Successful Immunization with a Single Injection of Non-integrating Lentiviral Vector

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We evaluated the ability of an integrase (IN)-defective self-inactivating lentiviral vector (sinLV) for the delivery of human immunodeficiency virus-1 (HIV-1) envelope sequences in mice to elicit specific immune responses. BALB/c mice were immunized with a single intramuscular injection of the IN-defective sinLV expressing the codon optimized HIV-$1_{JR\text{-}FL}$ gp120 sequence, and results were compared with those for the IN-competent counterpart. The IN-defective sinLV elicited specific and long-lasting immune responses, as evaluated up to 90 days from the immunization by enzyme-linked immunosorbent spot (ELISPOT) and intracellular staining (ICS) for interferon-γ (IFN-γ) assays in both splenocytes and bone marrow (BM) cells, chromium release assay in splenocytes, and antibody detection in sera, without integration of the vector into the host genome. These data provide evidence that a single administration of an IN-defective sinLV elicits a significant immune response in the absence of vector integration and may be a safe and useful strategy for vaccine development.

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INTRODUCTION

Despite the major recent developments in human immunodeficiency virus-1 (HIV-1) therapeutics, there were an estimated 4.1 million new cases in 2005 and the HIV-1 epidemic continued to grow, highlighting the need for an effective vaccine (World Health Organization 2006 report).[1] Several approaches based on viral vectors, including modified vaccinia virus Ankara, adenovirus, and adeno-associated virus expressing different combinations of viral antigens, have been used in various prime–boost protocols and have shown some level of control of viremia after challenge in simian models.[2–9] Unfortunately, to date only vaccination with live attenuated simian immunodeficiency virus (SIV) has actually prevented infection, in the macaque model, and this approach is not considered safe for humans.[10–13] Nevertheless, the utility of attenuated SIV as a vaccine in the primate model is an important observation, suggesting that presentation of viral epitopes within the context of the whole virus could elicit a protective immune response in certain hosts.[11]

One strategy to overcome some of the safety issues relating to an attenuated virus is to express multiple viral gene products in the context of SIV/HIV-derived non-replicating lentiviral vectors. Lentiviral vectors have been shown efficiently to transduce non-replicating cells *in vitro* as well as *in vivo*,[14,15] and, recently, we reported that a single intramuscular administration of a self-inactivating (sin) HIV-based lentiviral vector elicits strong cell-mediated immune responses.[16,17] However, safety concerns around the use of these vectors for delivery of therapeutic genes or as a vaccine include insertional mutagenesis and vector mobilization after viral infection. In particular, the use of integrating retroviral vectors has been associated with the development of leukemia-like conditions in children who received a retroviral vector as treatment for X-linked severe combined immunodeficiency disease (XSCID).[18,19]

To minimize the risk of insertional mutagenesis, integrase (IN)-defective lentiviral-based vectors can be engineered to present viral antigens in a similar but safer manner. This takes advantage of the presentation and prolonged expression of viral antigens from unintegrated extra-chromosomal forms of viral DNA (E-DNA) in the context of a non-replicating whole virus, which constitutes a major safety advantage compared with previously attenuated candidates. E-DNA, which derives from circularization of reverse-transcribed viral DNA, is produced naturally during lentiviral infections and contains either a single copy or a tandem double copy of the long terminal repeat (LTR) region, including the viral transcription initiation and termination control elements.[20] HIV-derived IN-defective viruses and lentiviral vectors are transcriptionally active, although at lower levels than the integrated wild-type counterpart, and produce E-DNA in the absence of noticeable amounts of integrated provirus;[21] moreover, they can be modified to mediate stable transduction *in vitro*[22–25] and *in vivo*.[26,27]

Here, we demonstrate that a single immunization with a non-integrating lentiviral vector carrying the codon optimized HIV-$1_{JR\text{-}FL}$ gp120 in mice results in a strong and sustained

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immune response characterized by the presence of anti-gp120 antibodies and effector-specific T cells and the generation of a specific T-cell memory. These results support the development of IN-defective lentiviral vectors as a vaccine delivery system, especially for HIV-1.

RESULTS

A single immunization with a non-integrating sinLV induces strong and durable cellular immune responses

The presence and persistence of the immune response elicited by a non-integrating sin lentiviral vector (sinLV) expressing the codon optimized HIV-$1_{JR\text{-}FL}$ gp120 (TY2-JRmZ/IN$^-$) was compared with the effects of the integration-competent sinLV counterpart (TY2-JRmZ/IN$^+$) in BALB/c mice at 30 and 90 days after a single intramuscular immunization. At day 30, all mice immunized with TY2-JRmZ/IN$^+$ displayed a high number of cells expressing interferon-γ (IFN-γ) in the presence of the H-2^d-specific JR-9-mer (the number of spot-forming cells (SFCs) per 10^6 cells ranged from 960 to 1,602, with a median value of 1,142; **Figure 1b**). This effective and specific immune response confirmed our previous data obtained with a similar HIV-$1_{JR\text{-}FL}$ gp120–expressing lentiviral vector.[15] A single immunization with the TY2-JRmZ/IN$^-$ vector also elicited a robust response (the number of SFCs per 10^6 cells ranged from 832 to 2,060, with a median value of 1,192; **Figure 1b**). This is the first demonstration that non-integrating lentiviral vectors can induce a specific immune response against an HIV antigen. No specific responses were detected in the control mice or in splenocytes pulsed with the H-2^d-matched unrelated green fluorescent protein (GFP)-9-mer peptide (**Figure 1b**).

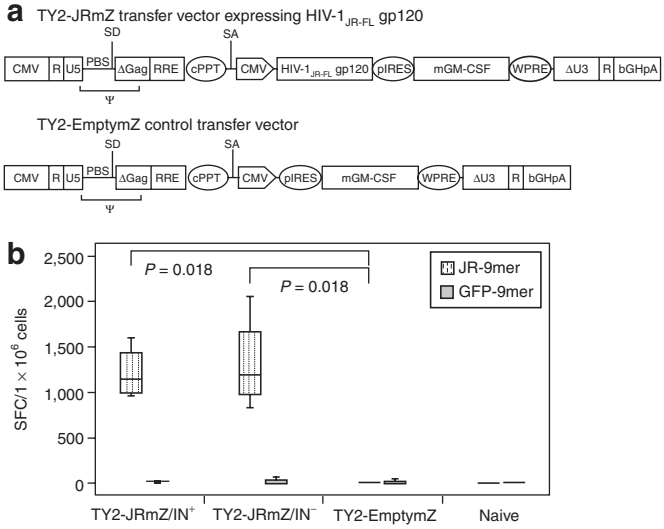

Figure 1 (a) Transfer vectors used in this study. The packaging signal (ψ), primer binding site (PBS), major splice donor (SD) and acceptor (SA) sites, cytomegalovirus promoter (CMV), bovine growth hormone polyadenylation signal (bHGpA), central polypurine tract (cPPT), internal ribosomal entry site (IRES) from poliovirus (pIRES), rev responsive element (RRE), mouse granulocyte monocyte–colony stimulatory factor (mGM-CSF), and woodchuck hepatitis post-transcriptional regulatory element (WPRE) are indicated. (**b**) Interferon-γ (IFN-γ) enzyme-linked immunosorbent spot assay on fresh splenocytes from the mice immunized with TY2-JRmZ/IN$^+$, TY2-JRmZ/IN$^-$, TY2-EmptymZ and in naïve mice 30 days after injection. Analysis was performed on splenocytes stimulated overnight with the indicated stimuli. IFN-γ-producing T cells are expressed as the number of spot-forming cells (SFCs) per 10^6 cells after background subtraction. The median for each dataset is indicated by the black center line of the box plots, and *P*-values are indicated for intergroup comparisons. GFP, green fluorescent protein; HIV, human immunodeficiency virus.

To verify the durability of the specific immune response, further groups of mice were killed at 90 days after the single injection of the sinLV.

All mice immunized with the TY2-JRmZ/IN$^+$ vector maintained a high level of specific antigen response, as determined by IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay on *ex vivo* splenocytes, with the number of SFCs per 10^6 cells ranging from 1,135 to 1,935, with a median value of 1,482 (**Figure 2a**). Splenocytes derived from TY2-JRmZ/IN$^-$-immunized mice also maintained a high level of specific antigen responsiveness, with the number of SFCs per 10^6 cells ranging from 585 to 1,675, with a median value of 839 (**Figure 2a**). To determine the phenotype of the IFN-γ-producing cells, an intracellular staining (ICS) for IFN-γ was performed. The percentage of antigen-specific CD8$^+$ cells expressing IFN-γ was 4 and 2.8% in the TY2-JRmZ/IN$^+$ and TY2-JRmZ/IN$^-$ vector–injected mice, respectively (**Figure 2b**). In both instances these cells (94.3 and 94.4%, respectively) were CD62L$^-$, indicating an effector memory phenotype (data not shown). The presence of cytotoxic T lymphocytes was investigated on splenocytes stimulated with irradiated naïve splenocytes pulsed with the JR-9-mer peptide. Specific lysis of target cells was high at all effector-to-target ratios analyzed in all the mice immunized with the TY2-JRmZ/IN$^+$ vector (average values of specific lysis from 60 to 40% at effector-to-target ratios from 40:1 to 10:1, respectively) (**Figure 2c**) and the TY2-JRmZ/IN$^-$ vector (average values of specific lysis from 35 to 11.5% at effector-to-target ratios from 40:1 to 10:1, respectively), although the figures for TY2-JRmZ/IN$^-$ were lower (**Figure 2c**). No specific responses were detected in the control mice or in splenocytes pulsed with the H-2^d-matched unrelated GFP-9-mer peptide.

Immune response in bone marrow cells

To check the presence of antigen-specific memory T cells in bone marrow (BM), both ELISPOT and ICS for IFN-γ were performed on BM-derived cells. As expected, TY2-JRmZ/IN$^+$-immunized mice showed high levels of antigen-specific IFN-γ-producing cells localized in BM (the number of SFCs per 10^6 cells ranged from 82 to 652, with a median value of 281; **Figure 3a**). Importantly, a high number of antigen-specific IFN-γ-producing cells were also detected in BM from all the TY2-JRmZ/IN$^-$-immunized mice (the number of SFCs per 10^6 cells ranged from 182 to 752, with a median value of 440; **Figure 3a**). This is particularly striking in light of the fact that only 2% of the cells derived from BM were CD8$^+$. Both groups of treated mice also showed high levels of CD8$^+$ IFN-γ-producing cells in the ICS assay upon stimulation with JR-9-mer peptide (9.97 and 8.05% of specific cells in TY2-JRmZ/IN$^+$- and TY2-JRmZ/IN$^-$-immunized mice, respectively; **Figure 3b**). As already observed in splenocytes, these cells (98.3 and 95.4%, respectively) were CD62L$^-$, indicating an effector memory phenotype (data not shown). No specific responses were detected in the control mice or in BM cells pulsed with the H-2^d-matched unrelated GFP-9-mer peptide (**Figure 3a** and **b**).

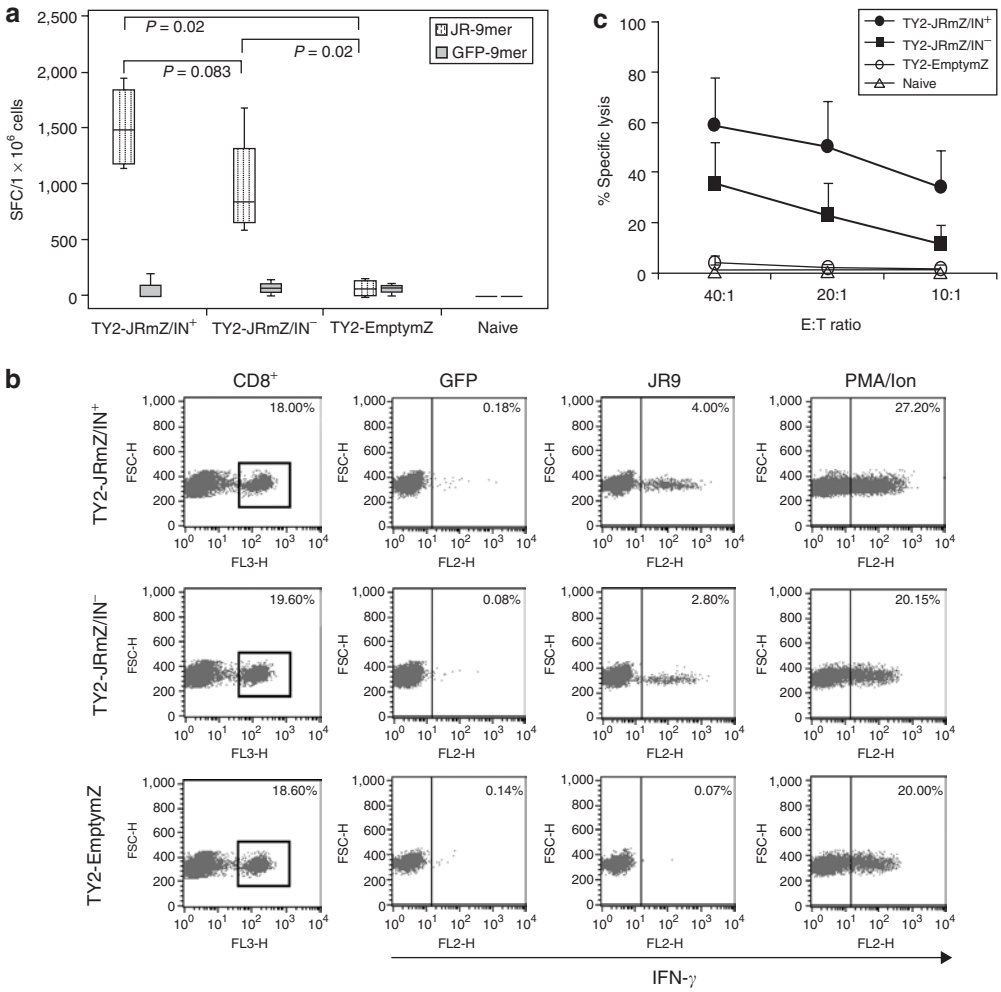

Figure 2 T-cell responses measured on splenocytes 90 days after immunization with self-inactivating lentiviral vector. (**a**) An interferon-γ (IFN-γ) enzyme-linked immunosorbent spot assay was performed on splenocytes stimulated overnight with the indicated stimuli. IFN-γ-producing T cells are expressed as the number of spot-forming cells (SFCs) per 10^6 cells after background subtraction. The median for each dataset is indicated by the black center line of box plots and *P*-values are indicated for intergroup comparisons. (**b**) Intracellular staining for IFN-γ production on gated $CD8^+$ T cells after incubation with the indicated stimuli. Percentages of $CD8^+$ IFN-γ-producing cells are indicated. (**c**) Anti-HIV-$1_{JR\text{-}FL}$ envelope-specific cytotoxic T-lymphocyte activity in mice immunized with TY2-JRmZ/IN^+ (filled circles), TY2-JRmZ/IN^- (filled squares), and TY2-EmptymZ (open circles), and in naive mice (open triangle). A ^{51}Cr release assay was performed after 4 days' *in vitro* culture, as described in Materials and Methods. The percentage of specific lysis is shown at three different effector-to-target ratios: 40:1, 20:1, and 10:1. GFP, green fluorescent protein; PMA/Ion, phorbal 12-myristate 13-acetate/ionomicin.

Humoral response

At both time points, mice injected with JREnvmZ/IN^+ vector displayed anti-gp120 immunoglobulin G titers significantly higher ($P < 0.05$) (median titer 1:9,600 at both time points) than those from the mice injected with JREnvmZ/IN^- vector (median titer 1:500 and 1:300 at 30 and 90 days, respectively) (**Figure 4a**). These results are consistent with the observation that IN-defective vectors express a lower amount of protein than IN-competent vectors.

Lentiviral vector sequence in the immunized mice

All DNA samples were subjected to glyceraldehyde 3-phosphate dehydrogenase amplification to ensure DNA integrity (**Figure 5d** and **h**). The presence of the vector at the injection site was confirmed in all muscle samples from the mice injected with the IN-competent and IN-defective vectors (**Figure 5a** and **e**). Differences in intensity of polymerase chain reaction (PCR) products observed at 90 days may reflect instability of the vector *in vivo* over time or the host's immune response to the cells containing the transgene (**Figure 5e**), in addition to sampling differences in size and amount of muscle collected at the time of killing. Similarly, all mice possessed unintegrated DNA at both time points, as evaluated by a 2-dLTR PCR assay, with the only exception of mouse 16, injected with JREnvmZ/IN^+ vector and evaluated 3 months after injection (**Figure 5b** and **f**). All of the PCR products from the DNA derived from IN-defective-injected mice were of the expected size, but the PCR products from DNA derived from the IN-competent-injected mice did not always correspond to the anticipated size. In particular, the DNA sample from mouse 14, injected with JREnvmZ/IN^+ vector and evaluated 3 months after injection, showed evidence of two separate PCR products. The 2-dLTR junctional sequences were confirmed for all

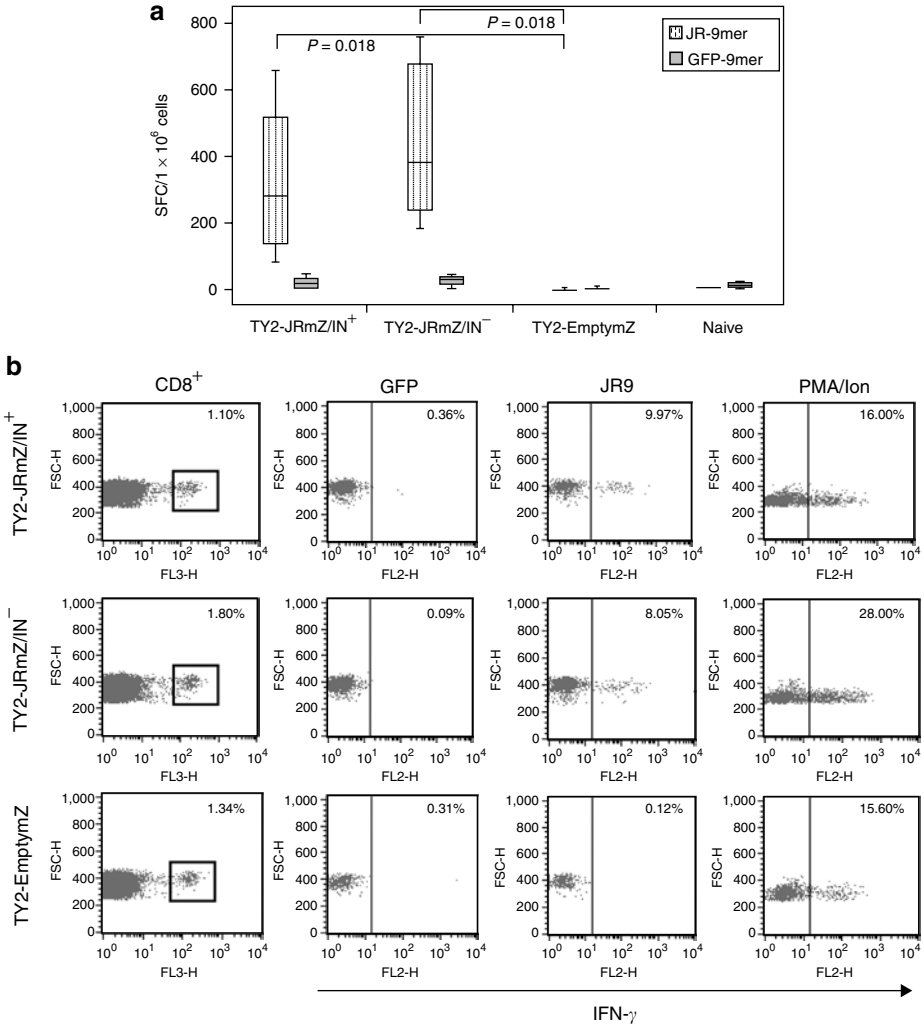

Figure 3 T-cell responses measured on bone marrow (BM) cells 90 days after immunization with self-inactivating lentiviral vector. (**a**) An interferon-γ (IFN-γ) enzyme-linked immunosorbent spot assay was performed on BM cells stimulated overnight with the indicated stimuli. IFN-γ-producing T cells are expressed as spot-forming cells (SFCs) per 10^6 cells after background subtraction. The median for each dataset is indicated by the black center line of the box plots and *P*-values are indicated for intergroup comparisons. (**b**) Analysis of IFN-γ production by intracellular staining on gated $CD8^+$ T cells after incubation with the indicated stimuli. Percentages of $CD8^+$ IFN-γ-producing cells are indicated. GFP, green fluorescent protein; PMA/Ion, phorbal 12-myristate 13-acetate/ionomicin.

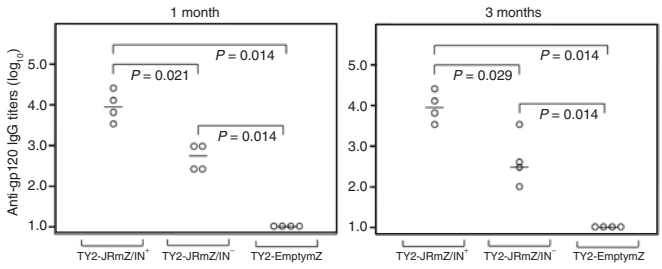

Figure 4 Anti-gp120 immunoglobulin G (IgG) measured by enzyme-linked immunosorbent assay in serum of mice at 30 (left) and 90 days (right) after immunization. Sera samples from immunized mice were analyzed separately. Lines represent the median values in each group, and *P*-values are indicated for intergroup comparisons.

PCR products, including the two separate bands from mouse 14 (see **Supplementary Figure S1**). We found a high number of miscellaneous sequences at the junction between the two LTRs in the PCR products from mice injected with the IN-competent vectors, whereas the majority of the samples from the mice injected with IN-defective vectors corresponded to the expected sequence, representing the joining of the two unprocessed LTR termini, consistent with an IN-defective phenotype *in vivo*. The D116N mutation present in the IN-defective vector abolishes IN function, including 3′-processing of the reverse transcribed viral DNA ends. Therefore the frequency of wild-type junctions was expected to be higher than that found in the IN-competent counterpart. Consistently, the frequency of the wild-type junctions was significantly higher in the IN-defective vector–inoculated mice (93.7%, corresponding to 15 out of 16 sequenced clones containing wild-type junctions; two clones per PCR reaction per mouse) than in the IN-competent vector–inoculated mice (12.5%, corresponding to 2 out of 16 sequenced clones containing wild-type junctions; two clones per PCR reaction per mouse).

Only samples derived from TY2-JRmZ/IN^+-inoculated mice showed clear evidence of integrated vector sequences, as evaluated by the B2-PCR assay (**Figure 5c** and **g**). There was no

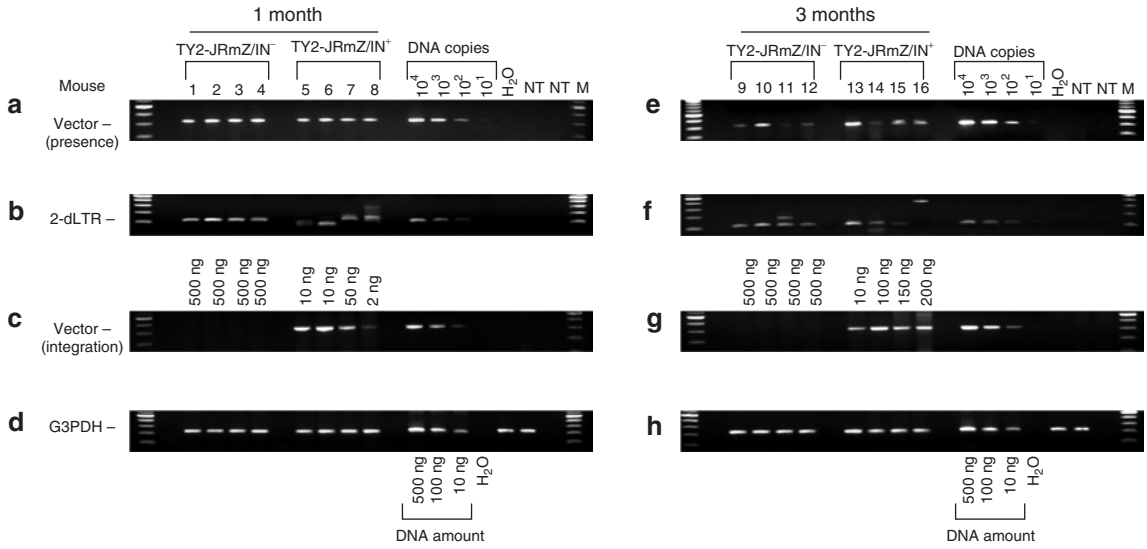

Figure 5 Lentiviral vector sequence in the immunized mice DNA polymerase chain reaction for evaluation of (**a** and **e**) vector presence on 200 ng of DNA, (**b** and **f**) 2-dLTR circles on 200 ng of DNA, (**c** and **g**) vector integration (shown are the amplification products on 500 ng of DNA for TY2-JRmZ/IN$^-$-inoculated mice and the lowest positive amount of DNA for TY2-JRmZ/IN$^+$-inoculated mice; range 2–200 ng), and (**d** and **h**) DNA integrity on 100 ng of DNA from the immunized mice. DNA from immunized mice was extracted at 30 days (left) and 90 days (right) from inoculum and amplified as described in Materials and Methods using the indicated primer pairs. The DNA amplification products were separated on a 2% agarose gel and stained with ethidium bromide. G3PDH, glyceraldehyde 3-phosphate dehydrogenase; LTR, long terminal repeat.

Table 1 Dose-dependent immune response in TY2-JRmZ/IN$^+$ vector–inoculated mice

TY2-JRmZ/IN$^+$ dose (RT counts)[a]	Number of positive mice	IFN-γ ELISPOT: SFCs per 10^6 cells; median (range)		Average CTL activity at 25:1 E:T ratio (%)	Anti-gp120 IgG; median (range)
		Spleen	BM		
4.5×10^6	3/3	610 (570–680)	144 (85–255)	26.5	3,200 (800–12,800)
4.5×10^5	3/3	780 (597–1,025)	164 (102–265)	24.7	400 (100–400)
4.5×10^4	0/3	10 (5–85)	7 (5–10)	2	1 (1–1)
4.5×10^3	0/3	5 (5–10)	5 (5–5)	1	1 (1–1)

Abbreviations: BM, bone marrow; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunosorbent spot; E:T ratio, effector-to-target ratio; IFN-γ, interferon-γ; IgG, immunoglobulin G; IN, integrase; SFCs, spot forming cells.
[a]Three mice per group were inoculated intramuscularly with a single dose of the indicated amount of TY2-JRmZ/IN$^+$.

evidence of integrated vector in up to 500 ng of genomic DNA from mice injected with the IN-defective vector; conversely, as little as 2 ng of genomic DNA derived from the IN-competent vector–injected mice had a positive signal after amplification in all the tested samples. A lower amount of input genomic DNA was required to identify integrated lentiviral sequences at 30 days after inoculation of the IN-competent vector (range 2–50 ng) than was required to obtain a positive signal at 90 days after injection (range 10–200 ng), consistent with the instability of the vector *in vivo* over time and/or a host immune response directed against cells expressing the transgene.

Residual integration activity of the IN-defective vector

Residual integration of the mutant IN vector was performed in HeLa cells using the IN-competent and IN-defective TY-DX-IRES-Neo sinLVs expressing the neomycin resistance gene downstream of the internal ribosomal entry site (IRES) sequence. HeLa cells were infected in 6-well plates with serial dilutions of each vector (range 1×10^5 to 1×10^1 reverse transcriptase (RT) counts for the IN-competent viruses and 1×10^6 to 1×10^2 RT counts for the IN defective viruses) and subjected to treatment with geneticin, as described in Materials and Methods. As expected, the final titers for the IN-competent vectors were high (2.7×10^4 and 4.7×10^4 colonies per 1×10^6 RT counts), whereas final titers for the IN-defective vectors were much lower (59 and 98 colonies per 1×10^6 RT counts for TY-DX-IRES-Neo/IN$^-$) (see **Supplementary Table S1**). At the indicated doses, the IN-defective vector integrated between 2.1×10^3 and 2.2×10^3 times less frequently than the IN-competent vector, demonstrating that it maintained the integration-deficient phenotype after transduction, with negligible amounts of residual integration activity.

Titration of IN-competent vector *in vivo*

Because the IN-defective virus was associated with very low levels of integration, it is possible that this minimal amount of integrated virus with IN-defective phenotype was responsible for the immune responses observed using TY2-JRmZ/IN$^-$. Consequently, we performed an *in vivo* titration experiment to determine the minimum dose of TY2-JRmZ/IN$^+$ required to induce immune responses. Groups of mice were inoculated once with serial dilutions of TY2-JRmZ/IN$^+$ (range 4.5×10^6 to 4.5×10^3 RT counts) and immune responses were evaluated

3 months after injection. As detected by IFN-γ ELISPOT assay on both splenocytes and BM, cytotoxic T-lymphocyte activity on splenocytes, and specific anti-gp120 immunoglobulin G on sera, immune responses were present in all mice immunized with the two highest doses of TY2-JRmZ/IN$^+$ vector (**Table 1**) but absent at the two lowest doses of TY2-JRmZ/IN$^+$ vector. The next-to-lowest dose of virus (4.5×10^4) that did not induce a specific immune response corresponds to 1/288th of the IN-defective viral dose used to inject mice that showed potent immune responses up to 3 months. This strongly argues against the possibility that the residual levels of integration are responsible for the immune responses detected in the TY2-JRmZ/IN$^-$ vector–inoculated mice.

DISCUSSION

We show that integration-deficient HIV-based vectors induce a strong and sustained immune response *in vivo* after a single injection. We recently reported that a single immunization with an integration-competent sinLV expressing HIV-Env or SIV-Gag generates long-lasting specific immune responses.[16,17] The use of a sinLV for efficient transgene expression has been well documented in several studies.[28,29] Importantly, recent studies have shown that IN-defective lentiviral vectors can mediate stable expression *in vitro* in non-dividing cells[24,27,30] and *in vivo* in the murine model.[26,27] In this study, the efficiency and the durability of the immune response were evaluated in mice by comparing a single injection of IN-competent and IN-defective lentiviral vectors expressing the codon optimized HIV-$1_{JR\text{-}FL}$ gp120. In an effort to improve the antigen-specific response upon immunization, the murine granulocyte monocyte–colony stimulatory factor transgene was also introduced into the lentiviral vector expression cassette.[31] At 30 days after administration of the vectors, cellular immune responses were similar in terms of antigen-specific IFN-γ-expressing cells in the two groups of mice. At 90 days after the single immunization, splenocytes from mice inoculated with the IN-defective lentiviral vector still maintained marked and specific immune responses, as evaluated by *ex vivo* IFN-γ production (ELISPOT and ICS assays). Data indicated that both vectors induced preferentially an effector memory response, as evaluated by CD62L expression on antigen-specific CD8$^+$ splenocytes and BM-derived cells. In addition, the presence of cytotoxic T lymphocytes indicates that the immunization generated functional effector cells. Notably, BM cells recovered from both groups of mice had high levels of antigen-specific cells, supporting the central role that the BM plays in the maintenance of memory T cells.[32,33] To rule out the possibility that DNA contamination carried over from viral preparations was inducing such strong immune responses, we quantified plasmid DNA contamination in the pelleted viral preparations and found it to be very low, in the range 0.2–200 ng per virus inoculum per mouse (data not shown). This amount of DNA is approximately 1,000-fold lower than is needed to induce a sustained immune response in mice.[34]

In terms of humoral responses, anti-gp120 antibodies were higher in mice injected with the IN-competent vector than in mice injected with the IN-defective vector at both 30 and 90 days after immunization, suggesting that although both vectors induce a sustained humoral response without any boost, the IN-competent vector is more efficient. This was not surprising, as in cell culture systems integration of the transducing vector results in prolonged high-level expression of antigen compared with a lower-level chronic expression in the absence of integration.[24,27,30]

Safety and efficacy are the main characteristics of a successful vaccine. Our results indicate that replication-incompetent non-integrating lentiviral vectors persist *in vivo* long enough to induce a long-lasting immune response after a single injection, without requiring any boosting. Importantly, we did not find evidence of integrated proviral sequences in the mice inoculated with the IN-defective vector, confirming earlier results using IN-defective lentiviral vectors *in vitro*[22,23,28] and *in vivo*[26,27] and parental IN-defective viruses *in vitro*.[24,35–41] This is an important safety feature in light of the clonal proliferative disorder, presumably owing to insertional mutagenesis, reported in children participating in a retroviral vector gene therapy protocol.[18,19]

Overall, the extent of the immune response associated with expression of gp120 from an IN-defective vector was unexpected given previous work performed in both primary cell cultures and continuous cell lines indicating that protein expression from unintegrated templates is at much lower levels than with the integrated counterpart.[21] Long-term gene expression from unintegrated templates is also dependent on the non-replicating status of the target cell population, as viral E-DNA is otherwise rapidly diluted out with rounds of cell division in the absence of an effective origin of replication.[22,25] However, a number of target cell types for vaccine delivery are relatively quiescent, including myocytes, dendritic cells, and macrophages, and are desirable targets for IN-defective lentiviral vectors because E-DNA might remain stable over a long period of time. It is therefore possible that expression from unintegrated templates persisted *in vivo* in certain cell types. An in-depth analysis of the cell type(s) targeted by the vectors used in this study is necessary to address this issue.

There is an urgent need to evaluate new HIV vaccine strategies that mimic the efficacy and improve on the safety of live attenuated SIV approaches.[11] Combining the high efficiency of gene transfer mediated by lentiviruses with a stable non-integrating vector system is highly attractive for clinical application of this delivery system for vaccine. Importantly, further validation of an HIV vaccine based on non-integrating lentiviral vectors will require the use of the non-human primate model and challenge with SIV/SHIV viral strains.

MATERIALS AND METHODS

Vector construction. A schematic depiction of the transfer vectors is provided in **Figure 1a**. Details on vectors construction are provided in **Supplementary Materials and Methods**. In brief, the sequence for the poliovirus IRES (pIRES) and the mouse granulocyte monocyte–colony stimulatory factor were obtained from plasmids pS1179D8 and pxLNC-mGM-CSF, respectively, a gift from Peter Searle (University of Birmingham, Edgbaston, UK); the sequence for the woodchuck hepatitis post-transcriptional regulatory element (WPRE) was obtained from plasmid R5SA-EFS-GFP-W, a gift from Didier Nègre (INSERM, Lyon, France). All fragments were introduced downstream of the HIV-1 $gp120_{JR\text{-}FL}$ coding sequence in the parental sinLV pTY2-JREnv plasmid.[16] The control transfer lentiviral vector plasmid pTY2-EmptymZ was obtained by digestion of pTY2-JRmZ with *Xho*I/*Sal*I, removing the HIV-1 $gp120_{JR\text{-}FL}$ coding sequence.

Plasmid TY-DX-IRES-Neo, containing the neomycin resistance gene, was obtained by cloning the IRES-Neo sequence from plasmid

pFB-Neo (Stratagene, La Jolla, CA) in the sinLV pTY-SIVGagDX vector.[16] The packaging plasmids IN competent (pCMVΔR8.2) and IN defective (pcHelp/IN$^-$, containing a D116N mutation in the IN genome) and the envelope expressing plasmid pMD.G have been described elsewhere.[42,43]

Production of recombinant vectors. The human HeLa and 293 cell lines were maintained in Dulbecco's modified Eagle's medium (Euroclone, Life Sciences Division, Pero, MI, Italy) supplemented with 10% fetal bovine serum (Euroclone, Life Sciences Division, Pero, MI, Italy) and 100 U/ml of penicillin–streptomicin–glutamine (Gibco Invitrogen, Paisley, UK). Recombinant lentiviral vectors were produced as described[16] and as detailed in **Supplementary Materials and Methods**. Viral titers were normalized by the RT[44] and p24 enzyme-linked immunosorbent assays (Innogenetics, Ghent, Belgium).

Mice immunization. Mice were kept in accordance with European Union guidelines and Italian legislation. All protocols were approved by the authors' Institutional Review Board. Six- to eight-week-old BALB/c mice were injected once intramuscularly (right thigh) with 1.3×10^7 RT units of each vector formulated in 0.2 ml of phosphate-buffered saline. Eight mice were injected for each group to perform analyses at days 30 and 90 after the immunization. Naïve, non-immunized mice were kept for parallel analysis. For titration experiments, groups of mice were inoculated once with serial dilutions of TY2-JRmZ/IN$^+$ (range 4.5×10^6 to 4.5×10^3 RT counts). At the time they were killed, mice were first bled orbitally under metaphane-induced anesthesia and sera were collected and kept at −80 °C. The muscle injection site was removed for DNA analysis and spleens were taken using sterile forceps and tweezers. A single-cell suspension was prepared by mechanical disruption and passage through cell strainers (BD Biosciences Discovery Labware, Bedford, MA). BM cells were obtained from tibias by syringe insertion into one end of the bone and flushing with Roswell Park Memorial Institute 1640 medium.

IFN-γ ELISPOT assay. The IFN-γ ELISPOT assay was performed using reagents from Mabtech (Mabtech AB, Gamla Värmdöv, Sweden) as described.[16] The 9-mer containing the H-2^d restricted HIV-1 gp120 V3 loop epitope (IGPGRAFYT) (UFPeptides, Ferrara, Italy)[45] and the H-2^d restricted GFP-9-mer peptide (HYLSTQSAL)[46] (UFPeptides, Ferrara, Italy) were used at 5 μg/ml as specific and unrelated stimuli, respectively. Medium alone and concanavalin A (Sigma Chemicals, St. Louis, MO) were used as negative and positive controls, respectively. From the assay values for the samples (specific and unrelated peptide–treated wells) were subtracted the values obtained for the medium-treated wells (background); the samples were scored positive when a minimum of 50 spots per 10^6 cells were present and the number was twofold of more higher than for the unrelated peptide.

Antibodies and ICS. Cells from each group were pooled and cultured with JR9-specific (5 μg/ml) or GFP2-unrelated (5 μg/ml) peptide in the presence of anti-mouse CD28 monoclonal antibody (clone 37.51) at 2 μg/ml. phorbol 12-myristate 13-acetate (50 ng/ml) in combination with ionomicin (2 μg/ml) was used as positive control. One hour after stimulation, 10 μg/ml of brefeldin A was added to the cultures to inhibit cytokine secretion. Cells were stained with phycoerythrin-Cy5 anti-mouse CD8 (clone 53–6.7), fluorescein isothiocyanate anti-mouse CD62L (clone MEL-14), or their isotype-matched monoclonal antibodies. Then cells were permeabilized and stained with phycoerythrin-labeled anti-mouse IFN-γ monoclonal antibody (clone XMG1.2) or its isotype-matched control in phosphate-buffered saline/0.5% saponin and analyzed by flow cytometry. All monoclonal antibodies were from BD Pharmingen (San Diego, CA) and all chemicals were from Sigma Chemicals (St. Louis, MO). Acquisition and analysis were performed using Cell Quest software (Becton Dickinson, Franklin Lakes, NJ).

In vitro *stimulation of effector cells and ^{51}chromium release assay.* Stimulator splenocytes from naïve BALB/c mice were pulsed for 1 hour with the appropriate peptides at 5 μg/ml in serum-free medium, irradiated with 30 Gy on a ^{60}Co source, washed once, and then added to effector splenocytes from immunized mice at an effector-to-stimulator ratio of 10:1. After 4 days of culture, effector cells were tested in a ^{51}chromium release assay for the analysis of antigen-specific T cells as described elsewhere[16] and as detailed in **Supplementary Materials and Methods**.

Antibody detection. Specific antibody titers against gp120 were measured by enzyme-linked immunosorbent assay as previously described[47] and as detailed in **Supplementary Materials and Methods**.

DNA isolation and PCR. DNA from the site of injection was extracted using the SV Total RNA Isolation System protocol, modified for DNA preparation (Promega Corporation, Madison, WI).[48] All samples supported the amplification of the mouse glyceraldehyde 3-phosphate dehydrogenase gene (G3PDH Control Amplimer Set; Clonthech, Mountain View, CA) and were included in subsequent PCR analysis to detect the presence of the vector DNA sequence using 200 ng of DNA and a primer pair spanning the WPRE-LTR region at the 3′ end of the vector (WPREbis: 5′-GAAGCCGAATTCTGCAGATATCC-3′; AA55: 5′-CTGC TAGAGATTTTCCACACTGAC-3′). The integrated vector sequence was evaluated using a modified B2-PCR assay.[49] In a first set of amplifications, one primer based on the murine B2 family of short interspersed elements[50] (B2AS: 5′-ATATGTAAGTACACTGTAGC-3′) was used with one primer in the central polypurine tract sequence of the vector (5′-TCAG TACAAATGGCAGTATTCATCC-3′). Reactions were performed on 2–500 ng of genomic DNA. A nested PCR was performed on 5 μl of the first PCR product using the two internal primers in the vector genome described above (WPREbis/AA55). The presence of 2-LTR (U3)–deleted circular forms (2-dLTR) was evaluated by nested PCR using 200 ng of DNA with 5 pmol each of outer primers (9600: 5′-GCTTAAGCCTCAATA AAGCTTGCCT-3′ and R485: 5′-AGAGAGCTCCCAGGCTCAG-3′) and 20 pmol each of inner primers (477: 5′-GTGACTCTGGTAACTAGAGA-3′ and R475: 5′-TCTGGTCTAACCAGAGAGAC-3′) spanning the junction between the two dLTRs (U5–ΔU3). PCR parameters and cycling conditions are detailed in **Supplementary Materials and Methods**.

Evaluation of residual integration activity. HeLa cells were seeded at 5×10^4 per well in 6-well plates. On the next day cells were transduced with serial dilutions of IN-competent TY-DX-IRES-Neo/IN$^+$ (10^5, 10^4, 10^3, 10^2, 10^1 RT counts per well) and IN-defective TY-DX-IRES-Neo/IN$^-$ (10^6, 10^5, 10^4, 10^2, 10^2 RT counts per well). The medium was removed 24 hours later and replaced with medium supplemented with 800 μg of geneticin (Gibco Invitrogen, Paisley, UK) every 3 days. Cells were grown for 2 weeks, and developed clones were fixed with methanol and stained with Giemsa. Clones on each well were counted and expressed as the number of colonies per 10^6 RT counts.

Statistical analysis. Statistical analyses were performed using the nonparametric Mann–Whitney *U* test. All *P*-values were two-tailed and considered significant at less than 0.05. All analyses were performed using SPSS for Windows version 13.0 (SPSS, Chicago, IL).

SUPPLEMENTARY MATERIAL

Figure S1. Nucleotide sequences at the 2-dLTR circle junction derived from DNA of PCR from **Figure 5b** and **f**.

Table S1. DNA recombination frequencies of IN-competent and IN-defective vectors.

Materials and Methods.

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