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.

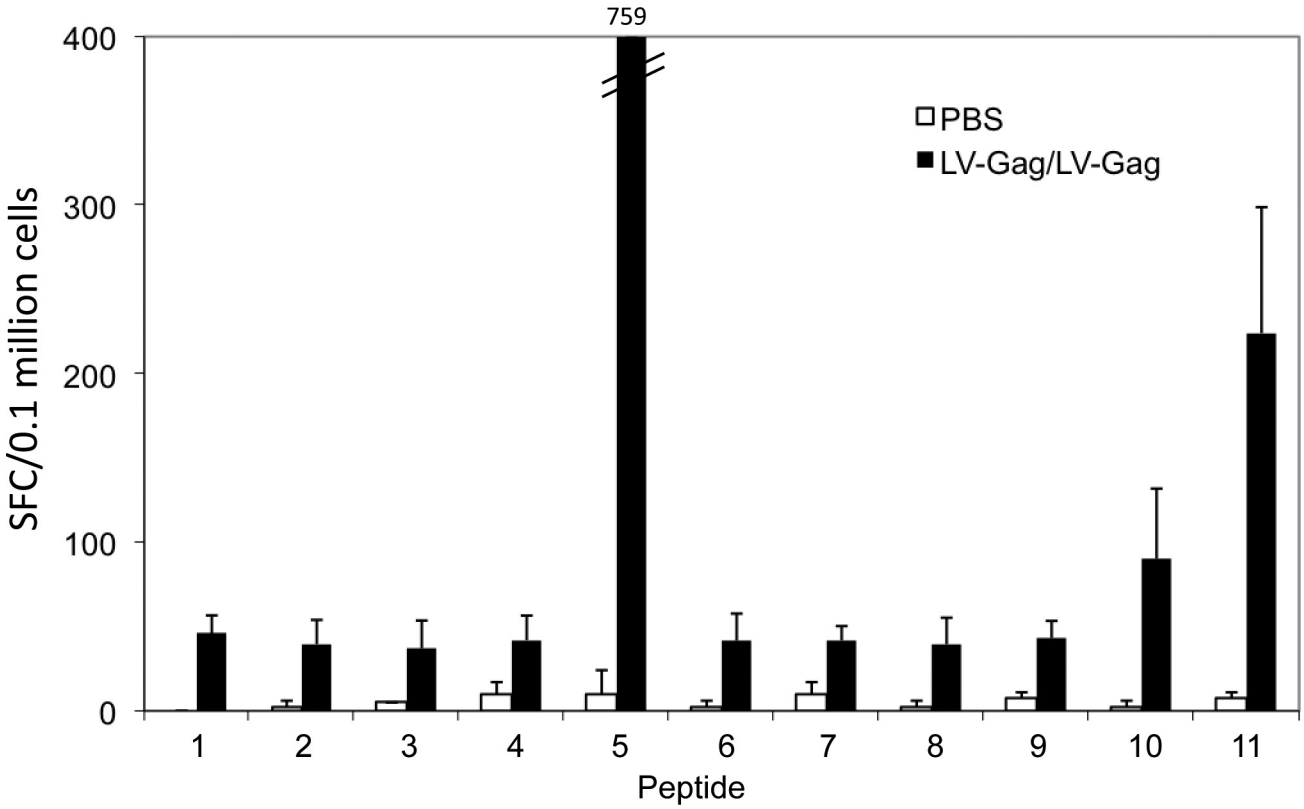

Fig. S1. Confirmation of the response of T cells from LV-Gag/LV-Gag mice to individual peptides derived from the peptide pool study. The amino acid sequences of these peptides are: 1, eeeqnkskkkaqqaa; 2, nkskkkaqqaaadtg; 3, kkaqqaaadtghsnq; 4, sqnypivqniqgqmv, 5, amqmlketineeaae; 6, lketineeaaewdrv;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

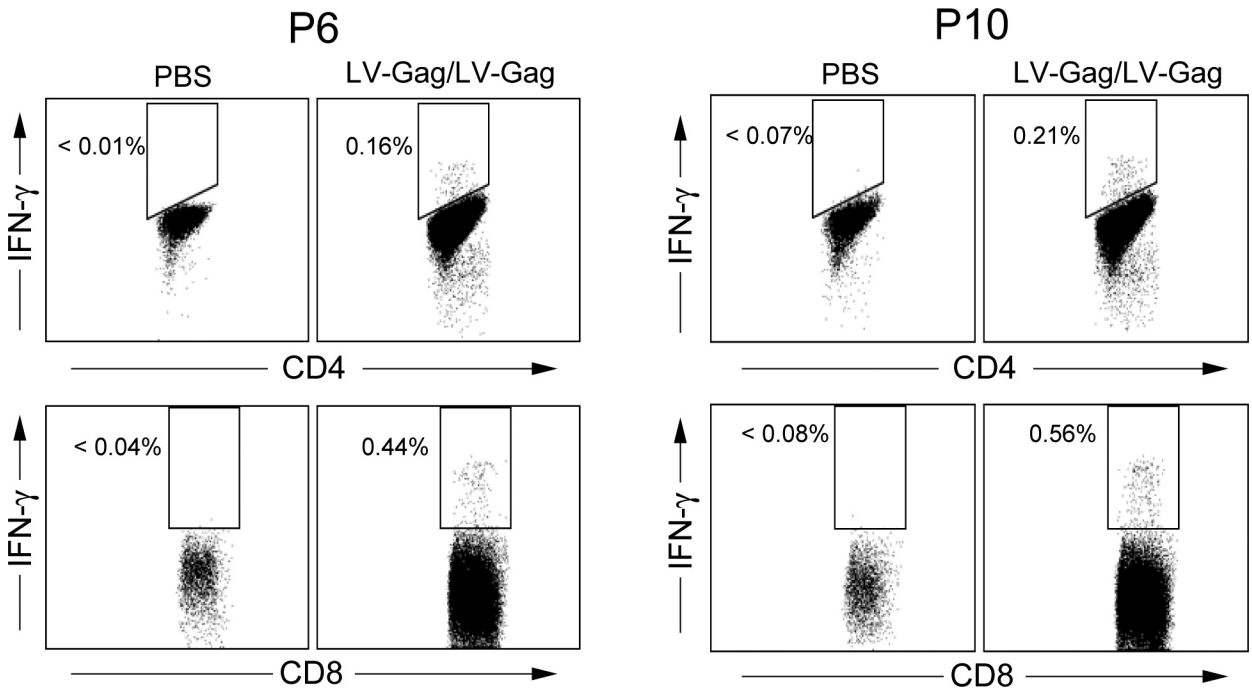

Fig. S2. Intracellular cytokine staining (ICCS) analysis of splenocytes harvested from LV-Gag/LV-Gag vaccinated mice and stimulated by two representative and highly reactive peptide pools (P6 and P10). Gag-specific CD4 and CD8 T-cell responses can be assessed by ICCS staining of IFN-γ along with the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