HIV-1 Gag-specific immunity induced by a lentivector-based vaccine directed to dendritic cells

Bingbing Dai[a], Lili Yang[b], Haiguang Yang[a], Biliang Hu[a], David Baltimore[b,1], and Pin Wang[a,1]

[a]Mork Family Department of Chemical Engineering and Materials Science, University of Southern California, Los Angeles, CA 90089; and [b]Division of Biology, California Institute of Technology, Pasadena, CA 91125

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Lentivectors (LVs) have attracted considerable interest for their potential as a vaccine delivery vehicle. In this study, we evaluate in mice a dendritic cell (DC)-directed LV system encoding the Gag protein of human immunodeficiency virus (HIV) (LV-Gag) as a potential vaccine for inducing an anti-HIV immune response. The DC-directed specificity is achieved through pseudotyping the vector with an engineered Sindbis virus glycoprotein capable of selectively binding to the DC-SIGN protein. A single immunization by this vector induces a durable HIV Gag-specific immune response. We investigated the antigen-specific immunity and T-cell memory generated by a prime/boost vaccine regimen delivered by either successive LV-Gag injections or a DNA prime/LV-Gag boost protocol. We found that both prime/boost regimens significantly enhance cellular and humoral immune responses. Importantly, a heterologous DNA prime/LV-Gag boost regimen results in superior Gag-specific T-cell responses as compared with a DNA prime/adenovector boost immunization. It induces not only a higher magnitude response, as measured by Gag-specific tetramer analysis and intracellular IFN-γ staining, but also a better quality of response evidenced by a wider mix of cytokines produced by the Gag-specific CD8$^+$ and CD4$^+$ T cells. A boosting immunization with LV-Gag also generates T cells reactive to a broader range of Gag-derived epitopes. These results demonstrate that this DC-directed LV immunization is a potent modality for eliciting anti-HIV immune responses.

AIDS vaccine | human immunodeficiency virus | lentiviral vector | T cell vaccine

Recombinant adenovirus-based vectors (rAd), used either alone or as a booster immunization after priming with a DNA plasmid, are among the most potent viral vectors for inducing human immunodeficiency virus (HIV)-specific T-cell responses in animals and humans (1, 2). However, a phase 2b trial that used recombinant adenovirus serotype 5 (rAd5) vectors as the HIV vaccine carrier failed to show efficacy (3–6). This trial result is consistent with a preclinical study in which a rAd5-based vaccine expressing a simian immunodeficiency virus (SIV) Gag antigen failed to lower setpoint viral loads after SIV challenge of rhesus monkeys (7). A recent study by Barouch and coworkers has shown that a heterologous prime/boost vaccine regimen using a newly identified rAd26 vector could elicit a strong and high quality immune response in non-human primates (NHP), resulting in markedly reduced viral loads and decreased AIDS-related mortality (8). This study highlights the importance of exploring viral vector-based vaccine modalities for development of an effective HIV vaccine.

Efficient antigen delivery to antigen-presenting cells (APCs) and their subsequent presentation to stimulate virus-specific T cells is vital for the success of a T-cell-based vaccine. Dendritic cells (DCs) are the most powerful APCs to initiate and maintain immune responses of T cells (9–11) and therefore become one of the major target cells for the HIV vaccine development (12, 13). Immunization by adoptive transfer of autologous DCs loaded in vitro with inactivated HIV particles induced anti-virus immunity in animals (14–16) and humans (17, 18). However, this is a labor-intensive, personalized medicine approach, which limits its prospect as a vaccine design to deal with the worldwide AIDS pandemic. A direct method is to target the delivery of HIV immunogens to DCs in vivo (19, 20). Steinman and coworkers reported a strategy to conjugate HIV Gag p24 and p41 onto an antibody to DEC-205, a relatively DC-restricted surface protein, as a means to load antigens into DCs in vivo for generating an immune response (21, 22). Although with coadministration of appropriate adjuvants, a strong Gag-specific CD4$^+$ T-cell response was elicited (21), it remains a challenge for this antibody fusion vaccine to evoke CD8$^+$ cytotoxic T cells, which are essential for controlling HIV replication.

We have developed a DC-targeted, lentivector (LV)-based system for delivery of genetic vaccines in vivo (23). LV is known to be an efficient vehicle for genetic modification of DCs in vitro (24), and direct injection with LV enveloped with glycoproteins with broad tropism is able to induce CD8$^+$ T-cell responses (25–30). To fully harness the immuno-stimulatory potency of DCs and mitigate off-target effects, we synthesized a LV enveloped with a Sindbis virus-derived glycoprotein engineered to be specific to the DC-specific surface protein DC-SIGN [also known as CD209 (31, 32)] (23); DC-SIGN has also been explored by others as the target receptor of DCs for protein antigen delivery (20, 33). In our prior studies, we used ovalbumin (OVA) as a model antigen and found that a single-round immunization with this vector could result in substantial antigen-specific T-cell and antibody response (23). In the present study, we show that the significant HIV Gag-specific immune response can be elicited by this DC-directed LV used alone or with other modalities.

Results

Immune Responses Generated by Various Routes of Vaccine Administration. We constructed a Gag-encoding lentiviral backbone plasmid by insertion of Gag cDNA into FUW (34) downstream of a human ubiquitin C promoter, a vector designated FUWGag (Fig. 1*A*). LV encoding the Gag immunogen and pseudotyped with the DC-directed envelope SVGmu was generated in 293T cells by transient transfection with appropriate combinations of various plasmids (see *Materials and Methods*) and is designated LV-Gag. We first tested a range of vector doses [$1.25 \times 10^6 \approx 10 \times 10^6$ transduction units (TU)] for immunization of naive mice through footpad injection and found that a dose of 5×10^6 TU generated the highest percentage of Gag-specific CD8$^+$ T cells 2 weeks after vaccination. We then assessed the immunogenic response to this vector dose via different administration routes. Naive mice were immunized with a single

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The authors declare no conflict of interest.

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[1]To whom correspondence may be addressed. E-mail: baltimo@caltech.edu or pinwang@usc.edu.

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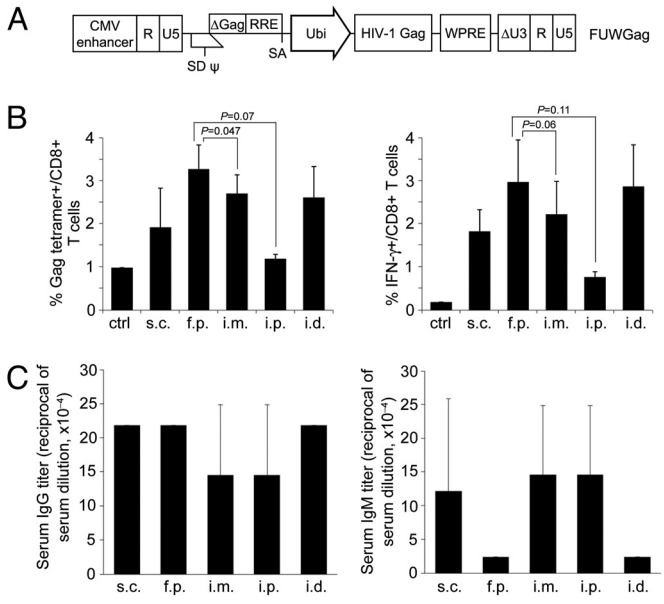

Fig. 1. Comparison of immune responses generated from different injection routes after a single immunization. (*A*) Schematic representation of a lentiviral backbone construct encoding the full sequence of a HIV-1 subtype B Gag antigen. R, U5, and ΔU3 are components of the long terminal repeat (LTR) and ΔU3 contains the self-inactivating deletion; SD: splicing donor; SA: splicing acceptor; ψ and ΔGag: the encapsulation sequence; RRE: the Rev-responsive element; Ubi: human ubiquitin-C promoter; WPRE: woodchuck hepatitis virus posttranductional regulatory element. (*B*) Five groups of BALB/c mice were immunized with 5×10^6 TU (Transduction Units) of LV-Gag by a s.c., footpad (f.p.), intramuscular (i.m.), i.p., or intradermal (i.d.) injection route. Two weeks postimmunization, spleen cells were harvested and analyzed for the frequency of Gag-specific $CD8^+$ T cells by H2-K_d-AMQMLKETI-PE tetramer and CD44 staining. Spleen cells were also restimulated in vitro with the HIV-1 Gag peptide (AMQMLKETI). Intracellular cytokine staining (ICCS) was performed to assess the IFN-γ response. (*C*) Sera from different groups of mice were harvested 2 weeks postimmunization. IgG and IgM antibody responses against HIV-1 Gag were detected by ELISA. Each group consisted of three mice.

injection by the s.c. (s.c.), footpad (f.p.), intramuscular (i.m.), i.p. (i.p.), or intradermal (i.d., at the base of tail) route. Gag-specific T-cell responses were monitored by tetramer analysis and intracellular cytokine staining (ICCS). The f.p. and i.d. injections resulted in the strongest Gag-tetramer$^+$ CD8 T-cell responses (≈3%, Fig. 1*B*, left) 2 weeks postimmunization, consistent with these being the best routes to target skin DCs. The i.p. injection gave the lowest responses. When splenocytes harvested from vaccinated animals were restimulated in vitro with the Gag-dominant peptide (AMQMLKETI), a similar trend for the pattern of IFN (IFN)-γ producing $CD8^+$ T cells was observed (Fig. 1*B*, right).

HIV Gag-specific serum IgG and IgM could be detected 2 weeks postimmunization with the LV-Gag. The highest IgG titers were obtained from the f.p., i.d., and s.c. routes (Fig. 1*C*, left). Interestingly, the IgM titer showed a reverse trend, in which f.p. and i.d. injections yielded lower IgM production (Fig. 1*C*, right). This suggests that immunization through these two injection routes yields a significant $CD4^+$ T-cell response, resulting in efficient isotype switching to convert IgM into IgG. Because of the superior response, the f.p. injection route was chosen for the subsequent prime/boost and other functional studies.

Enhanced Gag-Specific Immunity by Prime/Boost Regimens. To further characterize the efficacy of the LV-Gag immunization, four cohorts of mice were injected with PBS, empty LV (lacking the Gag transgene), bone marrow-derived DCs (BMDCs) loaded with the HIV-1 Gag dominant peptide and matured with lipopolysaccharide (LPS), or LV-Gag. Two weeks postinjection, we assessed IFN-γ-secreting $CD8^+$ T cells in freshly harvested splenocytes restimulated in vitro with the Gag peptide for all of the comparison groups. We observed that the LV-Gag-immunized mice displayed a significant fraction of $CD8^+$ T cells secreting IFN-γ, with a statistically significant difference ($P < 0.01$) when compared to the three comparison groups (Fig. 2*A*). The fact that the empty vector was not different from the PBS control suggests that the Gag-specific $CD8^+$ T cells elicited by LV-Gag results from the delivery of the vector-encoded transgene, rather than being elicited by Gag protein that might be carried within the vector particles. In addition, no significant level of epitope-specific responses was elicited by adoptive transfer of in vitro-loaded DCs, indicating that DC-directed delivery of Gag antigen by the LV in vivo is a much more potent vaccination method.

We next explored the utility of the LV-Gag vector in prime/boost settings. Three groups of mice received either one dose of LV-Gag vector, a dose of LV-Gag vector prime followed by a homologous LV-Gag vector boost, or a DNA prime followed by a LV-Gag vector boost. Two weeks after the final injection, tetramer-positive and IFN-γ-producing $CD8^+$ T cells were quantified by flow cytometry (Fig. 2*B*). Both assays showed an enhanced anti-Gag $CD8^+$ T cells response in the prime/boost animals compared to the single dose LV-Gag immunization group. Splenocytes from the different groups of animals were also cocultured with Gag peptide and then examined for IFN-γ production by an ELISPOT assay (Fig. 2*C*). Obvious enhancement of IFN-γ secretion was seen in the prime/boost groups, with 4- to 5-fold greater responses than for the single dose LV-Gag mice. We further measured the titers of HIV Gag-specific IgG and IgM antibodies in the sera from these animals and found that sera from LV-Gag/LV-Gag mice and DNA/LV-Gag mice showed higher responses to Gag protein than the single dose LV-Gag mice (Fig. 2*D*). Collectively, our data demonstrate that the DC-directed LV is an effective booster of responses initiated by either DNA or LV itself, enhancing $CD8^+$ T-cell as well as antibody responses.

Comparison of T-Cell Responses Elicited by Lentivector and Adenovector. We conducted experiments to compare DC-directed LV with the extensively studied rAd5 for their ability to induce the Gag-specific immune responses. Several groups of naive mice were immunized with a DNA prime/LV-Gag boost, LV-Gag prime/LV-Gag boost, or DNA prime/rAd5-Gag boost. Following the last immunization, comparable frequencies of IFN-γ-producing and Gag-specific $CD8^+$ T cells were detected in splenocytes of the prime/boost vaccine groups (Fig. 3*A*). Presumably due to the high rAd5 vaccine dose (10^{10} VP) used in this study, we found that a single rAd5-Gag immunization was about as good as the prime/boost regimens.

Kinetic analysis of the early responses to single immunization showed that the magnitude of Gag-specific $CD8^+$ T-cell immunity with LV-Gag immunization declined after week 2 to approximately 1% IFN-γ-producing $CD8^+$ T cells by 4 weeks postvaccination (Fig. 3*B*). In contrast, the primary response to the rAd5-Gag vaccine reached a higher level at week 2, and active T cells were continuously expanded through week 4 (Fig. 3*B*).

The memory phenotype of the Gag-specific $CD8^+$ T cells elicited by different regimens was studied by scoring the memory differentiation markers CD44 and CD62L. After gating on Gag-tetramer$^+$$CD8^+$ T cells among splenocytes from LV-Gag-vaccinated mice, approximately 30% of them exhibited the central memory phenotype of $CD44^{hi}CD62L^+$, which was higher than the approximately 12% obtained from mice immunized with the rAd5-Gag vector (Fig. 3*C*). The LV-Gag immunized cells also displayed a discrete very high CD62L population

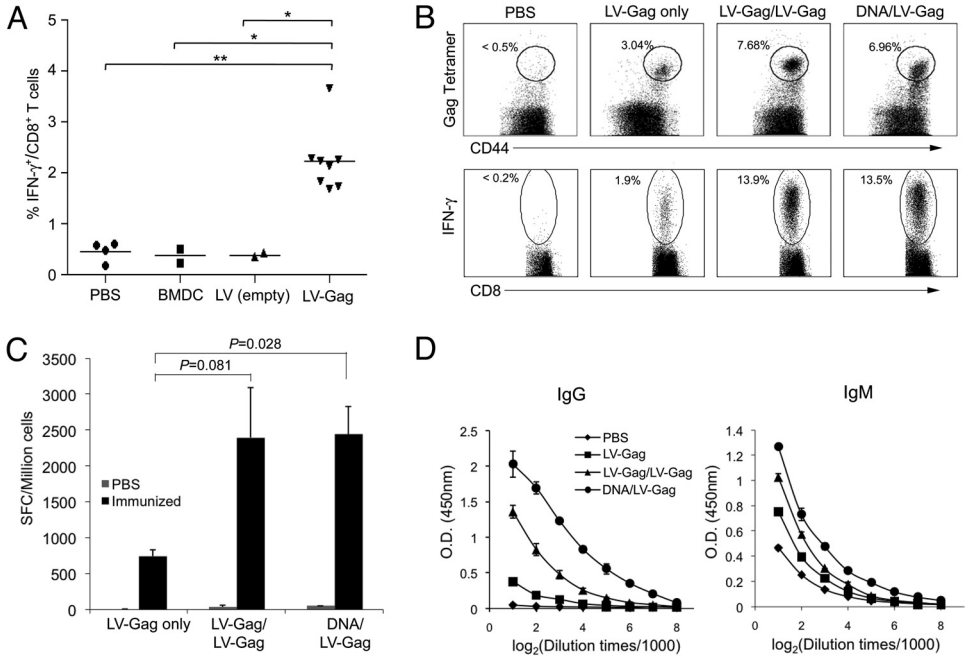

Fig. 2. DC-directed LV can effectively boost HIV-1 Gag-specific immune response. (*A*) BALB/c mice were immunized with PBS (●), BMDCs (1×10^{6}) loaded by the HIV-1 Gag peptide (AMQMLKETI) (■), empty LV lacking the Gag transgene (5×10^{6} TU) (▲), or LV-Gag (5×10^{6} TU) (▼). The immune responses of spleen cells upon restimulation with the Gag dominant peptide were estimated by IFN-γ ICCS 2 weeks postinjection (*, $P < 0.01$; **, $P < 0.001$) (*B–D*) Four vaccine groups received PBS, single immunization of LV-Gag (LV-Gag once), LV-Gag prime/LV-Gag boost (LV-Gag/LV-Gag), or DNA prime/LV-Gag boost (DNA/LV-Gag). Splenocytes from vaccinated animals were analyzed for Gag-specific response by H2-K_d-AMQMLKETI-PE tetramer staining (*B*), IFN-γ ICCS (*C*), and mouse serum ELISAs for IgG and IgM (*D*). The data shown are mean values of triplicates ± SD.

lacking in the rAd5-Gag immunized mice. This result suggests that the DC-targeted LV is more potent than rAd5 for induction of high quality memory T cells.

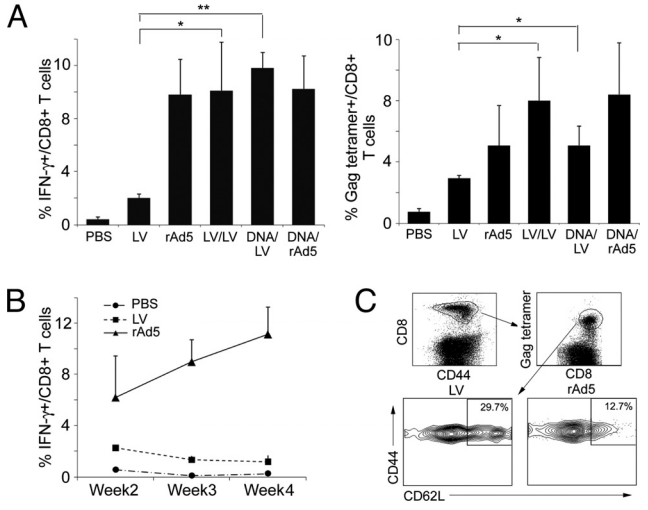

Fig. 3. Comparison of magnitude, kinetics and memory responses of Gag-specific $CD8^+$ T cells after immunization with LV-Gag and rAd5-Gag. Six groups of BALB/c mice received the following vaccination regimens: PBS, LV-Gag (LV, 5×10^{6} TU), rAd5-Gag (rAd5, 10^{10} VP), LV-Gag prime/LV-Gag boost (LV/LV), DNA prime/LV-Gag boost (DNA/LV), and DNA prime/rAd5-Gag boost (DNA/rAd5). Vaccine-induced HIV Gag-specific immune responses were analyzed by: (*A*) percentage of IFN-γ- or Gag-tetramer-positive $CD8^+$ T cells (*, $P < 0.05$; **, $P < 0.005$); (*B*) kinetics of the total frequency of IFN-γ-producing $CD8^+$ T cells of LV-Gag and rAd5-Gag groups on indicated time points after immunization; and (*C*) division of central memory (T_{CM}, $CD44^{high}CD62L^+$) and effector memory (T_{EM}, $CD44^{high}CD62L^-$) $CD8^+$ T cells of LV-Gag (LV) and rAd5-Gag (rAd5) groups by surface staining.

Multifunctional $CD4^+$ and $CD8^+$ T-Cell Responses Elicited by Lentivector. We examined the capacity of individual HIV-specific T cells to produce multiple cytokines, a parameter which was shown to correlate with a cell's ability to protect against infection in certain models (35). We selected the LV-Gag/LV-Gag, DNA/LV-Gag, and DNA/rAd5-Gag immunization regimens for the study because they were able to generate sufficiently high levels of responses to allow a reliable multifunctionality analysis. Splenocytes harvested from vaccinated animals were restimulated with a pool of 123 overlapping peptides covering the entire Gag protein. Intracellular cytokine levels were measured by multiparameter flow cytometry to assess the ability of single cells to produce various combinations of IFN-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-α. As shown in Fig. 4*A*, although the DNA/rAd5-Gag elicited $CD4^+$ T cells that were single-positive for IFN-γ, IL-2, or TNF-α, and double-positive for IFN-γ and TNF-α, there was no detectable level of these cells that were IFN-γ^+IL-2^+, IL-2^+TNF-α^+, or IFN-γ^+IL-2^+TNF-α^+. In contrast, both the LV-Gag/LV-Gag and DNA/LV-Gag regimens generated substantial percentages of $CD4^+$ T cells that were IFN-γ^+TNF-α^+, IFN-γ^+/IL-2^+, and IL-2^+TNF-α^+. Especially, the DNA/LV-Gag group induced a high frequency of $CD4^+$ T cells secreting three cytokines simultaneously (6.4% of the responding cells). The distribution of Gag-specific $CD8^+$ T cells by various regimens showed the same pattern (Fig. 4*A*). Compared with the DNA/rAd5-Gag immunization, a substantially greater proportion of the LV-Gag/LV-Gag- or DNA/LV-Gag-elicited $CD8^+$ T cells were able to secrete multiple cytokines, with approximately 3% of responding cells from the DNA/LV-Gag vaccine producing three cytokines. Interestingly, we found that $CD4^+$ T cells were more multipotent than $CD8^+$ T cells. Examining the IL-2-secreting $CD4^+$ T cells, we see that although the overall frequency of such cells was highest following DNA/rAd5-Gag immunization, only a slight portion of them secreted more than one cytokine (i.e., 2.8% of them are IL-

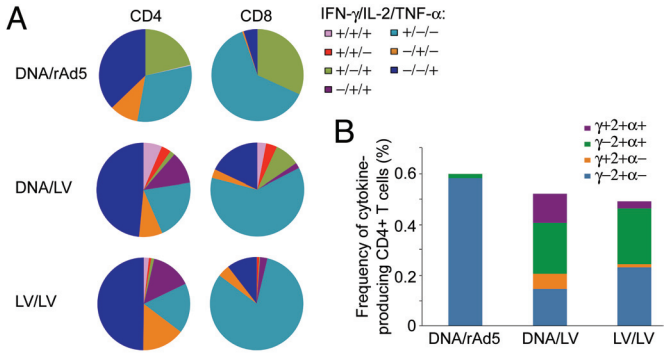

Fig. 4. Generation of multifunctional CD4$^+$ and CD8$^+$ responses by prime/boost immunization regimens. Splenocytes of DNA/rAd5-Gag (DNA/rAd5), LV-Gag/LV-Gag (LV/LV) and DNA/LV-Gag (DNA/LV) groups of BALB/c mice were stimulated with the pooled HIV-1 Gag peptides (2.5 μg/mL for each peptide) for 6 h, and analyzed by an eight-color ICCS assay to assess: (*A*) the fraction of total responding CD4$^+$ or CD8$^+$ T cells expressing each of the seven possible combinations of IFN-γ, IL-2, and TNF-α; and (*B*) the frequency and proportion of responding CD4$^+$ T cells expressing all three cytokines (IFN-γ^+IL-2$^+$TNF-α^+: γ+2+α+), two cytokines (IFN-γ^-IL-2$^+$TNF-α^+: γ–2+α+; or IFN-γ^+IL-2$^+$TNF-α^-: γ+2+α–), or one cytokine (IFN-γ^-IL-2$^+$TNF-α^-: γ–2+α–).

2$^+$TNF-α^+) (Fig. 4*B*). On the contrary, at least half of the IL-2-secreting CD4$^+$ T cells from both DNA/LV-Gag and LV-Gag/LV-Gag vaccination were multicytokine producers, and a notable portion of them were able to generate three cytokines. Thus, both the CD4$^+$ and CD8$^+$ T cells induced by immunization involving DC-directed LV were more polyfunctional than those generated by the DNA/rAd5-Gag vaccine.

Breadth of T-Cell Responses Induced by Various Immunization Regimens. To assess the breadth of the induced T-cell responses, we generated a peptide matrix as shown in Fig. 5*A* (36). A library of peptides covering the entire HIV-1 Gag protein was divided into 23 pools named P1-P23, with each peptide present in two independent pools. The splenocytes of mice immunized with LV-Gag/LV-Gag, DNA/LV-Gag, and DNA/rAd5-Gag were stimulated by one of the peptide pools, and then assayed by IFN-γ ELISPOT. In contrast to the T cells from DNA/rAd5-Gag immunized mice, those from LV-Gag/LV-Gag mice responded to many peptides. Taking an ELISPOT cut-off at 80 SFC (spot forming cells)/0.1 million cells, mice immunized with LV-Gag/LV-Gag responded to eight peptide pools (P4, P5, P6, P9, P10, P15, P17, and P18) (Fig. 5 *B* and *C*), while the DNA/LV-Gag and DNA/rAd5-Gag mice only vigorously responded to three pools (P4, P5, and P17) (Fig. 5 *B* and *C*). We were able to verify the response of LV-Gag/LV-Gag T cells to 11 individual peptides derived from these responding eight peptide pools (Fig. S1). However, when the DNA/rAd5-Gag did respond to a peptide pool, its response was higher than that of the mice receiving LV-Gag. There were 15 different peptide pools identified as nonreactive (refers to the ELISPOT reading <20 SFC/0.1 million cells) to cells from the DNA/rAd5-Gag immunized mice. However, none of the pools were found to be nonreactive for either the LV-Gag/LV-Gag or DNA/LV-Gag induced T cells (Fig. 5*B*). We also conducted an ICCS analysis of the LV-Gag/LV-Gag splenocytes stimulated by two representative peptide pools (P6 and P10) and found that the ratio of Gag-specific CD8 vs. CD4 T-cell responses for both pools was approximately 3:1 (Fig. S2). The above results indicate that the Gag-specific T cells generated by LV-Gag-involved vaccination regimens (LV-Gag/LV-Gag and DNA/LV-Gag) can recognize a broader range of epitopes as compared to the T-cell response induced by the DNA/rAd5-Gag strategy. The DNA/rAd5-Gag-immunized mice gave a high total response but one much more focused on immune-dominant determinants.

A

Pools	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
P13	1	2	3	4	5	6	7	8	9	10	11	12
P14	13	14	15	16	17	18	19	20	21	22	23	24
P15		25	26	27	28	29	30	31	32	33	34	35
P16	36		37	38	39	40	41	42	43	44	45	46
P17	47	48		49	50	51	52	53	54	55	56	57
P18	58	59	60		61	62	63	64	65	66	67	68
P19	69	70	71	72		73	74	75	76	77	78	79
P20	80	81	82	83	84		85	86	87	88	89	90
P21	91	92	93	94	95	96		97	98	99	100	101
P22	102	103	104	105	106	107	108		109	110	111	112
P23	113	114	115	116	117	118	119	120		121	122	123

B

No. of highly reactive peptide pools/No. of nonreactive peptide pools			
PBS	LV-Gag/ LV-Gag	DNA/ LV-Gag	DNA/ rAd5-Gag
0/23	8/0	3/0	3/15

Highly reactive peptide pools refer to those showing the ELISPOT reading higher than 80 SFC/0.1miilion cells, while the nonreactive peptide pools are defined as the ELISPOT reading lower than 20 SFC/0.1miilion cells.

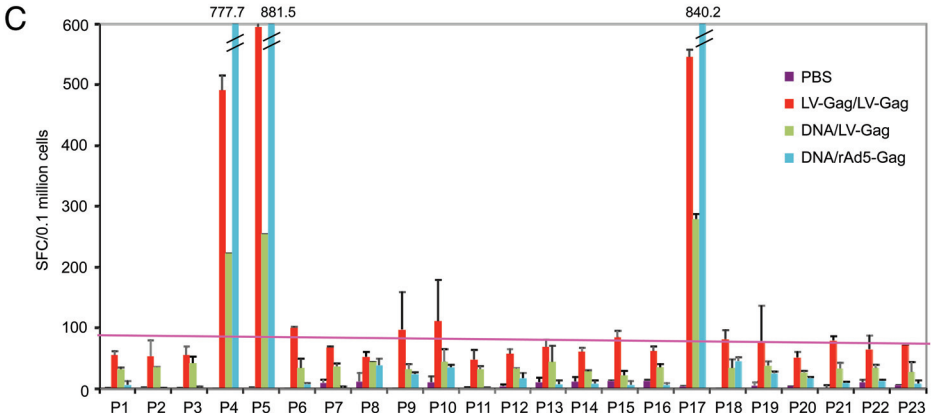

Fig. 5. Breadth of HIV-1 Gag-specific responses to LV-Gag-based vaccination. (*A*) A library of 123 15-mer peptides spanning the entire HIV-1 subtype B Gag sequence was divided into 23 pools (P1–P23) as indicated by the peptide matrix table. (*B* and *C*) Spleen cells of DNA/rAd5-Gag, LV-Gag/LV-Gag, and DNA/LV-Gag groups of BALB/c mice were harvested, stimulated with one of peptide pools for 18–24 h, and assayed by IFN-γ ELISPOT. Each group consisted of three mice. Number of highly reactive peptide pools versus number of nonreactive peptide pools for each group of mice was summarized as shown in (*B*). The threshold for defining a nonreactive peptide pool was based on the ELISPOT readout comparable with the control PBS group. A peptide pool was defined as highly reactive when its stimulated response was 5 times higher than that of the control PBS reading and could be obviously detected by an ICCS assay.

Discussion

We have previously shown that one can build a replication-deficient LV that targets a gene of interest directly to DCs in an animal to induce antigen-specific immune responses (23). In this study, we examine a special case, the production of T cells and antibodies against the Gag protein of HIV. This is of particular interest because attempts to make a vaccine against HIV have failed thus far and a new and more effective vector system is needed (5, 6). When this vector was used to deliver Gag immunogen (LV-Gag), a significant quantity of Gag-specific $CD8^+$ T cells could be detected upon a single injection. We performed a direct comparison of the LV-Gag with rAd5-Gag with the respect to magnitude, kinetics and the memory nature of the induced cellular immune response. A single round immunization of rAd5-Gag induced a stronger immunity than LV-Gag at the chosen doses. The time-course measurement showed that LV-Gag resembled a usual immune response of expansion and contraction, but rAd5-Gag provoked a persistent response. This persistent immunization of rAd-Gag vector might stem from the continuously active transcription of adenovirus vector at the site of injection (37) and could present a challenge for generating high quality memory T cells (2, 5). This unusually prolonged response of rAd5-Gag can compromise its utilities for repeated immunization [such a protocol was used by Merck's STEP trial (3, 4)] because the sustained APCs and neutralizing antibodies may inhibit responses from further homologous vaccination. As compared with the rAd5-Gag vector, LV-Gag induced a greater percentage of Gag-specific memory T cells that were of the central memory phenotype (T_{CM}: $CD44^{high}CD62L^+$).

Heterologous prime/boost strategies have been well-studied for AIDS vaccines, especially with a rAd5-based vector (38, 39). Unlike the adenoviral vector, the DC-directed LV is less likely to be restricted by the preexisting immunity, thus we tested its application for both homologous and heterologous vaccination regimens. We demonstrated that the DNA/LV-Gag as well as LV-Gag/LV-Gag displayed a remarkable enhancement of vaccine efficacy for generation of HIV-specific T-cell and antibody responses. Although generation of a robust $CD8^+$ T-cell response is one requirement for a HIV vaccine, the magnitude itself is not necessarily predictive of a superior control of HIV infection in many individuals (40). Therefore, we further investigated the functional potency and breadth of LV-Gag-induced T-cell responses. The $CD8^+$ T cells from the DNA/rAd5-Gag regimen were primarily IFN-γ^+, TNF-α^+, and IFN-γ^+TNF-α^+ cells, with few of them secreting IL-2. The ability of $CD8^+$ T cells to generate IL-2 could be significant because it should allow them to survive and expand (41). Moreover, the central memory $CD8^+$ T cells which home to lymphoid organs are thought to produce IL-2, while the effector counterparts are restricted to peripheral tissues and primarily secrete IFN-γ (42). Promisingly, our data indicate that there is a significant portion of HIV-specific $CD8^+$ T cells generated by either DNA/LV-Gag or LV-Gag/LV-Gag that are IL-2 producers. Notably, approximately 3% of the cytokine-producing DNA/LV-Gag-induced $CD8^+$ T cells produced three cytokines.

$CD4^+$ T cells, especially the polyfunctional ones, are of great importance to vaccine responses (43). Consistent with the results of Sun et al. (42), we observed that IFN-γ and TNF-α production dominated the $CD4^+$ T-cell population induced by DNA/rAd5-Gag immunization. The functional profiles of Gag-specific $CD4^+$ T cells elicited by DNA/LV-Gag and LV-Gag/LV-Gag were different from that of DNA/rAd5-Gag. This may not be too surprising, considering that LV-Gag and rAd5-Gag target different cell populations through distinct cellular receptors and are likely to mediate different forms of antigen presentations. IL-2^+TNF-α^+ cells represented the highest portion of $CD4^+$ T cells in the DNA/LV-Gag and LV-Gag/LV-Gag groups, with IFN-γ^+IL-2^+, IFN-γ^+TNF-α^+, and IFN-γ^+IL-2^+TNF-α^+ Gag-specific $CD4^+$ T cells at detectable levels. Several studies on the SIV-macaque model revealed that prolonged survival of infected monkeys was associated with the preservation of SIV-specific $CD4^+$ T cells producing IFN-γ, IL-2, and TNF-α (8, 40, 44). The frequency of $CD4^+$ T cells producing three cytokines simultaneously was also positively related to protection against *Leishmania major* infection (35). Furthermore, $CD4^+$ T cells were reported to be indispensable for secondary $CD8^+$ T-cell expansion, and the depletion of them during the priming phase led to deficient development of functional $CD8^+$ T-cell memory (41, 45). The role of $CD4^+$ T cells is particularly important in a prime/boost vaccination, because $CD4^+$ T cells help establishment of $CD8^+$ T-cell functionality and expansion in the boost phase of immunization (46). Balanced $CD8^+$ and $CD4^+$ T-cell responses are thought to be highly desirable for vaccine effectiveness, and were suggested by Liu et al. to explain an efficient priming by rAd26 vectors (8). This cytokine profile study reveals that DNA/LV-Gag is very effective regimen to produce both multifunctional $CD8^+$ and $CD4^+$ HIV-specific T cells in mice.

We further assessed the breadth of antigen recognition displayed by vector-induced T cells. Our experiments showed that there were three peptide pools that elicited the most vigorous responses for all three groups of mice given a prime/boost regimen. Although the magnitude of DNA/rAd5-Gag response to the three dominant peptide pools was the highest, there was a greater diversification of immunogen recognition by the DNA/LV-Gag and LV-Gag/LV-Gag regimens. We speculate that through the DC targeting, the vaccination involved with LV-Gag might load and present antigens more efficiently in the DCs, allowing the generation of broader responses (38). Our polyfunctional study also supports the notion that this wider epitope response might be the result of a better $CD4^+$ T-cell response.

In summary, we report an effort to evaluate an anti-HIV vaccination involving a LV directed to DCs. We found that both the DNA/LV-Gag and LV-Gag/LV-Gag vaccination regimens elicited multifunctional $CD4^+$ and $CD8^+$ Gag-specific T cells, and the DNA/LV-Gag method generated the highest frequencies of $CD4^+$ and $CD8^+$ cells secreting three cytokines simultaneously. Homologous or heterologous immunization using LV-Gag-induced T cells recognizing a wide range of Gag epitopes. This study in mice demonstrates that this DC-targeted LV is a promising vector system and should warrant further investigations in NHP to continue the evaluation of its potential for future human HIV/AIDS vaccine development.

Materials and Methods

Mice and Vaccination Procedure. Six- to eight-week-old female BALB/c mice were purchased from Charles River Laboratories. All animal procedures were performed in accordance with the guidelines set by the National Institutes of Health and the University of Southern California on the Care and Use of Animals. The details of immunization protocols are described in the *SI Text*.

Plasmid Construction and Vector Production. The plasmid encoding the DC-targeted envelope SVGmu was constructed as described in ref. 23. FUWGag was constructed by insertion of the cDNA of a HIV-1 subtype B Gag into the lentiviral backbone plasmid FUW (34) downstream of the human ubiquitin C promoter. The production of LV-Gag and rAd5-Gag vectors is described in the *SI Text*.

Tetramer Staining and Intracellular Cytokine Staining (ICCS). The details of tetramer staining, staining for phenotypic analysis, ICCS, and multiparameter ICCS are described in the *SI Text*.

Antibody ELISA and IFN-γ ELISPOT Assays. Antibody responses were assessed by ELISA using the method described before (see *SI Text*) (47). ELISPOT assays

were performed for IFN-γ using a kit from Millipore according to the manufacturer's instruction (see *SI Text*).

Statistical Analysis. The significance of the difference between groups was evaluated by analysis of variance followed by a one-tailed Student *t* test.

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1. Barouch DH, Nabel GJ (2005) Adenovirus vector-based vaccines for human immunodeficiency virus type 1. *Hum Gene Ther* 16:149–156.
2. Lasaro MO, Ertl HC (2009) New insights on adenovirus as vaccine vectors. *Mol Ther* 17:1333–1339.
3. Buchbinder SP, 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* 372:1881–1893.
4. McElrath MJ, et al. (2008) HIV-1 vaccine-induced immunity in the test-of-concept Step Study: A case-cohort analysis. *Lancet* 372:1894–1905.
5. Barouch DH (2008) Challenges in the development of an HIV-1 vaccine. *Nature* 455:613–619.
6. Walker BD, Burton DR (2008) Toward an AIDS vaccine. *Science* 320:760–764.
7. Casimiro DR, et al. (2005) Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with dna and recombinant adenoviral vaccine vectors expressing Gag. *J Virol* 79:15547–15555.
8. Liu J, et al. (2009) Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. *Nature* 457:87–91.
9. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. *Nature* 392:245–252.
10. Steinman RM, Banchereau J (2007) Taking dendritic cells into medicine. *Nature* 449:419–426.
11. Guermonprez P, et al. (2002) Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 20:621–667.
12. Dorrell L (2005) Therapeutic immunization strategies for the control of HIV-1. *Expert Rev Vaccines* 4:513–520.
13. Rinaldo CR (2009) Dendritic cell-based human immunodeficiency virus vaccine. *J Intern Med* 265:138–158.
14. Lapenta C, et al. (2003) Potent immune response against HIV-1 and protection from virus challenge in hu-PBL-SCID mice immunized with inactivated virus-pulsed dendritic cells generated in the presence of IFN-alpha. *J Exp Med* 198:361–367.
15. Yoshida A, et al. (2003) Induction of protective immune responses against R5 human immunodeficiency virus type 1 (HIV-1) infection in hu-PBL-SCID mice by intrasplenic immunization with HIV-1-pulsed dendritic cells: Possible involvement of a novel factor of human CD4(+) T-cell origin. *J Virol* 77:8719–8728.
16. Lu W, et al. (2003) Therapeutic dendritic-cell vaccine for simian AIDS. *Nat Med* 9:27–32.
17. Lu W, Arraes LC, Ferreira WT, Andrieu JM (2004) Therapeutic dendritic-cell vaccine for chronic HIV-1 infection. *Nat Med* 10:1359–1365.
18. Garcia F, et al. (2005) Therapeutic immunization with dendritic cells loaded with heat-inactivated autologous HIV-1 in patients with chronic HIV-1 infection. *J Infect Dis* 191:1680–1685.
19. Tacken PJ, Torensma R, Figdor CG (2006) Targeting antigens to dendritic cells in vivo. *Immunobiology* 211:599–608.
20. Tacken PJ, de Vries IJ, Torensma R, Figdor CG (2007) Dendritic-cell immunotherapy: From ex vivo loading to in vivo targeting. *Nat Rev Immunol* 7:790–802.
21. Trumpfheller C, et al. (2006) Intensified and protective CD4+ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. *J Exp Med* 203:607–617.
22. Trumpfheller C, et al. (2008) The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. *Proc Natl Acad Sci USA* 105:2574–2579.
23. Yang L, et al. (2008) Engineered lentivector targeting of dendritic cells for in vivo immunization. *Nat Biotechnol* 26:326–334.
24. Dullaers M, Thielemans K (2006) From pathogen to medicine: HIV-1-derived lentiviral vectors as vehicles for dendritic cell based cancer immunotherapy. *J Gene Med* 8:3–17.
25. VandenDriessche T, et al. (2002) Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. *Blood* 100:813–822.
26. Esslinger C, et al. (2003) In vivo administration of a lentiviral vaccine targets DCs and induces efficient CD8(+) T cell responses. *J Clin Invest* 111:1673–1681.
27. Palmowski MJ, et al. (2004) Intravenous injection of a lentiviral vector encoding NY-ESO-1 induces an effective CTL response. *J Immunol* 172:1582–1587.
28. Kim JH, et al. (2005) Induction of therapeutic antitumor immunity by in vivo administration of a lentiviral vaccine. *Hum Gene Ther* 16:1255–1266.
29. Iglesias MC, et al. (2007) Lentiviral vectors encoding HIV-1 polyepitopes induce broad CTL responses in vivo. *Mol Ther* 15:1203–1210.
30. Lopes L, et al. (2008) Immunization with a lentivector that targets tumor antigen expression to dendritic cells induces potent CD8+ and CD4+ T-cell responses. *J Virol* 82:86–95.
31. Geijtenbeek TB, et al. (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* 100:575–585.
32. Caminschi I, et al. (2006) Functional comparison of mouse CIRE/mouse DC-SIGN and human DC-SIGN. *Int Immunol* 18:741–753.
33. Tacken PJ, et al. (2005) Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti-DC-SIGN antibody. *Blood* 106:1278–1285.
34. Lois C, et al. (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295:868–872.
35. Darrah PA, et al. (2007) Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med* 13:843–850.
36. Gavioli R, et al. (2008) The Tat protein broadens T cell responses directed to the HIV-1 antigens Gag and Env: Implications for the design of new vaccination strategies against AIDS. *Vaccine* 26:727–737.
37. Tatsis N, et al. (2007) Adenoviral vectors persist in vivo and maintain activated CD8+ T cells: Implications for their use as vaccines. *Blood* 110:1916–1923.
38. Wu L, Kong WP, Nabel GJ (2005) Enhanced breadth of CD4 T-cell immunity by DNA prime and adenovirus boost immunization to human immunodeficiency virus Env and Gag immunogens. *J Virol* 79:8024–8031.
39. Cox KS, et al. (2008) DNA gag/adenovirus type 5 (Ad5) gag and Ad5 gag/Ad5 gag vaccines induce distinct T-cell response profiles. *J Virol* 82:8161–8171.
40. Kaufmann DE, et al. (2004) Comprehensive analysis of human immunodeficiency virus type 1-specific CD4 responses reveals marked immunodominance of gag and nef and the presence of broadly recognized peptides. *J Virol* 78:4463–4477.
41. Gattinoni L, et al. (2005) Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *J Clin Invest* 115:1616–1626.
42. Sun Y, et al. (2006) Virus-specific cellular immune correlates of survival in vaccinated monkeys after simian immunodeficiency virus challenge. *J Virol* 80:10950–10956.
43. Yamamoto T, et al. (2009) Polyfunctional CD4+ T-cell induction in neutralizing antibody-triggered control of simian immunodeficiency virus infection. *J Virol* 83:5514–5524.
44. Letvin NL, et al. (2006) Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. *Science* 312:1530–1533.
45. Janssen EM, et al. (2003) CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421:852–856.
46. Shedlock DJ, Shen H (2003) Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 300:337–339.
47. Kong WP, et al. (2003) Immunogenicity of multiple gene and clade human immunodeficiency virus type 1 DNA vaccines. *J Virol* 77:12764–12772.

Supporting Information

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SI Text

Vaccination Procedure. The DNA-based immunization protocol involved the injection of 50-μg Gag-encoded plasmid DNA intramuscularly three times successively at 3-week intervals (1). For immunization with viral vectors, mice were injected with replication-defective rAd5-Gag [1×10^{10} VP (Viral Particles)] via an f.p. route or LV-Gag [5×10^{6} TU (Transduction Units)] using the indicated routes. For the DNA/rAd5-Gag and DNA/LV-Gag immunization regimens, animals were given three consecutive intramuscular injections of plasmid DNA (50 μg each) as priming, followed by a booster injection of rAd5-Gag (1×10^{10} VP) or LV-Gag (5×10^{6} TU) f.p. 3 weeks after the last DNA injection. For the LV-Gag/LV-Gag homologous immunization, mice were primed with the LV-Gag (5×10^{6} TU) f.p. and boosted with the same vector and dose i.d. (at the base of tail) 4 weeks thereafter. The mice vaccinated by a single injection of LV-Gag were analyzed for their immune responses 2 weeks postinjection. The rAd5-Gag immunized mice were analyzed 3 weeks postinjection. The animals with prime/boost vaccinations were analyzed 2 weeks after the final injection.

Vector Production. The LV-Gag vector was produced by transient transfection of 293T cells using a standard calcium phosphate precipitation protocol. 293T cells cultured in a 15-cm tissue culture dish (BD Biosciences) were transfected with the following plasmids: the lentiviral backbone plasmid FUWGag (37.5 μg), the SVGmu-encoding envelope plasmid (18.75 μg), and the packaging plasmids (pMDLg/pRRE and pRSV-Rev, 18.75 μg for each). The viral supernatant was harvested twice at both 48 and 72 h posttransfection, combined and filtered through a 0.45-mm filter (Corning). The concentrated viral pellets were obtained after ultracentrifugation of the viral supernatants at $50{,}000 \times g$ for 90 min, and were then resuspended in an appropriate volume of cold PBS for in vivo study. The Gag-encoded and E1/E3-deleted adenovirus serotype 5 vector (rAd5-Gag) was constructed and produced according to the procedure provided by the manufacturer (ViraPower Adenoviral Expression System, Invitrogen). The Gag-encoding plasmid for the DNA vaccine was generously provided by Dr. Gary Nabel of the National Institutes of Health Vaccine Research Center (Bethesda, Maryland) and the endotoxin-free plasmid DNA was produced using a Qiagen kit (Qiagen) for the immunization of mice.

Gag Peptide and Peptide Pool. The immunodominant H2-K^d-restricted $CD8^+$ T-cell epitope (AMQMLKETI, amino acids 197–205) is derived from the p24 portion of the Gag protein. This peptide was synthesized (GenScript) and dissolved in dimethyl sulfoxide (DMSO) at 8 mg/mL. The Gag peptide pool includes 123 15-mer peptides overlapping by 10–11 amino acids and spanning the entire HIV-1 subtype B Gag sequence. Individual peptides in this library were dissolved in DMSO at 10 mg/mL, and stored at -80 °C.

Tetramer Staining and Phenotypic Analysis. The phycoerythrin (PE)-conjugated major histocompatibility complex (MHC) class I tetramer H2-K^d-AMQMLKETI was obtained from Beckman Coulter. At indicated time points after immunization, tetramer-specific and phenotypic properties were evaluated on spleen cells harvested from vaccinated and control mice. Surface staining was performed by blocking the Fcγ receptors of cells with an anti-mouse CD16/CD32 antibody (clone 2.4G2, BD Biosciences), followed by incubating the cells with tetramer along with other fluorochrome-conjugated antibodies, including FITC-, PE-Cy5- or APC- conjugated antibodies specific for mouse CD8, CD44, CD62L (BD Biosciences). The flow cytometry analysis was conducted using either the FACSort or the FACSCalibur instrument (BD Biosciences).

Intracellular Cytokine Staining (ICCS) and Multiparameter ICCS. Splenocytes from immunized or control mice (1×10^{6}/sample) were pooled and incubated with the HIV Gag peptide (AMQMLKETI) (4 μg/mL) in the presence of costimulatory anti-CD28 antibody (2 μg/mL, BD Biosciences) for 1 h at 37 °C in a 96-well round-bottom plate in RPMI medium supplemented with 10% FBS (Sigma), 10 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. Brefeldin A (BFA, Sigma) was added (10 μg/mL) to wells to inhibit cytokine exporting for another 4 h. Surface staining was performed by incubating restimulated cells with anti-mouse CD16/CD32 antibody, followed by anti-mouse CD8 and anti-mouse CD4 antibodies. Cells were then permeabilized in 100 μL Cytofix/Cytoperm solution (BD Biosciences) at 4 °C for 20 min, washed with Perm/Wash buffer (BD Biosciences), and followed by intracellular staining with PE-conjugated anti-mouse IFN-γ at 4 °C for 30 min. The flow cytometry analysis was carried out using the FACSort instrument from BD Biosciences. A similar procedure was used for the multiparameter ICCS, except that the splenocytes were stimulated by the pooled HIV-1 Gag peptides (2.5 μg/mL for each peptide) and the resulting cells were incubated with the viability dye (ViViD, Invitrogen) and stained with the following surface monoclonal antibodies: anti-CD4-PerCP, anti-CD8-APC-Cy7, anti-CD3-Alexa488, and intracellular monoclonal antibodies: anti-IL-2-PE, anti-IFN-γ-APC, and anti-TNF-α-PE-Cy7. ICCS data were acquired on a BD LSR II flow cytometer. All of the staining antibodies were purchased from (BD Biosciences).

ELISA. Antibody responses were assessed by ELISA using the method described before (2). PBST (PBS containing 0.2% Tween 20) was prepared as wash buffer, and three to five times of extensive washes were conducted each time before adding new reagents to plates. ELISA plates were coated with *Galanthus nivalis* lectin 100 μL/well (10 μg/mL, Sigma) overnight at 4 °C, followed by blocked with PBSS (PBS containing 10% FBS) at 200 μL/well, 2 h, and room temperature. To evaluate Gag-specific antibody response, 293T cells were transfected with FUWGag (100 μL) 48 h before the time of assaying ELISA. Supernatant of transfected cells was collected, and was added to the plates (100 μL/well) for 1 h of incubation at room temperature. Sera from immunized mice of different groups were diluted 2-fold serially (in PBSS) and 100 μL were added to each well for 1 h. The plates were then treated with 100 μL horseradish peroxidase (HRP)-labeled anti-mouse IgG or IgM antibody (1:10,000 dilution) for an additional 1 h. Finally, color development was accomplished by using 100 μL per well of tetramethylbenzidine (TMB) substrate solution (KPL) for 45 min at 37 °C, and stopped by 100 μL of 2 M H_2SO_4. Titer was reported as the reciprocal of endpoint dilutions, at which the absorbance readings at the wavelength of 450 nm (OD_{450}) were at least 0.2 OD greater than that of the control groups. The optical density was measured using a plate reader (Molecular Devices).

IFN-γ ELISPOT Assay. ELISPOT assays were performed for IFN-γ using a kit from Millipore according to the manufacturer's instruction. Briefly, anti-mouse IFN-γ antibody (10 μg/mL in PBS) was used as the capture antibody and plated 100 μL/well on 96-well MultiScreen-IP plates overnight at 4 °C. The plate was decanted and blocked with RPMI medium containing 10% FBS at 37 °C for 2 h. Splenocytes from mice were plated at 1×10^5 cells/well in 150 μL complete medium in company with stimulus, which are HIV-1 Gag single peptide (2 μg/mL) or pools of peptides (at a final concentration of 3 μg/mL for each peptide) for this assay. After 18 h incubation at 37 °C, cells were lysed by water and plates were detected by 0.5 μg/mL biotinylated anti-IFN-γ antibody (BD Biosciences) for 2 h at room temperature. Plates were further washed and incubated with the 1,000-fold-diluted streptavidin-alkaline phosphate conjugate for 45 min at room temperature. After a final extensive wash, spots were identified by addition of BCIP/NBTplus substrate, and the number of IFN-γ producing cells was quantified by an ELISPOT reader.

1. Wu L, Kong WP, Nabel GJ (2005) Enhanced breadth of CD4 T-cell immunity by DNA prime and adenovirus boost immunization to human immunodeficiency virus Env and Gag immunogens. *J Virol* 79:8024–8031.

2. Kong WP, et al. (2003) Immunogenicity of multiple gene and clade human immunodeficiency virus type 1 DNA vaccines. *J Virol* 77:12764–12772.

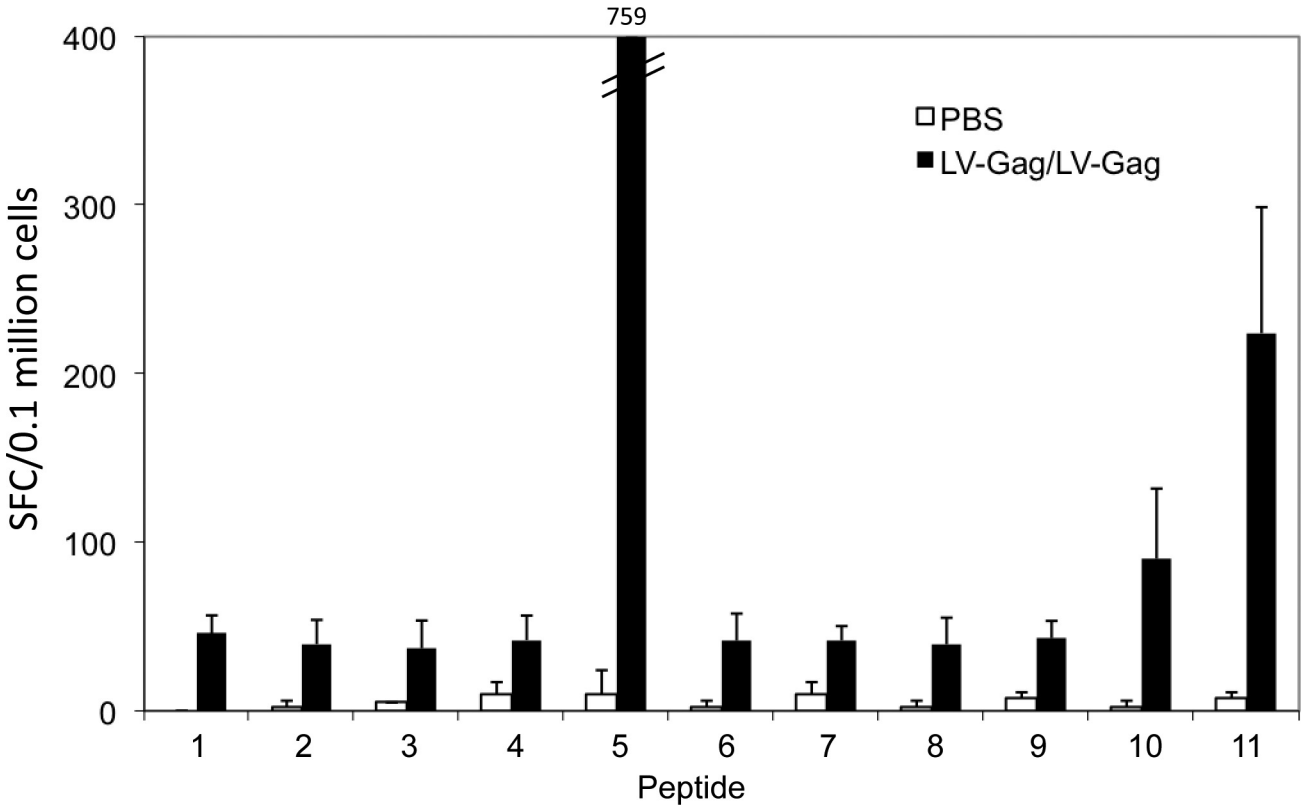

Fig. S1. Confirmation of the response of T cells from LV-Gag/LV-Gag mice to individual peptides derived from the peptide pool study. The amino acid sequences of these peptides are: 1, eeeqnkskkkaqqaa; 2, nkskkkaqqaaadtg; 3, kkaqqaaadtghsnq; 4, sqnypivqniqgqmv, 5, amqmlketineeaae; 6, lketineeaaewdrv; 7, drvhpvhagpiapgq; 8, pvhagpiapgqmrep; 9, stlqeqigwmtnnpp; 10, pvgeiykrwiilgln; and 11, iykrwiilglnkivr.

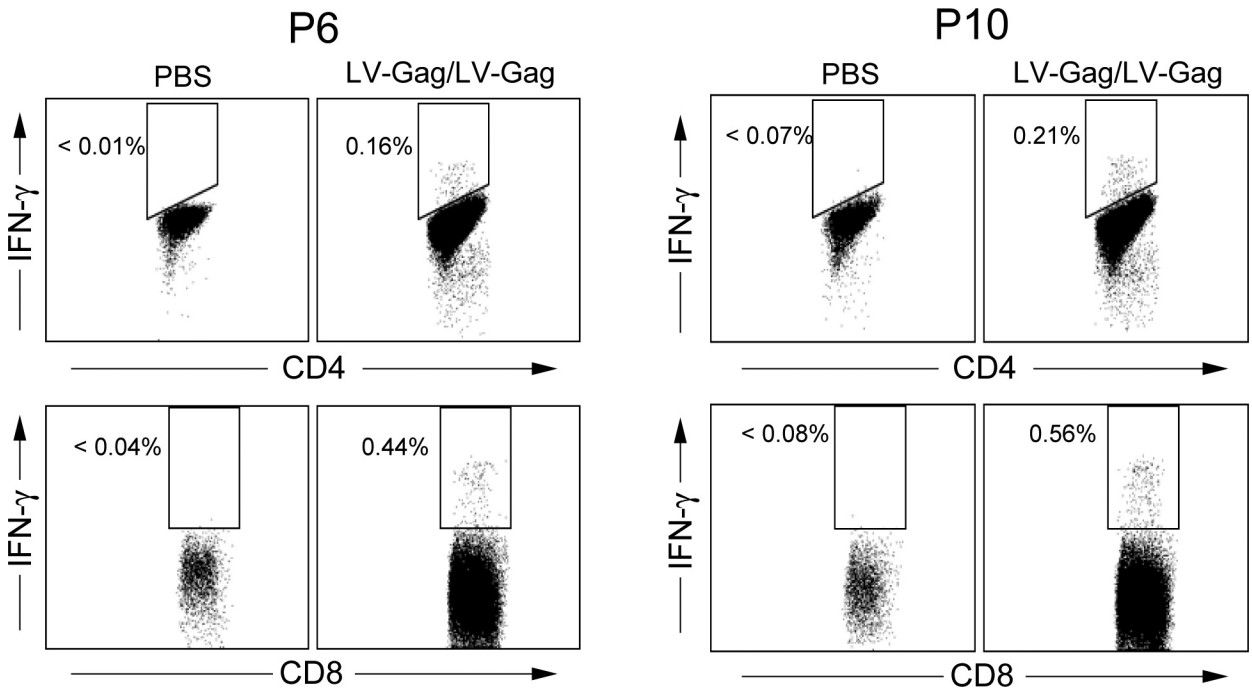

Fig. S2. Intracellular cytokine staining (ICCS) analysis of splenocytes harvested from LV-Gag/LV-Gag vaccinated mice and stimulated by two representative and highly reactive peptide pools (P6 and P10). Gag-specific CD4 and CD8 T-cell responses can be assessed by ICCS staining of IFN-γ along with the surface costaining of CD4 and CD8.