Consensus HIV-1 FSU-A Integrase Gene Variants Electroporated into Mice Induce Polyfunctional Antigen-Specific CD4+ and CD8+ T Cells

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Abstract

Our objective is to create gene immunogens targeted against drug-resistant HIV-1, focusing on HIV-1 enzymes as critical components in viral replication and drug resistance. Consensus-based gene vaccines are specifically fit for variable pathogens such as HIV-1 and have many advantages over viral genes and their expression-optimized variants. With this in mind, we designed the consensus integrase (IN) of the HIV-1 clade A strain predominant in the territory of the former Soviet Union and its inactivated derivative with and without mutations conferring resistance to elvitegravir. Humanized IN gene was synthesized; and inactivated derivatives (with 64D in the active site mutated to V) with and without elvitegravir-resistance mutations were generated by site-mutagenesis. Activity tests of IN variants expressed in E. coli showed the consensus IN to be active, while both D64V-variants were devoid of specific activities. IN genes cloned in the DNA-immunization vector pVax1 (pVaxIN plasmids) were highly expressed in human and murine cell lines (>0.7 ng/cell). Injection of BALB/c mice with pVaxIN plasmids followed by electroporation generated potent IFN-γ and IL-2 responses registered in PBMC by day 15 and in splenocytes by day 23 after immunization. Multiparametric FACS demonstrated that CD8+ and CD4+ T cells of gene-immunized mice stimulated with IN-derived peptides secreted IFN-γ, IL-2, and TNF-α. The multi-cytokine responses of CD8+ and CD4+ T-cells correlated with the loss of in vivo activity of the luciferase reporter gene co-delivered with pVaxIN plasmids. This indicated the capacity of IN-specific CD4+ and CD8+ T-cells to clear IN/reporter co-expressing cells from the injection sites. Thus, the synthetic HIV-1 clade A integrase genes acted as potent immunogens generating polyfunctional Th1-type CD4+ and CD8+ T cells. Generation of such response is highly desirable for an effective HIV-1 vaccine as it offers a possibility to attack virus-infected cells via both MHC class I and II pathways.

Introduction

34 million people worldwide are infected with human immuno-deficiency virus type 1 (HIV-1) [1]. Highly active antiretroviral therapy (HAART) significantly improves the prognosis for infected individuals but cannot exterminate the virus and in many cases does not suppress the virus load [2]. Furthermore, treatment leads to the development of drug resistance, which initiates the spread of drug-resistant HIV-1 strains. By now, the level of new infections with drug-resistant HIV-1 has reached 15% [3]. Both the acquired drug resistance and primary infections with drug-resistant HIV-1 strains and minority variants grossly limit the therapy options in acute primary as well as chronic HIV-1 infection [4], [5], [6], [7], [8].

Drug-resistant mutations often emerge in highly conserved domains indispensable for protein activity; further mutations in these regions (to mask the new epitopes) are restricted as deleterious to viral viability [9], [10], [11]. Thus, an escape from drugs makes virus vulnerable for the immune system. This is reflected by the changes in the properties of drug-resistant HIV-1 proteins: modified processing and presentation, shifts in the
epitope hierarchy, gain of new epitopes, and broadening of HLA-recognition of the mutated regions [12]. This makes drug-resistant HIV-1 proteins quite immunogenic in the natural infection [10], [13], [14]. It is logical to try to use these mutated antigens to induce an immune response against HIV-1 enzymes with the aim to suppress viral replication and limit the development of drug resistance under HAART. Strong immune response induced by drug-resistant HIV-1 antigens in the experimental settings would motivate their incorporation into therapeutic HIV-1 vaccine(s) aimed to support/complement antiretroviral treatment.

Years of HIV-1 vaccine trials and SIV pre-clinical studies showed that the control over viral replication strongly relies on the vaccine’s ability to elicit a multifunctional T cell response against multiple viral targets (multiple HIV and SIV epitopes) [15], [16], [17]. Such response can be effectively generated by genetic vaccination [18]. The latter can induce a protective immune response against viral infections in diverse, also large, species [19], [20], [21], [22], [23]. While early DNA vaccines exploited the genetic material of the microbes, modern vaccines use genetic information to build the synthetic immunogens, often quite different from the microbial genes. Variable pathogens, as HIV-1, are targeted by a specific cluster of synthetic gene vaccines, so-called consensus (inferred consensus, ancestral and center-of-tree) immunogens, often more potent than the expression-optimized genes [24], [25], [26], [27]. An encouraging example of their use is the protection against divergent influenza H1N1 viruses with genetic immunization with a Centralized Influenza Hemagglutinin [28]. Several consensus-gene based HIV-1 vaccines have already entered clinical trials [29], [30], [31], [32], [33].

With this in mind, we approached HIV-1 integrase, a key HIV-1 enzyme responsible for provirus integration into the host genome [34]; [35]. Early DNA vaccine trials avoided including HIV-1 integrase genes due to the fear of inducing genomic instability, with the exception of a single trial reporting high immunogenicity of expression-optimized integrase (as a part of the pol gene) in rodents and rhesus macaques [36]. Recent HIV-1 multigene vaccine trials included the IN gene but presented no details on the IN gene immunogenicity [37], [38], [39], [40]. This indicated both the feasibility of the IN gene application in preclinical as well as clinical trials, and the need to improve it to achieve better immunogenic performance.

Here, we have designed and tested the prototype immunogens based on the sequence of the wild-type integrase of HIV-1 FSU-A strain and its variant with elvitegravir-confering mutations, both devoid of the enzymatic activity. All consensus IN gene variants were found to be highly immunogenic in mice.

Results

Design of Consensus Integrase

Full-length sequences of 34 integrase genes of HIV-1 clade A prevalent in the territory of the former Soviet Union including Belarus, Estonia, Georgia, Russia, Ukraine, and Uzbekistan, [41], [42], [43], [44], and V. Lukashov, unpublished) were translated and aligned, and the amino acid consensus was created. The viral population was very homogeneous with 80% of the consensus fully conserved and an additional 10% having only five ambiguous positions of the total 287 (positions 48, 74, 134, 211, and 218). Consensus integrase sequence was modified to overcome the intrinsic instability due to phenylalanine residue on the N-terminus, which makes IN a physiological substrate of the N-end rule pathway [45], [46]. For this, IN was supplemented with the Met-Gly dipeptide prior to the N-terminal Phe. Extra glycine codon and the triplet ATT upstream of the AUG codon completed the Kozak’s consensus sequence (ANNATGG) required for the efficient initiation of IN gene translation [47]. An inactive form of consensus clade A integrase (IN_in) was created by mutating the first residue of the integrase catalytic triad motif D404 to V, as was earlier performed by Cherepanov P. et al [48]. Inactive IN was further supplemented with mutations H51Y, E92Q, S147G, and K160Q conferring resistance to elvitegravir [49] and a polymorphic mutation E157Q common for subtype A [50], which yielded IN_e3 (D64V+H51Y, E92Q, S147G, E157Q, K160Q). Amino acid sequences of IN variants are presented in Fig. 1.

Prokarytic Expression and in vitro Activity Tests of the N-terminal His-tagged IN Variants

IN genes cloned into pET15b vector directed high levels of prokaryotic expression of the N-terminal His-tagged IN variants; the levels of prokaryotic IN expression exceeded 10 mg per liter of culture of E. coli BL21(DE3) with pRARE plasmid (Fig. S1). His-tagged IN variants were purified by chromatography on the Ni-NTA–agarose to over 80% purity (Fig. 2A). All proteins had the expected molecular mass of 34 kDa and were stained specifically with polyclonal anti-IN antibodies (Fig. 2B).

Catalytic activities of the recombinant enzymes were evaluated using standard assays of 3'-processing and strand transfer using 3P-labelled oligodeoxyribonucleotide duplexes which mimicked the U5 region of HIV-1 LTR (Table 1). Endonuclease cleavage of the U5 duplex representing 3’-processing resulted in the removal of GT dinucleotide from the 3’-end of the processed strand U5B and formation of the pre-processed oligonucleotide U5B-2. ‘Self-insertion’ of the U5-2 duplex consisting of the pre-processed strand U5B-2 and U5A modeled the reaction of strand transfer (Fig. 3). IN_a performed both reactions with an efficiency higher than that of HBX2 HIV integrase (Fig. 3 A, B compared to C; Table 2). IN_in containing the inactivation mutation D64V could perform neither 3’-processing nor strand transfer, but possessed an exonuclease activity (Fig. 3 A, B, C). This activity was sequence-specific, since similar digestion patterns were seen after cleavage of the specific substrates U5 and U5-2 (Fig. 3A, B) and of the random DNA duplex (Fig. 3C). IN_in_e3 bearing both inactivating and drug resistance-conferring mutations was inactive (Fig. 3A, B, C; Table 2). To confirm this, IN_in_e3 was incubated with U5 duplex for 24 hours, but neither processing nor non-specific nuclease activities were detected (data not shown).

Expression of Integrase in Eukaryotic Cells

Next, “humanized” IN gene variants were cloned into eukaryotic expression vector pVax1. Human (HeLa, HEK) and mouse (NIH3T3) cell lines transiently transfected with pVaxIN plasmids expressed proteins with the expected molecular mass (33 kDa) specifically stained in Western blots with integrase-specific polyclonal antibodies (Fig. 4A). All IN genes were highly expressed in diverse eukaryotic cell lines (Fig. 4B, and data not shown). Having high expression levels and expected enzymatic properties (active or inactive), they fulfilled the prerequisites for using them as DNA immunogens.

Integrase Genes in pVax1 Induce Potent Cellular Immune Responses

The immunogenicity of integrase genes was assessed in BALB/c mice. For this, BALB/c mice were subcutaneously injected with pVaxIN variants with subsequent electroprotrusion (for description, see Materials and Methods). Blood was collected on day 15 after immunization, and PBMC were isolated and analyzed by dual

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IFN-γ/IL-2 Fluorospot for the capacity to secrete IFN-γ, IL-2 and both cytokines in response to stimulation with integrase-derived synthetic peptides. A similar assay was run on mouse splenocytes collected after the completion of immunization on day 22.

All IN variants induced an equally good immune response in terms of IFN-γ, IL-2 and dual IFN-γ/IL-2 production by T-cells in response to in vitro stimulation with IN-derived peptides, as manifested by 500 to 1000 cells per mln splenocytes producing IFN-γ or IL-2, and up to 500 cells producing IFN-γ and IL-2 in all three groups (Table 3; p < 0.05, Kruskal-Wallis test, except for IN_in_e3) (Fig. 5, and Fig. S2). IFN-γ and IL-2 were predominantly produced after stimulation of lymphocytes with peptides representing a cluster of human and murine CD4+ and CD8+ epitopes at aa 209-239, more precisely at aa 219-238 [10], [36], [51], [52], [53], [54] (peptides IN209 and MIN219, respectively, Table 3). IL-2 was also secreted after in vitro stimulation of splenocytes with peptides representing other known mouse epitopes (IN169; its shorter variant MIN169; a mixture of peptides MIN79; its IN_in_e3 derived variant MIN79e3; and MIN169; Table 3, Fig. 5). As could be expected, mouse T cells recognize neither the consensus IN-derived peptides corresponding to the known human CD8+ CTL epitopes of IN clade B (at aa 47–54, 66–74, 84–94, 92–102, 143–151, and 151–158; www.hiv.lanl.gov and www.immuneepitope.org), nor their variants with elvitegravir-resistance mutations (Fig. 5). T cell responses were highly specific as they were seen only in mice immunized with IN DNA (p < 0.05 compared to empty vector, Mann-Whitney test), whereas a T cell response against a peptide representing the CD8+ T cell epitope of luciferase (LUC) was seen in all mice (since Luc reporter gene was administered to all groups, including the controls; Fig. 5, Fig. S2).

The phenotype of responding cells was further evaluated by six-color flow cytometry assessing a surface expression of CD4+ or CD8+ and an intracellular expression of IFN-γ, IL-2, IL-4, and/or TNF-α. In this experiment, splenocytes were stimulated by a MIN peptide pool representing known CD4+ and CD8+ T-cell epitopes of mice (Pool_MIN, Table 3), LUC peptide to control the response to Luc reporter, ConA as a positive control, or medium alone.
Figure 3. Integrase activities: 3′-processing and DNA strand transfer by IN variants. Products of 3′-processing and strand transfer of the synthetic DNA duplexes with 32P-labeled B-strands (Table 1) by the consensus HIV-1 clade A integrase (IN_a), its inactivated variant (IN_in), and the inactivated variant with elvitegravir resistance mutations (IN_in_e3) were separated by gel electrophoresis and quantified using Image-QuantTM 4.1 software. The 3′-processing assay: US substrate in the absence of integrases (lane 1) and in the presence of IN_a, IN_in, and IN_in_e3 (lanes 2, 3, and 4, respectively) (A). The strand transfer reaction: US-2 substrate in the absence of integrases (lane 1) and in the presence of IN_a, IN_in, and IN_in_e3 (2, 3, and 4, respectively); T – the strand transfer products (B). Incubation of the non-specific DNA Ran in the absence of integrases (lane 1), and in the presence of IN_a, IN_in, and IN_in_e3 (2, 3, and 4, respectively) (C). Activities of HXB2 integrase, 3′-processing (1), strand transfer (2) (D). Tests were performed with 100 nM integrases and 10 nM DNA. Products were separated in denaturing 20% PAAG with 7M urea (see Methods for details). Data are representative of two independent experiments.

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Data from individual splenocytes collected by flow cytometry were subjected to the gating approach shown in Fig. 6A. A sample representative of cytokine expression by CD8+ T cells of IN_in_e3-immunized mice in response to in vitro stimulation with the MIN peptide pool, or medium is shown in Fig. 6B. No significant mouse-to-mouse difference in cytokine production was observed for unstimulated CD4+ or CD8+ T cells or for cells stimulated with mitogen ConA (Fig. 6B, and data not shown). Mouse groups were thus similar with respect to the levels of unspecific reactivities and cell viability. As expected, the CD4+ and CD8+ T cell response to LUC peptide was similar in all groups, and CD8+ T cells expressing single and multiple cytokines responses against the peptide pool represented significantly of CD4+ and CD8+ T cells (in all IN-immunized groups; Fig. 6D). None of the IN gene variants induced any detectable IL-4 production, and CD8+ T cells in this group significantly exceeded the respective numbers in the control animals (p<0.03, Mann-Whitney U-test). Inactivated consensus IN and its variant with elvitegravir resistance mutations demonstrated somewhat higher IFN-γ, IL-2 and TNF-α responses than the control mice, but the difference did not reach the level of significance (p>0.05; Fig. 6C, D). There were no difference in specific cytokine secretion between groups of mice immunized with different IN genes (p>0.1, Kruskal-Wallis test; Fig. 6 C, D).

Importantly, immunization with all three IN genes elicited a significant number of IN-specific CD4+ and CD8+ T cells which simultaneously produced IFN-γ, IL-2 and TNF-α (Fig. 6 E, F). The number of CD4+ and CD8+ T cells triple-positive for IFN-γ, IL-2 and TNF-α in mice receiving the IN genes was equally high in all three groups (p>0.1, Kruskal-Wallis test; Fig. 6 E, F), and significantly exceeded that in the control vector-immunized mice.

Data are representative of two independent experiments.

Table 1. Oligonucleotide duplexes used to assess integrase activities.

| Abbreviated name | Sequence | Function |
|------------------|----------|----------|
| USB | 5′-GTGGAAAATCTGATGATGATGT-3′ | Strand processed by integrase |
| USA | 3′-CACACCTTTTCTAGATCCCC-5′ | Complementary to USB |
| US | USA/USB duplex | Specific integrase substrate in 3′-processing |
| USB-2 | 5′-GTGGAAAATCTGATGATGATGT-3′ | Result of USB processing |
| US-2 | USA/USB-2 duplex | Integrase substrate in the strand transfer reaction |
| RanB | 5′-GGAAATTCAAGCCGCGTGTG-3′ | Complementary to RanA |
| RanA | 3′-CTCTGATGATGATGATGATGT-5′ | Complementary to RanB |
| Ran | RanB/RanA duplex | Non-specific DNA duplex |

*The dinucleotide removed by IN is underlined.

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(p<0.05 in pair-wise group comparisons by Mann-Whitney test; Fig. S4 A, B; Fig. 6 E, F).

**IN Gene Immunization Induces Specific Antibody Response**

Sera from BALB/c mice immunized with IN gene variants collected after the completion of immunization was subjected to indirect ELISA on plates coated with the IN variants. IN gene immunization was found to induce IN-specific IgG in the average titers from 500 to 2500 (Fig. 7). IN_a was equally well recognized in all three groups (p>0.1, Kruskal-Wallis test, Fig. 7), IgG titers varied from 200 to 3000. Interestingly, active consensus integrase was better recognized by the sera of mice immunized with the most divergent IN variant IN_in_e3: in this group the individual anti_IN_a titers reached 3000. Mice receiving IN gene variant IN_in_e3 demonstrated the lowest anti-IN clade B antibody titers (500; Fig. 7). This contrasted with their high ability to recognize the consensus active integrase of FSU-A strain. Titer of antibodies against IN of clade B in mice immunized with IN_in_e3 was lower than in mice receiving IN_in gene (p<0.05, Fig. 7). The overall antibody recognition of IN_clade B was weak with the average antibody titers less than 1500 (Fig. 7). Recognition of mutant FSU-A integrases IN_in and IN_in_e3 was tested only in mouse groups immunized with respective variants (the average titer from 1000 to 1500; Fig. 7).

**In vivo Assessment of the Effector Capacity of Anti-integrase Immune Response**

Next, we investigated whether the immunization with IN gene variants influences the in vivo expression of the transfected genes. For this, we followed the expression at the sites of immunization of the reporter gene encoding firefly luciferase (Luc) co-delivered as a 1:1 mixture with IN gene variants. By day 21, the expression of luciferase in mice receiving Luc and IN genes had significantly decreased, while little change was registered in mice receiving Luc gene together with an empty vector (p<0.00001; Fig. 8A, B). The decrease in the luminescent signal emitted from the sites of injection of the integrase and the reporter gene was similar for IN_a, IN_in and IN_in_e3 groups starting from day 9 and up to day 21 (p>0.1 in all these time points by both Kruskal-Wallis and Mann-Whitney tests; Fig. 8B). Luminescence on day 21 inversely correlated with the end-point (day 23) IFN-γ, IL-2 and dual IFN-γ/IL-2 production by CD4+ and with IFN-γ, TNF-α and dual IFN-γ/TNF-α production by CD8+ T-cells (all ps <0.05; Fig. 9A, B). Equally strong inverse correlations were found between the end-point luminescence and the magnitude of integrase-specific triple cytokine response of CD4+ and of CD8+ T-cells (Fig. 9A, B). Interestingly, luminescence at the early time points, as day 4, directly correlated with the end-point immune response (Fig. 9C). The latter indicated that the magnitude of T cell responses is predetermined by the efficacy of gene transfer and initial expression, while the induction of the integrase-specific multi-cytokine response of CD4+ and CD8+ T cells leads to the loss of luciferase reporter activity at the immunization sites.

**Discussion**

HIV-1 integrase inserts the proviral DNA into the host genome securing the life-long viral infection [27]; [34]; [35]. Alongside with reverse transcriptase and protease, it plays a key role in HIV-1 drug resistance [55]; [56]. The newest HIV-1 inhibitors targeting integrase have a low genetic barrier to resistance (one to two mutations are sufficient to make the enzyme drug-insensitive) [57] and it is only a matter of time before the

| Table 2. Catalytic activities of the recombinant integrases. |
|-------------------|-------------------|
| Integrase       | 3’-processing efficiency, % | Strand transfer efficiency, % |
|                  | measured | relative | measured | relative |
| IN (HXB2)        | 34±4    | 100      | 6,1±0,6  | 100      |
| IN_a             | 51±5    | 150±15   | 7,8±0,8  | 128±15   |
| IN_in            | Non-specific nuclease activity | undetectable | undetectable | undetectable |
| IN_in_e3         | undetectable | undetectable | undetectable | undetectable |

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A

B

C

(IFNγ/IL-2 sfu/min splenocytes

LUC IN66 IN169 IN219 MIN73 MIN79 MIN168 MIN219 pool_CTL pool_CTL e3 vector

p<0.05

p>0.1

p<0.1

p>0.1

p=0.1

p>0.1

p=0.1
resistance to integrase inhibitors reaches alarming levels [3]. Integrase induces a strong immunodominant CTL response [5], [36], but despite the immune pressure, remains highly conserved in order to preserve the activity (integrase activity depends on the full preservation of up to two-thirds of the protein; [60]). High conservation, immunogenicity and absence of counterparts in the cellular machinery positioned integrase as an ideal target for exerting bottle-neck immune pressure on the virus. We hypothesized that an effective immune response against HIV integrase including its drug-resistant forms may interfere with the viral evolution towards drug-resistant phenotype(s). This prompted us to design a series of novel integrase-based gene immunogens. Specifically, we constructed the consensus integrase of HIV-1 FSU-A based on 40 complete FSU-A pol gene sequences originating from the territory of the former Soviet Union. The amino acid sequences of FSU-A integrases appeared to be very homogeneous; 80% of the amino acid consensus was fully conserved. This consensus sequence was further modified to inactivate the enzyme, make it stable, and ensure its high-level expression. For this, the consensus IN gene was codon-optimized and modified toward stability. Viral IN has the N-terminal Phe residue, which makes it a substrate of the N-end rule pathway [46]. The N-terminal Phe was substituted with a dipeptide Met-Gly, since the N-terminal Met residue aids to the protein stability [45], [48], [61]. The Met-Gly-extension did not affect protein structure and folding as indicated by the consensus FSU-A enzymatic activity which exceeded the activity of the viral HIV-1 HXB2 integrase. To make the immunogen safe, the consensus IN was

| Abbreviated name of the peptide | Position in aa residues; positions of the mutated aa are given in brackets | Amino acid sequence of the peptide; mutated aa are shown in bold |
|---------------------------------|--------------------------------------------------------------------------|---------------------------------------------------------------|
| Peptides derived from consensus integrase of HIV-1 FSU-A* |                                                                 |                                                               |
| IN**66                          | 66–98                                                                    | THLEGKIVIAHVASYGIEAEVIPAETGQETA                               |
| IN169                          | 169–190                                                                   | AEHLKTAQOMAVF1HNFKRGGIGGYSYA                                   |
| IN209                          | 209–239                                                                   | QTKELQKIQIKQFVVYRYRDSRDPIWKGPA                                |
| Pool_MIN** (MIN79, MIN79e3, MIN169, MIN209, MIN219) contains*: |                                                                 |                                                               |
| MIN79                          | 79–98                                                                     | VASYGIEAEVIPAETGQETA                                            |
| MIN79e3                        | 79–98 (92)                                                                | VASYGIEAEVIPAETGQETA                                            |
| MIN169                        | 169–190                                                                   | AEHLKTAQOMAVF1HNFKRGGIGGYSYA                                   |
| MIN209                        | 209–228                                                                   | QTKELKQIQIKQFVVYRYRDSRDPIWKGPA                                |
| MIN219                        | 219–238                                                                   | KICNFVVYRRDSRDPIWKGPA                                           |
| Pool_CTL (CIN47, CIN66, CIN68, CIN84, CIN92, CIN143, CIN151) contains*: |                                                                 |                                                               |
| CIN47                          | 47–54                                                                    | GEAMHQQV                                                      |
| CIN66                          | 66–74                                                                    | THLEGKII                                                     |
| CIN68                          | 68–77                                                                    | LEGKIVIAV                                                     |
| CIN84                          | 84–94                                                                    | IEAEPVAETG                                                   |
| CIN92                          | 92–102                                                                   | ETQGQETAYFLL                                                |
| CIN143                         | 143–151                                                                  | YNPQSGQGV                                                   |
| CIN151                         | 151–158                                                                   | VESMNKEL                                                   |
| Pool_CTL (CIN47e3, CIN66, CIN68, CIN84e3, CIN92e3, CIN143e3, CIN151e3) contains***: |                                                                 |                                                               |
| CIN47e3                        | 47–54 (51)                                                                | GEAMHGQQV                                                  |
| CIN66                          | 66–74                                                                    | THLEGKII                                                     |
| CIN68                          | 68–77                                                                    | LEGKIVIAV                                                     |
| CIN84e3                        | 84–94 (92)                                                                | IEAEPVAPQTG                                                   |
| CIN92e3                        | 92–102 (92)                                                               | QTQGQETAYFLL                                                |
| CIN143e3                       | 143–151 (147)                                                            | YNPQSGQGV                                                   |
| CIN151e3                       | 151–158 (157)                                                             | VESMNKQQ                                                   |

*Choice of peptides done based on the epitopes mapped to these regions earlier [10], [36], [51], [52], [53], [54], [95]. Pool_CTL contains peptides representing human CTL epitopes mapped to the given regions.
**IN series includes peptides recognized by human T cells and MIN, by T cells of H2-Ka- restricted BALB/c mice.
***Peptides of pool_CTL with mutations of resistance to elvitegravir where applicable.

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Figure 5. IFN-γ/IL-2 Fluorospot assay of the splenocytes of mice immunized with IN gene variants. The results of IFN-γ/IL-2 Fluorospot performed on splenocytes of mice immunized with plasmids encoding consensus IN (IN_a), inactivated consensus IN (IN_in), inactivated consensus IN with mutations conferring resistance to elvitegravir (IN_in_e3), or empty vector. Splenocytes were stimulated in vitro with a Luc-derived peptide (LUC), and individual or pooled IN-derived peptides (Table 3) as described in Methods. IN-specific in vitro secretion of IFN-γ (A), IL-2 (B), and dual secretion of IFN-γ/IL-2 (C). Responses represent the average number of signal-forming units (sfu) per million cells in two independent experiment runs, each done in duplicate, +SD.

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inactivated (IN_in) by substituting Asp64 in the IN catalytic triad for Val, which prevents strand transfer activity [62], [63].

The inactivated IN was provided with mutations conferring resistance to elvitegravir, a novel strand transfer inhibitor currently in Phase III clinical trials [64]. For HIV-1 clade A, the main mutations of elvitegravir resistance are H51Y, E92Q, S147G, along with E157Q and a secondary nonpolymorphic mutation, K160Q, highly infrequent in integrase inhibitor-naive patients [49], [50]; introduction of these mutations generated IN derivative IN_in_e3.

Activity tests done on D64V-IN variants produced in E. coli demonstrated that they had no strand-transfer activity, and their genes are, therefore, safe to use in immunization. All three IN genes behaved as effective gene immunogens able to induce potent Th1-type T cell responses in both CD8+ and CD4+ T cells. Secretion of both IFN-γ and TNF-α was significantly expanded (Fig. 5). In aa 209–239 of consensus HIV-1 clade A appeared to contain a murine T cell epitope(s) (or epitope cluster). A strong T cell response against this region induced by all IN gene variants suggested its use as a lead-epitope to monitor integrase-specific T cell responses. Recognition of other peptides representing human and mouse T cell epitopes localized at aa 66–98 and 169–190 (IN69, MIN79, and IN/MIN169, respectively; Table 3) was weak and occurred mainly in the form of IL-2 production (Fig. 5).

T cell stimulation by IN-derived peptides was further analyzed by multiparametric FACS. In all groups receiving IN genes, stimulation by the pool of peptides representing mouse CD4+ and CD8+ T cell epitopes (pool_MIN; Table 3) triggered production of IFN-γ, IL-2, and/or TNF-α by 0.8 to 1.6% CD8+ T cells. Recognition of other peptides represented by effector CD8+ T cells (Fig 6). None of the stimulated T cells produced IL-4. IFN-γ/IL-2 response of murine PBMC against this cluster was registered already on day 15 after immunization (Fig. S2). By day 27, T cell responses of splenocytes against stimulation with IN209 and MIN219 had significantly expanded (Fig. 5). IN aa 209–239 of consensus HIV-1 clade A appeared to contain a murine T cell epitope(s) (or epitope cluster). A strong T cell response against this region induced by all IN gene variants suggested its use as a lead-epitope to monitor integrase-specific T cell responses. Recognition of other peptides representing human and mouse T cell epitopes localized at aa 66–98 and 169–190 (IN69, MIN79, and IN/MIN169, respectively; Table 3) was weak and occurred mainly in the form of IL-2 production (Fig. 5).

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Activity tests done on D64V-IN variants produced in E. coli demonstrated that they had no strand-transfer activity, and their genes are, therefore, safe to use in immunization. All three IN genes behaved as effective gene immunogens able to induce potent Th1-type T cell responses in both CD8+ and CD4+ T cells. Secretion of both IFN-γ and TNF-α by effector CD8+ T cells is critically important for protection against viral infections. Thus, all three synthetic IN genes behaved as effective gene immunogens able to induce potent Th1-type responses in both CD8+ and CD4+ T cells. Secretion of both IFN-γ and TNF-α by effector CD8+ T cells is critically important for protection against viral infections [66]. IL-2 supports the
secondary expansion of memory CD8+ T cells and generation of the long-term protective immunity [67], [68]. Generation of all three cytokines is considered to be a prerequisite for an efficient antiviral immunization.

Production of cytokines is hierarchical in character: most of the epitope-specific CTLs produce IFN-γ, some, IFN-γ+TNF-α, and still a smaller subset, referred to as “polyfunctional”, all three cytokines [69], [70]. Polyfunctional T cells have been associated with an effective control of intracellular infections, specifically of

Figure 8. Dynamics of bioluminescence at the sites of the IN gene and luciferase reporter gene co-administration. In vivo monitoring of luciferase activity at days 4, 9, 15 and 21 after the administration of plasmids encoding the consensus IN (IN_a), inactivated consensus IN (IN_in), inactivated consensus IN with elvitegravir resistance mutations (IN_in_e3), or empty vector pVax1, each mixed with Luc reporter gene (1:1). Images demonstrate two representative injection sites per group followed throughout the immunization. The scale to the right represents the strength of luminescent signal in pixels/sec/cm²/sr (A). Kinetics of the luciferase expression over time (four mice in each group; two independent experiments) (B).

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viral replication, and with strong protection in vaccination [71], [72], [73], [74], [75], [76]. In HIV-1 infection, multiple cytokine secretion by lymphoid cells has been associated with T cell suppressor activity, superior control of HIV-1 replication, and long-term non-progression to AIDS [71], [77], [78], [79]. In mice immunized with IN gene variants, all IL-2 positive CD8+ T cells stimulated with IN peptides secreted IFN-γ and TNF-α; 0.2% of CD8+ T cells co-expressed IFN-γ, IL-2 and TNF-α and thus belonged to the polyfunctional Tc1 phenotype. The majority of CD4+ T cells also co-expressed either two (IFN-γ and TNF-α, 0.02%) or all three cytokines (IL-2, IFN-γ and TNF-α; 0.06%) and thus belonged to the polyfunctional Tc1 phenotype. Co-expression of TNF-α and IFN-γ indicated that these IN-specific CD4+ T cells were the effectors acting through TRAIL-mediated apoptosis [80], [81], while co-secretion of IFN-γ, TNF-α and IL-2 identified the population of effector CD4+ T cells capable of perforin-mediated target cell killing [82]. The perforin- and cytokotoxic cytokines/ TRAIL-based killing account for the bulk of lytic activities of CD4+ T cells [80], [81]. Immunization with IN gene variants was apparently able to trigger at least one of the effector mechanisms.

Furthermore, IN gene immunization generated integrase-specific antibodies which recognized both the consensus FSU-A integrase and a clade B (HXB2) integrase with similar end-point titers. Thus, IN gene variants could induce antibodies against epitopes common for integrases of clade A and B.

Finally, we evaluated the capacity of the elicited anti-IN immune response to eliminate the transfected expressing cells from the immunization sites. This was done by assessing the level of expression in the injection sites of the reporter gene of firefly luciferase, co-delivered with the IN gene variants [83]. As we have recently shown, co-injection of Luc reporter gene with a potent gene immunogen results in a rapid loss of the in vivo reporter activity (decrease of bioluminescence) [83]. Here, co-delivery of Luc and IN genes led to a significant, 10- to 15-fold decrease in the total photon flux from the site of immunization three weeks post immunization. We found inverse correlations of luminescence with IFN-γ/TNF-α and IFN-γ/IL-2/TNF-α expression by CD8+ and with dual IFN-γ/IL-2 and triple IFN-γ/IL-2/TNF-α expression by CD4+ T cells (Fig. 9A, B). Correlations of luminescence with IFN-γ/TNF-α production by CD4+, and with IFN-γ/IL-2 production by CD8+ T cells did not reach the level of significance indicating that to affect the luminescence, CD4+ T cells depended on IL-2, and CD8+ T cells, on TNF-α, each featuring the respective effector (lytic) T cells. This supported the concept of luminescent “fading” being due to the T-cell mediated clearance of the expressing cells from immunization sites. Further, this indicates the role in clearance of immunogen/reporter expressing cells of the lytic CD4+ Th1 cells. Lytic CD4+ T cell clones can suppress replication of HIV and SIV in both CD4+ T cells and macrophages [84], [85]. Induction of such effectors would offer a possibility to attack virus-infected cells via both MHC class I and MHC class II pathways. Generation of such polyfunctional T cells is highly desirable for an effective HIV-1 vaccine as it would offer a possibility to attack virus-infected cells via both MHC class I and MHC class II pathways. Generation of such polyfunctional T cells is highly desirable for an effective HIV-1 vaccine [86]. Several recent HIV-1 multigene vaccine trials have included the IN gene [39], [40], [87] which supports its perspectivity for immune therapy of HIV/AIDS, specifically, the immune prevention of drug-resistance. Our consensus HIV-1 clade A immunogens would be specifically adapted to hinder epidemics caused by HIV-1 strains with low genetic diversity as in the Russian Federation [41], [42], [43].

Methods
Ethics Statement
All experiments were approved by the Northern Stockholm’s Unit of the Ethics of Animal Research on 2010-08-26, ethical permission N197/10 “Evaluation of the new generation of vaccines against highly dangerous infectious diseases and cancer”. The experiments conveyed under this ethical permission aimed to develop new vaccines and new vaccination strategies against cancer and serious viral infections as HIV, and to advance new treatment protocol for further clinical applications. Vaccine candidates to test under the application included naked DNA vaccines, proteins, peptides and viral vectors administered with or without adjuvants. Immunization were allowed by intramuscular, subcutaneous and intraderal injections, inoculations with Biojector with or without electroporation, and nasal immunization with drops. All injections, biojections and electroporation were made under the inhalation anesthesia with a mixture of air and 1.5 to 3% isofluorane.

All procedures were evaluated as having low to average degree of difficulty. Methods of immunization and follow-up were not painful; earlier done experiments had shown no effect of the procedures on the gain of the weight, water or food consumption or overall mouse behavior. Possible mouse discomfort under immunization monitoring and sample collection was relieved by the inhalation anesthesia. Animals were sacrificed by cervical dislocation.

In animal experiments conveyed in the present study, BALB/c (H2-Dd) mice (females, 8 week old) were purchased from Charles River Laboratories (Sandhofer, Germany) and housed at the Astrid Fagrius Laboratory, Karolinska Institute, Stockholm, Sweden. Mice were contained in the environment-enriched cages, 5-8 animals per cage. Food and water were supplied ad libitum. Animals were regularly controlled for the food and water intake, weight development, skin and fur changes and microscopical alterations at the site of immunization. Gene injections were made intradermally with ≤30 G needles with volume never exceeding 20 microliters. To screen the immune response, mice were bled from the tail vein two and four weeks after immunization. Gene expression was assessed with the help of reporters using in vivo imaging technique (IVIS). IVIS monitoring was performed in the thermoregulated dark chamber for ten to sixty seconds. Prior to intradermal injection, electroporation, bleeding, and during live imaging, the mice were anesthetized with a mixture of air and isofluorane, 2-2.5% during induction and 1.5–3% thereafter.
A Correlations of average radiance at the IN gene injection site on the MIN-peptide pool specific IFN-γ, IL-2, dual IFN-γ/IL-2 and triple IFN-γ/IL-2/TNF-α secretion by CD4+ T cells on day 21 from the immunization onstart

B Correlations of average radiance at the IN gene injection site on the MIN-peptide pool specific IFN-γ, TNF-α, dual IFN-γ/TNF-α and triple IFN-γ/IL-2/TNF-α secretion by CD8+ T cells on day 21 from immunization onstart

C Correlations of average radiance at the IN gene injection sites on days 4, 9, 15 and 21 from immunization onstart to the MIN peptide pool-specific triple IFN-γ, IL-2 and TNF-α secretion by CD4+ T cells by the end of experiment
Cloning of IN Genes for eu- and Prokaryotic Expression

IN_a, IN_in, IN_a_e3 and IN_in_e3 coding sequences were cloned into a pVax1 vector (Invitrogen) using BamHI and EcoRI restriction sites generating plasmids pVaxIN_a, pVaxIN_in and pVaxININ_e3, respectively. To create prokaryotic expression vectors, IN_a, IN_in, and IN_in_e3 coding sequences were PCR-amplified from the pVax1-based plasmids using Pfu polymerase (Promega) and primers: forward 5’-TGACCA-TATGGGCTTCCTGGAGGG-3’ and reverse 5’-TGACG-GATCCTAGTCTCTCAGG3’ containing NdeI and EcoRI restriction sites. PCR products were digested with NdeI and EcoRI (Fermentas, Vilnius, Lithuania) and ligated into the NdeI/EcoRI-cleaved plasmid pET15b-IN (a kind gift of Prof. J-F Mouscadet, Cachane, France) in frame with the codons for the N-terminal 6His tag in replacement for the coding sequence of HIV-1 IN. Ligation mixtures were transformed into competent OneShoTop10 E.coli cells (Invitrogen, Sweden) by heat shock. Clones obtained on the selective media were screened by PCR using cloning primers. All pVax1- and pET-based plasmids were purified using a miniprep kit (Qiagen, Sweden) and sequenced (Eurofins MWG Operon, Germany).

Prokaryotic Expression and Purification of Integrases

Integrase variants of HIV-1 subtype A bearing a 6His tail were expressed in E. coli BL21(DE3) host strain (Novagen®, Merck Millipore, Darmstadt, Germany and Billerica, MA, USA) with pRARE plasmid from Rosetta (DE3) strain (Novagen). Protein expression was induced by adding IPTG, and integrases were purified by affinity chromatography, as described previously [91]. Fractions were analyzed by electrophoresis in 12% SDS-PAGE with subsequent Western blot using polyclonal anti-IN rabbit sera. Quantitative image analysis of the Coomassie-stained gels with Image-QuantTM 4.1 software (Amersham Biosciences Corp, Piscataway, NJ) revealed each IN preparation to be at least 80% pure. Protein concentration in purified IN preparations was determined by micro-Bradford assay (BioRad, USA). Fractions were aliquoted and frozen at –80°C.

Integrate Activity Assays

DNA duplexes for assessing integrate activity. Integrate activities were assessed using synthetic DNA duplexes (Table 1). DNA duplex U5 consisting of the oligonucleotides U5B and U5A, which mimicked the end of HIV-1 U5 LTR, served as a substrate for 3’ processing activity. Duplex U5-2, formed by U5B and U5A, was used as a substrate for strand transfer and duplex RanB formed by oligonucleotides RanB and RanAn, to verify the specificity of 3’-processing. To measure integrate catalytic activities, the oligonucleotides U5B, U5B-2, and RanB (10 pmol each) were labeled using T4 polynucleotide kinase and 50 μCi of [γ-32P]ATP (3000 Ci/mmol). After 1 hour of incubation at 37°C, EDTA was added to the final concentration of 50 mM, and the reaction mixture was heated for 5 minutes at 65°C to inactivate the kinase. Labeled oligonucleotides were supplemented with equimolar amounts of unlabeled complementary oligonucleotides
and annealed by first heating for 3 minutes at 90°C and then cooling slowly to room temperature. Resulting duplexes were purified using Micro Bio-Spin columns P-6 (Bio-Rad, Berkeley, CA).

3'-end processing and strand transfer reactions. All assays were carried out as described previously [92]. DNA duplexes (10 nM) were incubated for 2 hours with 100 nM IN in 20 μl of the buffer containing 20 mM Hepes, pH 7.2, 7.5 mM MgCl₂, and 1 mM DTT, at 37°C. DNA fragments were precipitated with ethanol and separated in denaturing 20% polyacrylamide gels. Gels were analyzed on a Storm 840TM PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and quantified with Image-QuantTM 4.1 software (Amersham Biosciences Corp, Piscataway, NJ). Integrase activity was defined as percent substrate converted to a product; activities of IN variants were quantified relative to IN-a values. Each experiment was repeated at least three times with convergent results.

Eukaryotic Expression of Integrases

HEK293, HeLa and NIH3T3 cells (ATCC, Manassas, VA) were cultured in the Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (PBS) at 37°C in 5% CO₂ humidified atmosphere. Cells were transfected with pVaxIN-a, pVaxIN-in, pVaxIN-in-e3, or empty vector pVax1 using Lipofectamine LTX (Invitrogen Corporation, Carlsbad, CA). At hour 48 post-transfection, cells were harvested, lysed and analyzed by electrophoresis in 12% SDS-PAGE with subsequent Western blotting (Bio-Rad, Hercules, CA, USA), using for staining polyclonal anti-IN rabbit sera (1:10000). Binding was visualized by secondary HRP-conjugated anti-rabbit antibody (DAKO; 1:5000). The membrane was developed using the ECL plus western blotting detection system (GE Healthcare Amersham Biosciences, USA). To normalize for the total protein content, membranes were stripped according to the ECL protocol and re-stained with monoclonal mouse anti-actin antibody (Sigma-Aldrich, St. Louis, MO, USA; 1:5000), followed by the HRP-conjugated anti-mouse antibody (DAKO; 1:5000). Films were scanned and the relative intensity of the bands was estimated using ImageJ software (http://rsweb.nih.gov/ij/). To assess the level of IN expression per cell, the percent of cells expressing IN was estimated from the efficacy of transfection established in a control sample of recombinant IN in a range from 0.1 to 10 ng were scanned and the relative intensity of the bands was estimated using ImageJ software (http://rsweb.nih.gov/ij/). To assess the level of IN expression per cell, the percent of cells expressing IN was estimated from the efficacy of transfection established in a control sample of recombinant IN in a range from 0.1 to 10 ng were scanned and the relative intensity of the bands was estimated using ImageJ software (http://rsweb.nih.gov/ij/).

IFN antibody response at each time-point was set to the mean OD-values demonstrated by the sera of the vector-immunized mice at this time-point ± 3 SD. For positive sera showing OD values exceeding the cut-off, end-point dilution titers were established from the titration curves.

Assays of Antibody Response

Maxisorb 96-well microtiter plates (Nunc Maxisorp, Denmark) were coated with an IN protein variant in PBS at 0.3 μg/ml and incubated overnight at 6–8°C. Plates were washed six times with PBS containing 0.05% Tween-20. Individual mouse sera diluted step-wise from 1:100 in HIV-Scan Buffer (HSB; 2% normal goat serum, 0.5% BSA, 0, 05% Tween-20, 0, 01% sodium merthiolate) were applied and incubated overnight at 6–8°C. Plates were washed as above and HRP-conjugated goat anti-mouse IgG antibody (Sigma) diluted in HSB was applied and incubated for 15 minutes at 37°C. Plates were washed as above and developed with 3,3’,5,5’-tetramethylbenzidine solution (TMB; Medico-Diagnostic Laboratory, Moscow, Russia). The reaction was stopped by 50 μl 2.5M sulfuric acid, and optical density (OD) was measured at a dual wavelength of 450–620 nm. The cut-off for specific anti-IN antibody response at each time-point was set to the mean OD-values demonstrated by the sera of the vector-immunized mice at this time-point ± 3 SD. For positive sera showing OD values exceeding the cut-off, end-point dilution titers were established from the titration curves.

Assays of T-cell Responses

Blood samples collected on day 15 were pooled group-wise and peripheral blood mononuclear cells (PBMC) were purified by gradient centrifugation in Ficoll-Plaque Plus (GE Healthcare Amersham Biosciences, USA) as described [94]. Individual mouse spleens collected in day 21 were homogenized to obtain splenocytes. Single-cell spleen suspensions were treated with Red Blood Cell lysing buffer and re-suspended in RPMI supplemented with 2 mM L-glutamine, 2 mM Penicillin-Streptomycin (all from

and voltage was applied using DermaVax electroporator (Cellectis) in a regimen optimal for small rodents [93]. On days 4, 9, 15 and 21 after the injection, mice were subjected to in vivo imaging of the reporter expression. At day 15, the mice were bled, and at day 22, bled and sacrificed, and spleens were collected. Prior to intradermal injection, electroporation, bleeding, and during live imaging, the mice were anesthetized with 2–2.5% isoflurane/air delivered in the inhalation chamber or via nasal masks (Baxter Medical AB, Kista, Sweden). All experiments were approved by the Swedish National Board for Laboratory Animals, ethical permission N197/10.

In vivo Imaging of Reporter Expression after DNA Vaccination

To monitor luciferase expression in vivo, mice were injected i.p. with 15 mg/ml solution (100 μL per 10 g body weight) of D-luciferin potassium salt (Caliper Life Science, formerly Xenogen, Alameda, CA) in PBS; and let to move freely for 5 minutes. After that, mice were anesthetized for 5 min with 2–2.5% isoflurane in the inhalation chamber, and transferred into the in vivo imager (IVIS200, Caliper Life Science). Assessment of photonic emissions (photons/s/cm²) was performed for 1 minute. Luminescent and photographic images were captured by an in-built CCD camera and overlayed using Living Image software. A square-shaped frame was selected that engulfed each of the photon-emitting areas registered throughout the experiment cross groups and time-points (normally 10 x 10 mm). The frame was applied to all images in the series, and photons emitted from this area per minute were acquired as radiance per area (RAD, photons/s/cm²) using Living Image software version 2.50.1 (Caliper Life Science). Bioluminescence at one time-point was presented as an average of two sites in one mouse and as an average of all sites in a group.

DNA Immunization of Mice

BALB/c (H2-Dd) mice (females, 8 week old) were purchased from Charles River Laboratories (Sandhofer, Germany) and housed at the animal facility of the Karolinska Institute, Stockholm, Sweden. Groups of mice (4 per group) were immunized subcutaneously with pVaxIN-a, pVaxIN-in, pVaxIN-in-e3, or pVax1 (total of 20 μg in 20 μl PBS each) mixed with an equal amount of pVaxLuc reporter. Plasmids were delivered as two intradermal injections with a 29G insulin-grade syringe (MicroFine U-100; BD Consumer Healthcare, Franklin Lakes, NJ) on the lower back to the left and to the right of the base of the tail. Immediately after, a needle array electrode (1.5 x 4 mm gaps; Cellectis, Romainville, France) was placed over the injection site
High Immunogenicity of Consensus HIV Integrase DNA

Sigma-Aldrich, St. Louis, MO) and 10% FBS (Gibco, Invitrogen, Carlsbad, California) (complete media).

**Fluorospot assay.** Fluorospot was performed on pooled PBMC or individual mouse splenocytes using an IFN-γ/IL-2 Fluorospot kit (Mabtech, Stockholm, Sweden) as described by the manufacturer. In brief, Fluorospot plates were treated with 35% ethanol, washed and coated with a mixture of monoclonal antibodies to IFN-γ and IL-2. 250,000 cells were added per well and stimulated with peptides (single, or pooled in equimolar amounts at a total concentration of 10 μg/ml), recombinant IN (6 and 12 μg/ml), medium alone, and Concanavalin A (Con A, 5 μg/ml) as a positive control. Plates were developed using specific monoclonal detection antibodies and fluorophore-conjugated secondary reagents. Finally plates were treated with a Fluorescence enhancer (Mabtech) to optimize detection and then dried. The number of cytokine-producing spot-forming cells (SFC) per million was evaluated using the AID iSpot FluoroSpot Reader System (AID GmbH, Strassberg, Germany). A net SFC/10⁶ cells in response to each antigen was calculated by subtracting the background response detected in the medium alone. The response to an antigen was considered specific if it exceeded the mean net response to the antigen in the empty vector-immunized mice +3SD.

**Intracellular cytokine staining (ICCS).** All reagents used in ICCS were from BD Biosciences (Franklin Lakes, NJ, US) if not mentioned otherwise. Splenocytes of immunized or control mice (3±10⁵/well) were stimulated for 4 hours at 37°C and 5% CO₂ with recombinant IN protein (10 μg/ml), an equimolar mixture of peptides representing mouse CD4⁺ and CD8⁺ epitopes Pool_MIN (Table 1) with a total peptide concentration of 10 μg/ml, luciferase peptide LUC (10 μg/ml), or medium alone. Concanavalin A (Con A, 2 μg/ml) served as a positive control. All stimuli were diluted in RPMI 1640 supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/mL streptomycin, and 0.3 mg/ml glutamine (all from Gibco, Life Technologies Co), in the presence of GolgiPlug containing Brefeldin A. Ten minutes before the end of incubation, anti-mouse CD16/CD32 antibody was added to block non-antigen-specific binding of immunoglobulins to Fcy receptors. Surface staining was performed by incubating restimulated cells with Pacific Blue (PB)-conjugated anti-mouse CD68 and peridinin chlorophyll protein complex (PerCP)-conjugated anti-mouse CD4 antibodies. Cells were then fixed and permeabilized at room temperature for 20 minutes in 100 μl Cytofix/Cytoperm solution, washed with Perm/Wash buffer, and stained at 4°C for 30 minutes with fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ, allophycocyanin (APC)-conjugated anti-IL-2, phycoerythrin (PE)-conjugated anti-IL-4, and phycoerythrin-cyanine dye 7 (PE-Cy7)-conjugated anti-TNF-α anti-mouse antibodies. Samples were acquired on a FACS Canto flow cytometer (BD Biosciences). The flow cytometry analysis was carried on FlowJo software (Tree Star Inc, Ashland OR, USA). The gating approach is illustrated in Fig. 6A. A general lymphocyte area was defined and single living cells within this population were defined by their expression of CD4 or CD8, and further, by production of IFN-γ, IL-2, IL-4, and TNF-α. Frequencies of CD8⁺ and CD4⁺ cells producing cytokines in response to IN- or Luc-specific stimulation were quantified, and values for unstimulated cells were subtracted.

**Software and Statistics**

Integrase consensus was created using BioEdit software (Ibis Biosciences, Carlsbad, GA). The design of humanized genes included modeling of the RNA folding done using the web services at http://genomes.urv.es/OPTIMIZER/, http://www.invitrogen.com, http://mfold.rna.albany.edu/, and http://genomes.urv.es/ OPTIMIZER/. Radioactive signals were quantified using ImageQuantTM 4.1 (Amersham Biosciences Corp, Piscataway, NJ); Western blot images, using ImageJ [http://rsb.info.nih.gov/ij]; and luminescent images, using Living Image software version 2.50.1 (Caliper Life Science). Continuous but not normally distributed variables, such as the antibody levels, number of cytokine-producing spot-forming cells, or radiance per area, were compared by the nonparametric Kruskal-Wallis and Mann-Whitney U tests. The Spearman rank-order correlation coefficient was calculated to characterize linear correlations between variables. Calculations were done using STATISTICA AXA 10.0 (StatSoft Inc, OK, USA).

**Supporting Information**

**Figure S1 Expression of the IN genes in E. coli.** Expression of the consensus integrase of HIV-1 FSU-A strain (IN_a), inactivated consensus IN (IN_in), inactivated consensus IN with elvitegravir resistance mutations (IN_in_e3), in empty vector. PBMC were pooled group-wise, and stimulated with the Luc derived peptide (LUC), pooled peptides representing mouse CD4⁺ and CD8⁺ epitopes (Pool_MIN), and recombinant integrase of HXB2 (ProtIN) [see Methods for details]. IN-specific in vitro secretion of IFN-γ (A), IL-2 (B) and dual secretion of IFN-γ/IL-2 (C). Responses represent the average number of signal-forming units (sfu) per mln cells expressed as the mean ± SEM for four mice in each group in two independent experiments. (TIF)

**Figure S2 IN-specific responses in PBMC of BALB/c mice immunized with IN genes analyzed by IFN-γ/IL-2 Fluorospot.** Dual IFN-γ/IL-2 Fluorospot was performed on PBMC of mice immunized with consensus IN (IN_a), inactivated consensus IN (IN_in), inactivated consensus IN with elvitegravir resistance mutations (IN_in_e3), or empty vector. PBMC were pooled group-wise, and stimulated with the Luc derived peptide (LUC), pooled peptides representing mouse CD4⁺ and CD8⁺ epitopes (Pool_MIN), and recombinant integrase of HXB2 (ProtIN) [see Methods for details]. IN-specific in vitro secretion of IFN-γ (A), IL-2 (B) and dual secretion of IFN-γ/IL-2 (C). Responses represent the average number of signal-forming units (sfu) per mln cells expressed as the mean ± SEM for four mice in each group in two independent experiments. (TIF)

**Figure S3 T cell responses elicited in BALB/c mice by Luc reporter gene visualized by multiparametric FACs.** In vitro stimulation by LUC peptide (Table 3) of the total CD4⁺ positive (A, C) and total CD8⁺ positive (B) cells resulting in the secretion of single (A, B) or multiple (C, D) cytokines given as % of responding cells. Data are expressed as the mean ± SEM for four mice in each group in two independent experiments. Statistical comparison by Kruskal-Wallis and F-tests of the percent of CD4⁺ (E) and CD8⁺ (F) T cells simultaneously secreting IFN-γ, IL-2 and TNF-α in mice immunized with IN genes or empty vector (each mixed with Luc reporter plasmid). (TIF)

**Figure S4 Percent T-cells co-secretting IFN-γ/IL-2/TNF-α in response to IN-specific stimulation in the IN gene and vector-immunized mice.** Comparison by the Kruskal-Wallis and F-tests of the mean percent of CD4⁺ (A) and CD8⁺ (B) T cells simultaneously secreting IFN-γ, IL-2 and TNF-α after in vitro stimulation with MIN peptide pool (Table 3) in mouse groups immunized with plasmids encoding the consensus HIV-1 FSU-A integrase (IN_a), inactivated integrase (IN_in), inactivated integrase with elvitegravir resistance mutations (IN_in_e3) or empty vector. Analysis is performed in the data from two independent experiments.
25. Abdulhaqq SA, Weiner DB (2008) DNA vaccines: developing new strategies to enhance immune responses. Immunol Res 42: 219–232.

26. Arenas M, Posada D (2010) Computational design of centralized HIV-1 genes. Curr HIV Res 8: 613–621.

27. Yao J, Ceobin N, Pankouf P, Shin T, Khan A, et al. (2011) Immunogenicity of a novel engineered HIV-1 clade C synthetic consensus-based envelope DNA vaccine. Vaccine 29: 7173–7181.

28. Weaver EA, Rubrum AM, Webby RJ, Barry MA (2011) Protection against divergent influenza H1N1 virus by a centralized influenza hemagglutinin. PLoS One 6: e18314.

29. Mcaule M, Cebere I, Sutton J, Chakoti P, Winstone N, et al. (2004) A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans. J Gen Virol 85: 911–919.

30. Harro CD, Robertson MN, Lally MA, O’Neill LD, Edupuganti S, et al. (2009) Safety and immunogenicity of adenos virus-vectorized near-consensus HIV type 1 clade B gag vaccines in healthy adults. AIDS Res Hum Retroviruses 25: 103–113.

31. Murashev B, Kazennova E, Kozlov A, Murasheva I, Dkhovkina E, et al. (2007) Immunogenicity of candidate DNA vaccine based on subtype A of human immunodeficiency virus type 1 clade A in cynomolgus monkeys. Cancer Res 67: 5662–5668.

32. Samri A, Haas G, Duntze J, Bouley JM, Calvez V, et al. (2000) Immunogenicity of an HIV-1 DNA vaccine encoding the HA or NP gene protects ponies from equine influenza virus infection. Vaccine 18: 3139–3147.

33. Boberg A, Isaguliants M (2008) Vaccination against drug resistance in HIV-1 infected patients. Expert Rev Anti Infect Ther 6: 269–280.

34. Asante-Appiah E, Skalka AM (1999) HIV-1 integrase: structural organization, conformational changes, and catalysis. Adv Virus Res 52: 667–699.

35. Delelis O, Carayon K, Sabh A, Depraz E, Mousadket JF (2008) Integrase and integrase–inhibitor complex structures. Curr Opin Chem Biol 12: 305–312.

36. Buchbinder SP, Melchotra DV, Duerr A, Fitzgerald DW, Mogg R, et al. (2008) Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet 372: 1081–1083.

37. McLaird MJ, De Rosa SC, Moodie Z, Dubey S, Kierstead L, et al. (2008) HIV-1 vaccine-induced immune responses in the test-of-concept Step Study: a case–control analysis. Lancet 372: 1194–1195.

38. Li F, Fuminohe AC, Dubey SA, Koter BT, Sinzinger J, et al. (2011) Mapping HIV-1 vaccine-induced T-cell responses: bias towards less-conserved regions and potential impact on vaccine efficacy in the Step Study. PLoS One 6: e20479.

39. Stephenson KE, Sanniguel A, Simmons NL, Smith K, Lewis MG, et al. (2012) Full-Length HIV-1 Immunogens Induce Greater Magnitude and Comparable Breadth of T Lymphocyte Responses to Conserved HIV-1 Regions Compared with Conserved-Region-Only HIV-1 Immunogens in Rhesus Monkeys. J Virol 86: 11434–11440.

40. Bokbok AF, Kazennova EV, Selimova LM, Khainova TA, Rabyovs GL, et al. (2004) Temporal trends in the HIV-1 epidemic in Russia: predominance of subtype B. J Med Virol 74: 191–196.

41. Thomson MM, Vinogradova A, Delgado E, Rakhmanova A, Yakovlev A, et al. (2004) Impact of vaccine-induced mucosal high-avidity CD8+ CTLs in a delay of AIDS viral discrimination from mucosa. Blood 107: 3258–3264.

42. Samir A, Haas G, Duntze J, Bouley JM, Calvez V, et al. (2000) Immunogenicity of mutations induced by nucleoside reverse transcriptase inhibitors for human immunodeficiency virus type 1. Curr Opin Infect Dis 13: 513–522.

43. Samir A, Haas G, Duntze J, Bouley JM, Calvez V, et al. (2000) HIV-1 integrase DNA vaccine induces strong, polyfunctional T-cell responses. Aids 24: 1095–1105.

44. Frahma N, Bakera B, Brandera C (2008) Identification and Optimal Definition of the HA or NP gene protects ponies from equine influenza virus infection. Vaccine 26: 1083–1092.

45. Mulder LC, Muesing MA (2000) Degradation of HIV-1 integrase by the N-end rule pathway. J Biol Chem 275: 29749–29753.

46. Harro CD, Robertson MN, Lally MA, O’Neill LD, Edupuganti S, et al. (2009) Safety and immunogenicity of adenos virus-vectorized near-consensus HIV type 1 clade B gag vaccines in healthy adults. AIDS Res Hum Retroviruses 25: 103–113.

47. Letvin NL, Rao SS, Montefiori DC, Seaman MS, Sun Y, et al. (2011) Immune evasion of CD8 T-cell responses and associated with reduced treatment efficacy. PLoS One 6: e1585.

48. Hutnick NA, Myles DJ, Hirao L, Scott VL, Ferraro B, et al. (2012) An optimized strategy against cancer. J Biomed Biotechnol 2010: 174378.

49. Owen RE, Heitman JW, Hirschkorn DF, Lanteri MC, Biswas HH, et al. (2010) Temporal trends in the HIV-1 epidemic in Russia: predominance of subtype B. J Med Virol 74: 191–196.

50. Belyakov IM, Kuznetsov VA, Kelsall B, Klinman D, Moniuszko M, et al. (2006) New shutoff function of human CD9 for scientific advice and critical reading of the manuscript.

51. Young B, Fransen S, Greenberg KS, Thomas A, Martens S, et al. (2011) Immunogenicity of an HIV-1 DNA vaccine encoding the HA or NP gene protects ponies from equine influenza virus infection. Vaccine 29: 7173–7181.

52. Spector SA, Weiner DB (2008) DNA vaccines: developing new strategies to enhance immune responses. Immunol Rev 219: 219–232.

53. Letvin NL, Rao SS, Montefiori DC, Seaman MS, Sun Y, et al. (2011) Immune evasion of CD8 T-cell responses and associated with reduced treatment efficacy. PLoS One 6: e1585.

54. Santra S, Korber BT, Muldoon M, Barouch DH, Nabel GJ, et al. (2008) A cell-based gene gun that modulates transgene expression in murine immune responses in tumors. Proc Natl Acad Sci U S A 105: 10499–10494.

55. Abdulhaqq SA, Weiner DB (2008) DNA vaccines: developing new strategies to enhance immune responses. Immunol Res 42: 219–232.

56. Meloni CA, Kwan YP, Fragoso R, de Carvalho DOM, Loeb JR, et al. (2003) High Immunogenicity of Consensus HIV Integrase DNA
