primer pair FORN1/REVN1 (FORN1: 5'-GTG ACT CCA CGC TTG TTT GC-3'; REVN1: 5'- CTC CTG TGC CTC ATC TGA TAC C-3') and inner primer pair SIVforN3/SIVU3rev (SIVforN3: 5'-AAGACCTCTTCAATAAAGCTGCC- 3'; SIVU3rev: 5'-CATAGCCAGCCAAATGTCTTTGG-3'), as described [28]. All the above PCR products were analyzed by gel electrophoresis on ethidium bromide 2% agarose gel.

2.9. Cloning and sequencing of the 2-dLTR circle junctions

After purification from agarose gel, 2-dLTR containing PCR products were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA), sequenced with T7AS primer using an ABI/Prism-377 DNA sequencer (Perkin Elmer) and subjected to alignment using the AlignX program of the Vector NTI Advance suite from Invitrogen (Carlsbad, CA, USA).

2.10. Statistical analysis

Statistical analyses were performed by non parametric Mann-Whitney U test. All *P* values were two tailed and considered significant if less than 0.05. All analyses were performed using SPSS for Windows version 15.0 (SPSS Inc., Chicago Illinois US).

3. Results

3.1. In vitro expression of SIV-derived IN defective vectors and residual integration activity

Previous research has shown that the HIV-1_{IN/D64V} mutant completely abolish viral DNA integration of HIV-1 and HIV-derived lentiviral vectors without affecting expression from unintegrated templates [10,21,22]. In order to confirm this in the SIV system, the IN defective and IN competent recombinant vectors expressing eGFP (GAE-GFPW/IN- and GAE-GFPW/IN+, respectively) were tested for their ability to express over time in non dividing primary murine macrophages. Macrophages transduced with IN competent and IN defective recombinant viruses expressed eGFP at both early and late time points (Fig. 1b). Expression from the IN defective lentiviral vector was lower than that of the wild type counterpart (Fig. 1c), in agreement with previous data in the HIV-1 system [29,30]. As expected, expression in cycling human 293T and murine C26 cells infected with the IN defective vector was transient and lasted only a few days after infection (data not shown).

In order to evaluate the residual integration activity of the mutant IN defective lentiviral vector, IN competent and IN defective recombinant viruses expressing the neomycin resistance gene (GAE-GFP-IRES-Neo/IN+ and GAE-GFP-IRES-Neo/IN-) were generated and used to transduce human HeLa and murine C26 cells. Both cell types were infected with serial dilutions of each vector and subjected to selection with Geneticin. As expected, the number of colonies in the IN competent vector selected cells were significantly greater (4.01×10^5 and 8.72×10^5 colonies per 1×10^6 RT counts in HeLa cells and 9×10^5 and 8×10^5 colonies per 1×10^6 RT counts in C26 cells), than for the IN defective vectors (338 and 368 colonies per 1×10^6 RT counts in HeLa cells and 294 and 147 colonies per 1×10^6 RT counts in C26 cells) (Table 1). At the indicated doses, the IN defective vector integrated between 1.8×10^{-4} and 8.4×10^{-4} times less frequently than the IN competent vector, demonstrating the IN defective vector maintained the integration deficient phenotype following transduction of both murine

and human cells, with negligible amounts of residual integration activity; these results are consistent with what has been found in the HIV system [7-9].

3.2. In vivo presence and persistence of the immune response

Following the *in vitro* evaluation of expression of the IN defective lentiviral vector, the aim of the subsequent study was to evaluate the *in vivo* strength and persistence of immune response following a single intramuscular inoculum of the IN defective SIV-based lentiviral vector in comparison with the IN competent counterpart. We selected eGFP protein as model antigen, based on its high immunogenicity in BALB/c mice [26].

3.2.1. IFN- γ ELISPOT Response—At day 30 after the inoculum, in order to analyze the eGFP specific T cell response, IFN- γ (IFN- γ) ELISPOT assay was performed on ACK-treated whole blood cells stimulated with the H-2^d specific GFP-9mer peptide or with the H-2^d-matched JR-9mer unrelated peptide. Results showed that a strong eGFP specific response was present on cells derived from the mice immunized with both the integrating and non-integrating GAE-GFPW lentiviral vectors (Fig. 2). Importantly, blood from mice immunized with the IN defective vector displayed a high number of IFN- γ producing cells (Spot Forming Cells, SFC/10⁶ cells ranged from 914 to 1704, with an average value of 1383). This effective and specific immune response confirmed our previous data obtained with a similar but HIV-based IN defective lentiviral vector [9]. As expected, in the IN competent transduced mice the antigen specific response was maintained at high levels as well (SFC/10⁶ cells ranged from 1276 to 1794, with an average value of 1452). No statistical differences were seen between the two vaccinated groups ($P = 0.629$). No specific responses were detected in the control mice (inoculated with the control GAE-EmptyW vector or in naïve mice) or in PBMC pulsed with the H-2^d-matched unrelated JR-9mer peptide (Fig. 2).

At 90 days after the inoculum, mice were sacrificed and *ex vivo* splenocytes, bone marrow (BM) cells and draining lymph nodes cells were evaluated for immunological responses.

As detected by IFN- γ ELISPOT, splenocytes derived from mice immunized with the IN defective vector specifically produced IFN- γ (SFC/10⁶ cells ranged from 85 to 1308, with an average value of 641), indicating that the antigen-specific immune response was still present 3 months after the single inoculum. As expected, in mice immunized with the IN competent vector the antigen specific response was maintained at higher levels (SFC/10⁶ cells ranged from 990 to 1095, with an average value of 1061) (Fig. 3a).

Analysis of the presence of antigen-specific memory T cells in BM at 90 days from the inoculum confirmed the results observed in spleens. In fact, a high number of IFN- γ producing cells was detected in BM from all the IN defective vector immunized mice upon stimulation with the GFP-9mer peptide (SFC/10⁶ ranged from 55 to 740, with an average value of 308) (Fig. 3b). The IFN γ production from BM cells of the mice immunized with the IN competent vector remained at high levels as well (SFC/10⁶ cells ranged from 573 to 815, with an average value of 668).

We also evaluated epitope-specific IFN- γ production in the lymphocytes from the draining lymph nodes (Fig. 3c). Pooled cells from each group were stimulated with specific GFP-9mer peptide or JR-9mer unrelated peptide. Both the IN defective and IN competent immunized mice responded to the GFP-9mer peptide; in particular the average value in the IN competent group and IN defective group was 490 SFC/10⁶ cells and 223 SFC/10⁶ cells respectively.

Specific responses were absent in splenocytes, BM cells and lymph nodes cells derived from mice immunized with the control vector and from naïve mice. The use of the H-2^d-matched unrelated JR-9mer peptide did not show as well any response (Fig. 3).

3.2.2. Analysis of multifunctional cells—Multifunctional GFP-specific CD8⁺ T cell responses were evaluated in splenocytes and BM cells by intracellular cytokine staining (ICS) for IFN- γ , Interleukin-2 (IL-2) and TNF- α (TNF- α , following stimulation with the specific GFP-9mer and the unrelated JR-9mer peptides.

The IN competent and IN defective vectors elicited a strong CD8⁺ GFP-specific T cell response in both splenocytes and BM cells, when compared to control mice. In particular, double positive IFN- γ /IL-2 CD8⁺ cells were 1.18% and 0.61% in splenocytes and 0.79% and 0.49% in BM cells derived from the IN competent and the IN defective immunized mice, respectively (Fig. 4, left panels). A much stronger response was evident when evaluating double positive IFN- γ /TNF- α producing CD8⁺ T cells; dual producing IFN- γ /TNF- α CD8⁺ T cells were 2.66% and 1.64% in splenocytes and 4.89% and 4.21% in BM from the IN competent and the IN defective immunized mice, respectively (Fig. 4, right panels). All these results indicate that non-integrating lentiviral vectors are able to induce polyfunctional antigen-specific CD8⁺ cells in mice.

No specific responses were detected in the splenocytes and BM cells derived from the control or the naïve mice pulsed with GFP-9mer, and the use of an H-2^d-matched unrelated JR-9mer did not reveal any response, indicating the specificity of the immune response (Fig. 4 and data not shown).

3.2.3. Lentiviral vector sequence in the immunized mice—Mice were evaluated at 90 days after vectors administration for presence and persistence of integrated and unintegrated lentiviral vector sequences at the injection site as described in the Experimental Procedures. Following DNA extraction, all samples were subjected to GAPDH amplification to ensure DNA quality and integrity (Fig. 5d). Next, the presence of the vector at the injection site was confirmed in all muscle samples from both the IN competent and IN defective inoculated mice (Fig. 5a). All IN defective immunized mice possessed unintegrated DNA as evaluated by a 2-dLTR PCR assay using a primer pair spanning the junction between the two dLTR (Fig. 5b). Conversely, not all the IN competent immunized mice presented episomal DNA forms. In particular, mice 6 and 8 did not show presence of a PCR product, while DNA samples from mice 5 and 7 showed evidence of additional PCR products. Identity of 2-dLTR circles junction sequences from all PCR reactions was confirmed by sequencing of the PCR products (Fig. 6). A mixed-sequence phenotype was found at the junction between the two dLTR in the PCR products of the IN competent group, while the majority of the samples from the IN defective group contained the expected sequence, corresponding to the joining of the unprocessed two dLTR termini, indicative of the presence of an ineffective IN protein in the transducing vector also *in vivo*. Since the D64V mutation present in the IN defective vector was supposed to abolish IN function, consequently abolishing 3' processing of the reverse transcribed viral DNA ends, the frequency of wild-type junctions was expected to be higher than in the IN competent counterpart. Consistently, the frequency of the wild-type junctions was significantly higher in the IN defective (87.5%, corresponding to 7 out of 8 sequenced clones containing wild type junctions [2 clones per PCR reaction per mouse]) than in the IN competent inoculated mice (16.6%, corresponding to 1/6 sequenced clones containing wild type junctions [3 clones per PCR reaction per PCR positive mouse]). This indicates that absence of 3' processing *in vivo* in the GAE-GFPW/IN- leads to an increase in the frequency of 2-dLTR circles containing wild-type junctions and that the vector maintained the integration defective phenotype *in vivo*.

More importantly, evaluation of integration with the B2-PCR assay indicated that while all samples derived from the mice inoculated with the IN competent vector showed clear evidence of integrated vector sequences (Fig. 5c), all those derived from the mice inoculated with the IN defective vector did not show evidence of integrated vector sequences in up to 1 μ g of genomic DNA in any of the tested samples at 90 days from the injection (Fig. 5c).

4. Discussion

In this report we show that a single administration of a SIV-based IN defective lentiviral vector expressing a model antigen provides sustained immunization over a three-month period in mice. This is in agreement with recent data indicating that HIV-based IN defective lentiviral vectors were expressed *in vivo* [7,8,10,11] and were successful in inducing immunization in the mouse model [9,12,13]. The development and evaluation of the SIV-based IN defective vector was deemed necessary for its subsequent use for immunization purposes in the NHP model of SIV/SHIV infection. In fact, due to an innate post-entry restriction mechanism, HIV-1 and HIV-derived vectors cannot be proficiently used in commonly used NHP model of AIDS such as rhesus and cynomolgus macaques [17] (and references therein) and they would consequently not perform well in an *in vivo* setting. This appears to be partly in contrast with a recent report suggesting that rhesus macaques derived dendritic cells (DC) are susceptible to infection by HIV-derived vectors *in vitro* [31]. However, the same report demonstrated that primary rhesus macaques macrophages and T cells are resistant to transduction with HIV-derived vectors, thus suggesting that other antigen presenting cells might be resistant as well following *in vivo* transduction with HIV-derived vectors. In this context, a side-by-side comparison of the two different vector system in an *in vivo* setting would be important.

In the first set of experiments we have shown that the SIV-based IN defective vectors behave as their cousin HIV-based counterpart. In particular, they were able to stably transduce non dividing primary macrophages while expression in cycling cells was transient and rapidly lost following cellular division. Similarly to the HIV-derived vectors, expression from transduced macrophages was lower than that from macrophages transduced with the parental IN competent vector. This was expected and in accordance with a recent report using an IN mutant of wild type SIV [18] since transcription from E-DNA is lower than that from integrated templates. Importantly, residual integration levels, namely the ability of E-DNA from IN defective vectors to recombine with host genomic DNA, was negligible, and similar to that previously reported in the HIV-based vector system [7-9,13]. In the next set of experiments we evaluated the strength and durability of the immune response induced in the mouse model following a single injection with the IN defective vector and whether the IN defective phenotype we found *in vitro* was maintained in the *in vivo* model system as well.

Results indicated that a single *in vivo* immunization with SIV-derived non-integrating lentiviral vector coding for the eGFP protein stimulated a specific and long-lasting cellular immune response in BALB/c mice. At 30 days following vectors administration cellular immune responses were similar in terms of antigen specific IFN- γ expressing cells in whole blood from both groups of mice. The immune response was maintained at 90 days after the single immunization, as splenocytes, BM and lymph node cells from mice inoculated with the IN defective lentiviral vector were still showing marked and specific immune responses. Overall, immune responses were generally higher following immunization with the IN competent vector. This was expected, due to vector integration, and comparable to that reported in the HIV system [9,12,13]. Importantly, CD8+ T cells were polyfunctional since they were able to secrete IFN- γ in conjunction with TNF- α and IL-2, as evaluated by ICS analysis. Notably, ICS analysis of the antigen-specific cellular responses recovered at 90 days in BM cells from the IN defective group indicated that they were present at levels comparable to those recovered in the IN competent inoculated mice and at levels more pronounced than those recovered in spleens, confirming the assumption that BM plays a dominant role in the maintenance of memory T cells [32]. We focused on IL-2, IFN- γ and TNF- α production because T cell responses to vaccination or infection are characterized by populations of cells that produce various combinations of these cytokines [33-35]. These differences have profound implications for effector function and development into memory cells. For example, multifunctional cells that secrete all three cytokines have been shown to produce higher levels of cytokine per cell

[36-38] and the frequency of such multifunctional cells has been shown to be a correlate of non-progressor status in SIV and HIV-1 infection [39-41].

Different routes of injection have been used in various non-integrating lentivector immunization studies, including subcutaneous (s.c) [13], intramuscular (i.m.) [9,13] and intraperitoneal (i.p.) injection [12]. In all these studies sustained humoral and cellular immune responses have been detected, suggesting that non-integrating lentiviral vectors were able to proficiently transduce antigen presenting cells (APC) at the different sites of injection, resulting in a strong antigen-specific immune response. We could hypothesize that in the case of intramuscular injection long-lived differentiated cells, such as resident professional APC and muscle cells themselves, are potential target of non-integrating lentiviral vectors, both HIV- and SIV-based, as already described for DNA immunization [42-44] and for non-integrating lentiviral vectors [10]. In the case of subcutaneous or intraperitoneal immunization, the probable target cells are represented by numerous resident professional APC, such as skin-derived dendritic cells or macrophages, respectively.

Importantly, while E-DNA was present and intact in the IN defective inoculated mice, we did not find evidence of integrated proviral sequences in the mice inoculated with the IN defective vector, thus demonstrating that this vector maintains its integration deficient phenotype *in vivo* and confirming earlier results using HIV-based IN defective lentiviral vectors *in vivo* [8, 9].

The present report provides the first demonstration that an IN defective SIV-based lentiviral vector induces an effective immunization. These results emphasize the potential of the non-integrating lentiviral vectors as a new and safe vaccine delivery tool for vaccination (alone or in combination) against infectious diseases, including HIV-1, warranting further testing in NHP models, and calling for the optimization of the vector construction and immunization strategy in order to improve the immunological response. Although their evaluation in human clinical trials is still a long way off, optimization of the vaccination strategy and rigorous preclinical safety studies in NHP models should be performed to achieve this aim.

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Figure 1.

Vectors used and *in vitro* GFP expression. (a) Schematic depiction of the SIV-based lentiviral vectors used in this study. Ψ , indicates the packaging signal; PBS, the primer binding site; SD and SA major splice acceptor site, respectively; bGHpA, bovine Growth Hormone polyadenylation signal; cPPT, central polypurine tract; CMV, cytomegalovirus promoter; WPRE, Woodchuck hepatitis post-transcriptional regulatory element. The lentiviral vector GAE-GFP-IRES-Neo, containing the IRES internal ribosome entry site, expresses the neomycin resistance gene conferring resistance to Geneticin treatment. (b) eGFP expression in macrophages infected with IN defective (left) and IN competent (right) vectors analyzed at the indicated time points by light microscopy and fluorescent microscopy. (c) Mean fluorescence intensity (MFI) of vectorencoded eGFP expression levels in primary macrophages transduced with IN-competent and IN-defective vectors at 7 days post-transduction was determined by flow cytometry.

Figure 2.

IFN- γ responses 30 days after immunization. IFN- γ ELISPOT assay was performed on whole blood cells from the mice immunized with GAE-GFPW/IN+, GAE-GFPW/IN-, GAE-EmptyW and in naive mice 30 days after the injection. Cells were stimulated overnight with the specific GFP-9mer and the unrelated JR-9mer. The number of IFN- γ producing cells is expressed as SFC/10⁶ cells. Error bars represent the SE of the average. The *P* values are indicated for intergroup comparisons.

Figure 3.

IFN- γ responses 90 days after immunization. IFN- γ ELISPOT assay was performed on (a) fresh splenocytes, (b) BM cells and (c) lymph nodes cells from the mice immunized with GAE-GFPW/IN+, GAE-GFPW/IN-, GAE-Empty and in naive mice. Analysis was performed on cells stimulated overnight with the indicated stimuli. The number of IFN- γ producing cells is expressed as SFC/10⁶ cells after background subtraction. The average for each dataset is indicated by the columns and *P* values are indicated for intergroup comparisons.

Figure 4.

Analysis of antigen specific CD8⁺ response by intracellular staining assay for IFN- γ , IL-2 and TNF- α productions. Analysis of intracellular cytokine production was performed on gated CD8⁺ T cells. Splenocytes or BM-derived cells were cultured with the indicated stimuli for 6 hrs (see Materials and Methods section), labelled with anti-IFN- γ and IL-2 or IFN- γ and TNF- α and analysed with FACSanto. Percentages of dual-producing CD8⁺ T cells are indicated on top right of each panel.

Figure 5. Lentiviral vector sequences in the immunized mice. PCR analysis for evaluation of (a) vector presence, (b) 2-dLTR circles presence, and (c) vector integration in DNA extracted from muscle samples at the vector injection site. DNA quality and integrity of all samples was evaluated by PCR amplification of GAPDH of 100 ng of DNA (d). All PCR samples were run on a 2% agarose gel.

Figure 6. Nucleotide sequence at the 2-dLTR circle junction. The sequences were aligned to the corresponding U5-ΔU3 2-dLTR junctional sequence of GAE-GFPW reference sequence using the AlignX program of the Vector NTI Advance suite (Invitrogen, Carlsbad, CA, USA). The upper panel shows the sequences corresponding to the PCR products of the IN defective vector, while the lower panel shows those corresponding to the PCR products of the IN competent vector. Dots correspond to identical residues in the reference sequence while dashes represent gaps introduced in the figure for alignment purposes. In the reference sequence the outer and inner primers used in the nested PCR are indicated. The invariant GAT and AC nucleotides are shown in bold. Nucleotides in lower case are those derived from the tRNA primer binding site or from the PPT next to the deleted U3.

Table 1

DNA recombination frequencies of SIV-based IN competent and IN defective vectors

Vector (cell type)		Number of colonies/ 1×10^6 RT counts		Ratio
		wild type	D64V	D64V/WT
GAE-GFP-IRES-Neo (HeLa)	Exp1	$4,01 \times 10^5$	338	$8,4 \times 10^{-4}$
	Exp2	$8,72 \times 10^5$	368	$4,2 \times 10^{-4}$
GAE-GFP-IRES-Neo (C26)	Exp1	9×10^5	294	$3,2 \times 10^{-4}$
	Exp2	8×10^5	147	$1,8 \times 10^{-4}$