Comparative Immunogenicity of Human Immunodeficiency Virus Particles and Corresponding Polypeptides in a DNA Vaccine

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The immunogenicity of a plasmid DNA expression vector encoding both Gag and envelope (Env), which produced human immunodeficiency virus (HIV) type 1 virus-like particles (VLP), was compared to vectors expressing Gag and Env individually, which presented the same gene products as polypeptides. Vaccination with plasmids that generated VLP showed cellular immunity comparable to that of Gag and cell-mediated or humoral responses similar to those of Env as immunization with separate vectors. These data suggest that DNA vaccines encoding separated HIV polypeptides generate immune responses similar to those generated by viral particles.

Alternative strategies have been explored to generate immune responses to human immunodeficiency virus (HIV) that are important for the development of AIDS vaccines. Among these approaches, immunization with inactivated virus or virus-like particles (VLP) may allow generation of immunity to conformational epitopes and native virus structures, while presentation of individual gene products through the class I and II major histocompatibility complexes induces T-cell responses to peptides implicated in the control of HIV replication (1, 2, 12, 23). Vaccination with live attenuated viruses (10) and inactivated viruses (11, 16, 19, 22) and DNA vaccination with the proviral genome are among the approaches used to generate noninfectious viral particles (1, 2, 12). Live attenuated vaccines have proven highly effective in a simian immunodeficiency virus (SIV) nonhuman primate model (10), but they present significant safety concerns for human use (4, 5). Because expression vector plasmids can give rise to noninfectious VLP that mimic natural virus, they may have advantages over attenuated viruses, but comparisons of cellular and humoral immune responses to VLP or single-gene immunogens have not been made. In this study, we compared the immunogenicity of DNA vaccines encoding VLP to that of the corresponding separate polypeptides.

Construction and production of VLP. A hybrid CCR5-tropic Env protein gp160 modified by deletion of the cleavage site (C), the fusion peptide (F), and the interspace (I) between the two heptad repeats (Δ CFI) to stimulate high levels of antibody production without compromising the cytotoxic T-lymphocyte response (7, 24) was further altered by deletion of V1 and V2 (Δ V₁V₂) loop regions to expose core conserved determinants (Fig. 1A, gp145 Δ CFI Δ V₁V₂). The gp145 Δ CFI Δ V₁V₂ cDNA was inserted downstream of the Rous sarcoma virus (RSV) enhancer-promoter, linked to a human T-cell leukemia virus type 1 R-region translational enhancer (3), and is designated Env here. This plasmid had been prepared by insertion of the

AflIII/Klenow/HpaI-digested modified RSV promoter fragment into pVRC1012 (24) that had been digested with SpeI and HpaI and blunted with the Klenow fragment. The polyadenylation signal from herpes simplex virus thymidine kinase (GenBank accession number U40398), amplified with the sense primer 5'-CCGGATCCGTCGACCGGGAGATGGGGGAG3' and the antisense primer 5'-AACCAGGCCATGATGGCCACTTGGGGGGTGGGGTGGGG3', was digested with BamHI and SfiI and inserted into those sites in the plasmid. An XbaI-to-BamHI fragment of gp145 Δ CFI Δ V₁V₂ (24) was inserted into the modified RSV promoter vector digested with the same enzymes (Fig. 1B, Env). A codon-modified Gag known to induce cellular immunity (13) was also used (Fig. 1B, Gag). To compare these immune responses to those induced by VLP, a dual expression vector was made from the above plasmid by digesting the Env expression vector with MscI upstream of the RSV promoter and inserting the SpeI and KpnI Klenow-blunted Gag expression cassette in the same orientation (Fig. 1B, pVLP_{gp145}). Gag and Env showed comparable expression in these different plasmids after transfection of human embryonic kidney (293) cells and analysis by Western blotting (Fig. 1C).

To determine whether the dual expression plasmid VLP vector could give rise to pseudoparticles, pVLP_{gp145} was transfected into 293 cells and mouse embryonic fibroblasts (NIH 3T3), and its ability to produce VLP was assessed by electron microscopy as described previously (14). 293 cells were ~100-fold more transfectable than NIH 3T3 cells, but when standardized for transfection efficiency, the yields of VLP differed by less than twofold between the two cell types (Fig. 2, legend), suggesting that there was no block to VLP formation using codon-modified expression vectors in murine cells. The dual expression plasmid produced VLP with an average diameter of 100 nm in both human and mouse cell lines (Fig. 2A and B). Buoyant density gradient sedimentation (14, 24) of supernatants obtained from 293 cells transfected with dual expression plasmid vector VLP_{gp145} showed incorporation of Env into VLP, with a peak of activity detected at a density of 1.1 g/ml (Fig. 2C), comparable to that reported for HIV type 1 (HIV-1) viral particles. The relative amounts of Env per particle were quantified by measuring Gag and Env protein levels by en-

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FIG. 1. Schematic representation of HIV gene and immunization vectors. (A) The major structural features of the hybrid CCR5-tropic Env protein gp160, modified Env gp145ΔCFIΔV₁V₂ (7, 24), and Gag (13) used in the present study are shown. V₁, V₂, V₃, and V₄ indicate the respective variable regions. TM, transmembrane domain. (B) Schematic representation of immunization plasmids. The Gag expression plasmid containing a cytomegalovirus promoter (13) and Env gp145ΔCFIΔV₁V₂ (24) inserted into a modified RSV promoter were used as separate immunogens. The dual expression plasmid pVLP_{gp145} gives rise to VLP. CMV, cytomegalovirus. (C) Comparable expression of HIV-1 Gag, Env gp145ΔCFIΔV₁V₂, and the dual vector in 293 cells. Cells (2×10^6) were transfected with 5 μ g of Gag plus 5 μ g of pVRC1012 vector (lane 1), 5 μ g of Env plus 5 μ g of vector (lane 2), 5 μ g of Gag plus 5 μ g of Env (lane 3), 10 μ g of dual expression VLP vector (lane 4), and 10 μ g of vector (lane 5). Expression was measured 48 h after transfection by Western blotting as described previously (7).

zyme-linked immunosorbent assay (ELISA) and Western blot analysis, respectively. The VLP from the peak of fraction 6 (Fig. 2C) contained ~ 0.45 pmol of Gag and 0.065 pmol of Env, and the Gag/Env ratio was $\sim 7:1$. Assuming $\sim 2,000$ Gag molecules per particle, we estimate that these are ~ 285 Env's or ~ 95 trimeric Env spikes per particle of VLP. VLP incorporated Env more efficiently than native virus, estimated to have ~ 10 trimers per virion (9), possibly because codon-modified Env expression vectors driven by a strong promoter may allow for greater Env synthesis than native virus. Characterization of these modified Env's has been published previously (7), where they have been shown to form predominantly trimers. They would be expected to behave similarly in these VLP.

pVLP_{gp145} elicits cellular immune responses to Gag and Env similar to those elicited by separate Gag and Env injections. To determine whether pVLP_{gp145} enhanced CD4⁺ and CD8⁺ T-cell responses, mice were immunized with either the dual expression plasmid or individual vectors expressing Gag and Env injected at separate sites. Briefly, female 6- to 8-week-old

BALB/c mice were injected in the right and left quadriceps muscles with a total of 50 μ g of purified plasmid DNA suspended in 200 μ l of normal saline. In the group receiving two separate plasmids expressing Gag and Env, mice were injected in the right quadriceps muscle with 25 μ g of the Gag plasmid and in the left quadriceps muscle with 25 μ g of Env plasmid. In the pVLP_{gp145} group, mice received 25 μ g of the dual expression plasmid on both sides. Each group ($n = 5$) was injected three times at weeks 0, 3, and 6. Ten days after the final DNA immunization, animals were euthanized, and splenocytes were incubated with overlapping Gag and Env peptide pools. Intracellular gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF- α) expression in stimulated CD4⁺ or CD8⁺ lymphocytes was analyzed by intracellular cytokine staining as described previously (15).

The CD4⁺ and CD8⁺ responses to Gag peptides induced by administration of Gag alone, separate injections of Gag and Env, or pVLP_{gp145} vaccination increased relative to the empty vector control; however, there was no statistical difference in

FIG. 2. Production of virus-like particles shown by electron micrographs and buoyant density gradient analysis. (A) Human 293 cells were transfected with Env, Gag, and pVLP_{gp145}. Arrows indicate VLP from Gag and pVLP_{gp145}. The yield of the VLP was 1.46 $\mu\text{g/ml}$ measured by p24 ELISA as described previously (25). The experiment was performed in triplicate. (B) Mouse NIH 3T3 cells were transfected with pVLP_{gp145}. The cells were harvested 48 h after transfection and observed by electron microscopy. Arrows indicate VLP. The yield of the VLP was 0.024 $\mu\text{g/ml}$ as measured by p24 ELISA, but the transfection frequency was 100-fold lower, indicating 1.64-fold higher levels of VLP formation when normalized for transfection. There was therefore no block to VLP formation with these vectors in murine cells. This experiment was performed in triplicate. (C) OptiPrep (Invitrogen) gradient analysis of VLP encoded by pVLP_{gp145}. This vector was transfected into 293 cells, and supernatants were collected after 3 days, followed by density gradient centrifugation as previously described (24).

FIG. 3. CD4⁺ and CD8⁺ T-cell responses against HIV-1 Gag as determined by intracellular cytokine analysis. CD4⁺ (left) and CD8⁺ (right) intracellular cytokine staining for IFN- γ - and TNF- α -positive cells was performed by a standard method (15). Mice (five per group) were immunized with 50 μ g of DNA. The groups included mice injected with 50 μ g of vector pVRC1012 with no insert (Vector) (7) or those injected with 25 μ g of vector and 25 μ g of the single-gene plasmid expressing Gag (Gag), 25 μ g of vector and 25 μ g of the single-gene plasmid expressing Env (Env), two individual plasmids at separate sites (Gag [25 μ g] in the right quadriceps muscle and Env [25 μ g] in the left quadriceps muscle) (separate Gag+Env), or 25 μ g of VLP-forming dual expression vector bearing Gag+Env (VLP) on each side (total of 50 μ g). Splenic cells were harvested 10 days after the final immunization and sensitized with a Gag peptide pool. The cells were fixed, stained with monoclonal antibodies, and analyzed by fluorescence-activated cell sorting to detect the IFN- γ - and TNF- α -positive cells in the CD4⁺ and CD8⁺ populations shown. The symbols show the individual results for the five mice in each group. The thin horizontal bar represents the average of the five data points; bars show the standard deviation. The *P* values comparing separate and VLP groups against the Gag peptide pool are shown. The *P* values comparing the vector and separate groups for CD4 and CD8 response are 0.04 and 0.07, respectively.

CD4⁺ or CD8⁺ responses to Gag between the separate Gag and Env- and pVLP_{gp145}-injected mice (*P* values of 0.25 and 0.86, respectively; nonparametric Wilcoxon rank sum test) (Fig. 3). Although the CD4 responses to Env were not detectable, they were not different among groups (Fig. 4A, left panel). We have typically seen lower signals in CD4⁺ cells than in CD8⁺ cells (15), consistent with previous observations that CD4⁺ responses are lower than CD8⁺ responses by the intracellular cytokine staining assay (6, 17, 21), possibly because relevant posttranslational modifications are lacking in the peptides, because γ -IFN and TNF- α are less efficiently synthesized in these CD4⁺ cells than in CD8⁺ cells, or because antigen-presenting cells are limiting. In contrast, the CD8⁺ responses to Env were detectable and showed no statistical difference between separate Gag and Env and pVLP groups (*P* = 0.69) (Fig. 4A, right panel).

Antibody and neutralizing responses in mice injected with VLP or Env plasmids. Sera from mice immunized with pVLP_{gp145} and the individual plasmid groups were tested for antibody responses using a lectin-capture HIV-1 Env protein ELISA (15). The sera from the groups immunized with Env

FIG. 4. CD4⁺ and CD8⁺ T-cell responses and antibody titers to HIV-1 Env as determined by intracellular cytokine analysis, ELISA and neutralization assays. (A) CD4⁺ (left) and CD8⁺ (right) T-cell responses against Env determined by intracellular cytokine analysis for IFN- γ - and TNF- α -positive cells as previously described (15). Each group was immunized with control vector alone (Vector), Gag and control vector (Gag), Env and control vector (Env), Gag and Env in separate sites (separate Gag+Env), or pVLP_{gp145} (VLP). The symbols show the individual results for the five mice in each group. The thin horizontal bar represents the average of the five data points; bars show the standard deviations. The *P* values comparing the separate Gag+Env and VLP groups against the Env peptide pool are shown. (B) Antibody titer against Env in the immunized mouse sera as determined by ELISA. ELISA results represent endpoint dilution titers of Env-specific antibodies in mouse sera as determined by optical density. Sera from the five groups of mice were collected 10 days after the third immunization. The *P* value for the difference between the separate and VLP groups is shown. (C) Neutralizing antibody responses of the indicated mice were measured. IgG was purified from sera using the Nab protein A purification kit (Pierce, catalog number 45200). Purified IgG (12.5 μ g/ml) was incubated with HXB2 pseudotyped lentiviral vectors expressing a luciferase reporter gene for 30 min before infection of human T-cell line and M8166 cells. After 48 h, luciferase activity was analyzed as described previously (25). The experiment was performed in duplicate.

plasmid vector, separate Gag and Env, or pVLP_{gp145} showed significant responses against Env compared to the vector control ($P < 0.05$) (Fig. 4B); however, there was no statistically significant difference between responses to the separate Gag and Env and pVLP_{gp145} plasmids ($P = 0.91$) (Fig. 4B). The neutralizing antibody response was measured using an HXB2 pseudotyped lentiviral vector expressing a luciferase reporter gene. Immunoglobulin G (IgG) from mice immunized with these plasmids was purified to avoid the nonspecific inhibition sometimes observed with mouse antisera. Mice immunized with vector alone showed no neutralizing antibody responses. Two mice in each group immunized with separate Gag and Env or pVLP_{gp145} showed a strong anti-Env antibody response by ELISA and were analyzed in the neutralization assay. Antisera from the mice in both groups mediated neutralization ($P < 0.05$) (Fig. 4C); however, there was no statistical difference between separate Gag and Env and pVLP_{gp145} vaccination ($P = 0.69$). There was therefore no significant change in antibody titers against Env protein or neutralization, regardless of whether the dual VLP or the separate Env plasmid was used for vaccination.

In this study, we show that immune responses induced by separate vaccination injections of plasmid DNA with Gag and Env were comparable to those elicited by a plasmid encoding both proteins that gave rise to VLP. The cellular immune responses to Gag were similar (Fig. 3). Further, there was no major difference in the cellular or humoral immune response to Env elicited by VLP and separated plasmids (Fig. 4). In murine cells that express human CD4, CCR5, and cyclin T1, infection by wild-type HIV-1 tends to integrate and express viral proteins, but few infectious viral particles are produced. This block is postulated to be due to a decrease in viral entry, viral RNA synthesis, and the failure of Gag to target to the membrane (8, 18). In the present study, VLP were observed to bud efficiently from the membranes of both 293 cells and NIH 3T3 cells (Fig. 2A and B). This finding suggests that the block to assembly of wild-type HIV-1 in murine cells (20) is overcome by expression of codon-modified synthetic viral genes, likely because of altered mRNA structure that is no longer Tat- or Rev-dependent and because of the strong enhancers that drive gene expression. These VLP are indistinguishable from immature wild-type virus by electron microscopy.

Inactivated HIV-1 and SIV particles treated with 2,2'-dithiodipyridine (aldrithiol-2) have been used to boost recombinant vaccinia viruses expressing HIV Env or SIV Gag/Pol in macaque models (23). Vaccinated monkeys produced anti-Env and -Gag antibodies after being given a booster with inactivated particles, experienced less viremia after challenge, and showed no significant loss of CD4⁺ T cells. DNA vaccination producing viral particles from proviral DNA using a long terminal repeat or cytomegalovirus promoter have proven to be partially protective in chimeric simian-human immunodeficiency virus and SIV challenge models (1, 2, 11); however, the use of proviral genes presents safety concerns related to reversion and recombination with natural virus. Production of HIV VLP from a single plasmid system that expresses both Gag and Env variants using synthetic codons addresses these safety concerns. In addition, it provides the advantage of producing VLP from antigen-presenting cells that may take up these plasmids, not only cells naturally infected by virus, as is the case with live

attenuated vectors. However, we find that the immunogenicity of a DNA vaccine encoding HIV Env in a VLP is similar to that of individual HIV gene products in terms of quantitative cellular and humoral immune responses. Though it remains possible that VLP may prove useful for AIDS vaccine development, the present study suggests that individual plasmid DNA expression vectors can generate similar immunity. Vaccination with combinations of such gene products using DNA vaccines may add breadth to the antiviral immune response without the requirement for formation of particles *in vivo*, thus facilitating the development of multivalent AIDS vaccines.

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REFERENCES

1. Akahata, W., E. Ido, H. Akiyama, H. Uesaka, Y. Enose, R. Horiuchi, T. Kuwata, T. Goto, H. Takahashi, and M. Hayami. 2003. DNA vaccination of macaques by a full-genome simian/human immunodeficiency virus type 1 plasmid chimera that produces non-infectious virus particles. *J. Gen. Virol.* **84**:2237–2244.
2. Akahata, W., E. Ido, T. Shimada, K. Katsuyama, H. Yamamoto, H. Uesaka, M. Ui, T. Kuwata, H. Takahashi, and M. Hayami. 2000. DNA vaccination of macaques by a full genome HIV-1 plasmid which produces noninfectious virus particles. *Virology* **275**:116–124.
3. Attal, J., M. C. Theron, G. Kann, P. Bolifraud, C. Puissant, and L. M. Houdebine. 2000. The stimulation of gene expression by the R region from HTLV-1 and BLV. *J. Biotechnol.* **77**:179–189.
4. Baba, T. W., Y. S. Jeong, D. Pennick, R. Bronson, M. F. Greene, and R. M. Ruprecht. 1995. Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* **267**:1820–1825.
5. Baba, T. W., V. Liska, A. H. Khimani, N. B. Ray, P. J. Dailey, D. Penninck, R. Bronson, M. F. Greene, H. M. McClure, L. N. Martin, and R. M. Ruprecht. 1999. Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques. *Nat. Med.* **5**:194–203.
6. Betts, M. R., D. R. Ambrozak, D. C. Douek, S. Bonhoeffer, J. M. Brenchley, J. P. Casazza, R. A. Koup, and L. J. Picker. 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4⁺ and CD8⁺ T-cell responses: relationship to viral load in untreated HIV infection. *J. Virol.* **75**:11983–11991.
7. Chakrabarti, B. K., W. P. Kong, B.-Y. Wu, Z.-Y. Yang, J. Friberg, Jr., X. Ling, S. R. King, D. C. Montefiori, and G. J. Nabel. 2002. Modifications of the human immunodeficiency virus envelope glycoprotein enhance immunogenicity for genetic immunization. *J. Virol.* **76**:5357–5368.
8. Chen, B. K., I. Rouso, S. Shim, and P. S. Kim. 2001. Efficient assembly of an HIV-1/MLV Gag-chimeric virus in murine cells. *Proc. Natl. Acad. Sci. USA* **98**:15239–15244.
9. Chertova, E., J. J. Bess, Jr., B. J. Crise, R. C. Sowder, I. L., T. M. Schaden, J. M. Hilburn, J. A. Hoxie, R. E. Benveniste, J. D. Lifson, L. E. Henderson, and L. O. Arthur. 2002. Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus. *J. Virol.* **76**:5315–5325.
10. Daniel, M. D., F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers. 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* **258**:1938–1941.
11. Gorelick, R. J., R. E. Benveniste, T. D. Gagliardi, T. A. Wiltout, L. K. Busch, W. J. Bosche, L. V. Coren, J. D. Lifson, P. J. Bradley, L. E. Henderson, and L. O. Arthur. 1999. Nucleocapsid protein zinc-finger mutants of simian immunodeficiency virus strain Mne produce virions that are replication defective *in vitro* and *in vivo*. *Virology* **253**:259–270.
12. Gorelick, R. J., R. E. Benveniste, J. D. Lifson, J. L. Yovandich, W. R. Morton, L. Kuller, B. M. Flynn, B. A. Fisher, J. L. Rossio, M. Piatak, Jr., J. W. Bess, Jr., L. E. Henderson, and L. O. Arthur. 2000. Protection of *Macaca nemestrina* from disease following pathogenic simian immunodeficiency virus (SIV) challenge: utilization of SIV nucleocapsid mutant DNA vaccines with and without an SIV protein boost. *J. Virol.* **74**:11935–11949.
13. Huang, Y., W. Kong, and G. J. Nabel. 2001. Human immunodeficiency virus type 1-specific immunity after genetic immunization is enhanced by modification of Gag and Pol expression. *J. Virol.* **75**:4947–4951.
14. Huang, Y., L. Xu, Y. Sun, and G. J. Nabel. 2002. The assembly of Ebola virus

- nucleocapsid requires virion-associated proteins 35 and 24 and posttranslational modification of nucleoprotein. *Mol. Cell* **10**:307–316.
15. Kong, W. P., Y. Huang, Z. Y. Yang, B. K. Chakrabarti, Z. Moodie, and G. J. Nabel. 2003. Immunogenicity of multiple gene and clade human immunodeficiency virus type 1 DNA vaccines. *J. Virol.* **77**:12764–12772.
 16. Lu, W., X. Wu, Y. Lu, W. Guo, and J. M. Andrieu. 2003. Therapeutic dendritic-cell vaccine for simian AIDS. *Nat. Med.* **9**:27–32.
 17. Maecker, H. T., H. S. Dunn, C. J. Pitcher, E. Khatamzas, C. J. Pitcher, T. Bunde, N. Persaud, W. Trigona, T. M. Fu, E. Sinclair, B. M. Brecht, J. M. McCune, V. C. Maino, F. Kern, and L. J. Picker. 2001. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. *J. Immunol. Methods* **255**:27–40.
 18. Mariani, R., B. A. Rasala, G. Rutter, K. Wieggers, S. M. Brandt, H. G. Krausslich, and N. R. Landau. 2001. Mouse-human heterokaryons support efficient human immunodeficiency virus type 1 assembly. *J. Virol.* **75**:3141–3151.
 19. Moss, R. B., M. R. Wallace, W. K. Giermakowska, E. Webb, J. Savary, C. Chamberlin-Brandt, G. Theofan, R. Musil, S. P. Richieri, F. C. Jensen, and D. J. Carlo. 1999. Phenotypic analysis of human immunodeficiency virus (HIV) type 1 cell-mediated immune responses after treatment with an HIV-1 immunogen. *J. Infect. Dis.* **180**:641–648.
 20. Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim. 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* **418**:646–650.
 21. Trigona, W. L., J. H. Clair, N. Persaud, K. Punt, M. Bachinsky, U. Sadasivan-Nair, S. Dubey, L. Tussey, T.-M. Fu, and J. Shiver. 2003. Intracellular staining for HIV-specific IFN- γ production: statistical analyses establish reproducibility and criteria for distinguishing positive responses. *J. Interferon Cytokine Res.* **23**:369–377.
 22. Wagner, R., V. J. Teeuwssen, L. Deml, F. Notka, A. G. Haaksma, S. S. Jhagjhoorsingh, H. Niphuis, H. Wolf, and J. L. Heeney. 1998. Cytotoxic T cells and neutralizing antibodies induced in rhesus monkeys by virus-like particle HIV vaccines in the absence of protection from SHIV infection. *Virology* **245**:65–74.
 23. Willey, R. L., R. Byrum, M. Piatak, Y. B. Kim, M. W. Cho, J. J. Rossio, Jr., J. J. Bess, Jr., T. Igarashi, Y. Endo, L. O. Arthur, J. D. Lifson, and M. A. Martin. 2003. Control of viremia and prevention of simian-human immunodeficiency virus-induced disease in rhesus macaques immunized with recombinant vaccinia viruses plus inactivated simian immunodeficiency virus and human immunodeficiency virus type 1 particles. *J. Virol.* **77**:1163–1174.
 24. Yang, Z.-Y., B. K. Chakrabarti, L. Xu, B. Welcher, W.-P. Kong, K. Leung, A. Panet, J. R. Mascola, and G. J. Nabel. 2004. Selective modification of variable loops alters tropism and enhances immunogenicity of human immunodeficiency virus type 1 envelope. *J. Virol.* **78**:4029–4036.
 25. Yang, Z.-Y., Y. Huang, L. Ganesh, K. Leung, W.-P. Kong, O. Schwartz, K. Subbarao, and G. J. Nabel. 2004. pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced by dendritic cell transfer through DC-SIGN. *J. Virol.* **78**:5642–5650.