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Design and immunogenicity assessment of HIV-1 virus-like particles as a candidate vaccine

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The rapid growth of the global HIV/AIDS epidemic makes it a high priority to develop an effective vaccine. Since a live attenuated or inactivated HIV vaccine is not likely to be approved for clinical application due to safety concerns, HIV virus like particles (VLPs) offer an attractive alternative because they are considered safer since they lack viral genome. We got a stable eukaryotic cell line by G418 resistance selection, engineered to express the HIV-1 structure protein Gag and Env efficiently and stably. We confirmed the presence of Gag and Env proteins in the cell culture supernatant and that they could self-assemble into VLPs. These VLPs were found to be able to elicit specific humoral and cellular immune response after immunization without any adjuvant.

HIV-1, cotransfection, stable cell line, virus-like particles (VLPs), vaccine

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The development of an effective, safe, and affordable vaccine for human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) is needed to control the worldwide HIV pandemic. At present, there is no vaccine against HIV approved for clinical use. There are three obstacles that hinder creation of an HIV-1 vaccine: viral diversity, immune system evasion, and the lack of appropriate animal models for vaccine evaluation [1]. A major approach used to overcome viral immune evasion is to exploit the conserved regions of HIV-1 proteins, for example, the conserved sites of envelope glycoprotein gp120 critical for binding to CD4 as well as co-receptors, or the envelope (transmembrane) protein gp41 co-receptor binding site [2–5]. Inducing production of broadly reactive Mabs could potentially counteract HIV-1 diversity. These two potential solutions of HIV-1 vaccine development could potentially be realized by exploiting prophylactic HIV-1 virus-like particle (VLP) vaccines [6].

HIV-1 VLPs possess a complex structure based on viral gag proteins that self-assemble into particular structures analogous in size and morphology to immature HIV-1 particles. As non-infectious, replication-deficient particles, VLPs are much safer compared to traditional vaccines made by chemically inactivated or attenuated live viruses. The different forms of VLPs have distinct properties [7–10]. Only enveloped VLPs display full-length proteins with proper folding on their surface and closely mimic native envelope trimeric structures [7,11,12]. The immune system responds well to particular antigens that are similar to immature HIV-1 viruses [13]. VLPs antigens are processed and present

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antigens through the major histocompatibility (MHC) class II pathway as well as the MHC class I endogenous pathway, inducing both CD4- and CD8-T-cell-mediated immune responses [14–16]. Therefore, VLPs can effectively induce both humoral and cellular immune reactions. It is also widely accepted that a rational vaccine design should have the potential to block the initial step of infection. In this respect, VLP-based vaccines are a promising type of recombinant protein vaccine.

VLPs are now considered one of the most attractive candidate vaccines for HIV and multiple VLP-based vaccines for HIV-1 are currently under investigation [12,17–22]. However, it is still generally desirable to enhance VLP immunogenicity. In this study, we report the construction of VLPs by cotransfecting two kinds of plasmids into HEK293 cells to obtain a mammalian cell line that efficiently and stably expresses the HIV-1 structural proteins Gag, pol, and Env. The VLPs induce specific humoral and cellular immune responses after immunization, without any adjuvant.

1 Materials and methods

1.1 Materials

Plasmid pcDNA3.1(–) and Lipofectamine-2000 were purchased from Invitrogen (Carlsbad, CA, USA). The plasmids D-GPEi, pcDNA 3.1, EnvB/C, pNL4-3.Luc.R–E and HOS-CD4-CCR5 cells, HEK293 cells were obtained from our lab. The plasmids were purified using a Qiagen Plasmid Giga Kit (Qiagen, Venlo, Netherlands), and the final product was endotoxin free (<2.5 units mg^{-1}). HIV-1 positive serum was obtained from a Guangxi AIDS patient. Anti-human-HRP-IgG was purchased from Jackson Immuno Research Laboratory, Inc. (West Grove, PA, USA) and G418 was purchased from Invitrogen.

1.2 Production of recombinant HEK293 cells and HIV-1 VLPs

Recombinant HEK293 cells expressing the HIV-1 structural proteins Gag and Env were obtained by cotransfecting plasmid D-GPEi and pcDNA3.1(–) into 293 cells. The plasmid D-GPEi was composed of the CMV promoter, a kanamycin resistance gene, prokaryotic cell high copy factors, and intron A. The main structural proteins Gag, pol and Env of a common China HIV-1 strain were cloned into this expression vector, with Gag fused to the N terminal of Pol as described [23]. All plasmid cloning was consistent with USA FDA clinical trial safety controls. Briefly, HEK293 cells were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT, USA). D-GPEi and pcDNA3.1(–) plasmids were cotransfected into 293 cells using Lipofectamine-2000. Adherent G418 resistant cells were trypsinized and serial diluted into medium containing G418 48 h after transfection. Stable 293 cell lines were maintained in standard growth medium with G418 (1 mg mL^{-1}). Cells were harvested and assayed by Western blot after 2–3 weeks to assess clonality. The positive supernatants were collected by centrifugation at 4000 r min^{-1} for 30 min and passed through a 0.22 μm filter. VLPs were pelleted at 26000 r min^{-1} for 1.5 h at 4°C in a Beckman SW28 rotor through a 30% sucrose cushion twice, and their assembly was confirmed by standard electron microscopy analysis and Western blot analysis.

1.3 Immunization experiments

Female BALB/c mice, in groups consisting of six animals 6–8 weeks of age, were administered 0.5, 2.5, 12.5, and 25 μg purified VLPs plus DNA (100 μg) by intramuscular injection. Control mice were treated with endotoxin-free phosphate-buffered saline (PBS). The immunization schedule was based on a 3-dose regimen, where the boost inoculations were administered at weeks 3 and 5 after the primary immunization. All immunization protocols were performed in two independent experiments without addition of adjuvants.

1.4 Sample collection and processing

Blood samples were collected by tail bleeding from each animal one week after the last immunization. Serum was obtained by standard methods and stored at −80°C until use.

1.5 TEM observation

The VLPs were prepared by dropping the micelle solution on a copper grid. After a few minutes, excess solution was removed with a strip of filter paper. A JEOL-8100 electron microscope operated at 200 kV was used for transmission electron microscopy (TEM).

1.6 Detection of cytotoxic T lymphocytes (CTL)

Spleens from immunized mice were harvested two weeks after the fourth immunization. Approximately 1×10^6 P815/BALB target cells were sensitized with synthetic peptide at a concentration of 1 mmol L^{-1}, for 2 h at 37°C. Cytotoxicity was measured by a standard lactate dehydrogenase (LDH) release assay with a CTL kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

1.7 ELISPOT

Splenic HIV-1 antigen-specific T cells were identified indirectly using cytokine ELISPOT by measuring induced IFN-γ production (BD, Franklin Lakes, NJ, USA). Briefly,

nitrocellulose bottomed 96-well plates were coated overnight with 5 μg mL^{-1} anti-IFN-γ antibody at 4°C, and nonspecific binding was blocked for 1 h at 37°C. Purified splenocytes were dispensed in triplicate at a predetermined density. The plates were washed and the biotinylated detection antibody was added for another 1.5 h at 37°C. A 1/1000 dilution of horseradish peroxidase-streptavidin was added to the wells and incubated for 1 h, after which horseradish peroxidase-streptavidin substrate was added. After 30 min, the colorimetric reaction was terminated by washing with tap water. The spots were counted after drying.

1.8 Neutralization assays

The plasmid pcDNA 3.1 EnvB/C and pNL4-3.Luc.R–E were cotransfected into 293T cells. Supernatants were collected 48 h after transfection incubated with blood at a final dilution of 1∶1, 1∶2, 1∶4 and 1∶8 for 1 h at 37°C. The mixture infected CD4-CXCR4-expressing HOS cells by DEAE. HOS cells were harvested, lysed, and tested after 72 h using the luciferase assay system (Promega) according to the manufacturer's instructions. The extent of luciferase expression was quantitated in a Dynex MLX luminometer and recorded in relative light units (r.l.u.). An inhibitory neutralization concentration, the concentration required to neutralize the preincubation mixture, was calculated by linear regression analysis.

1.9 Statistical analysis

ANOVA (i.e., one-way ANOVA followed by Fisher's LSD) was employed using SPSS 13.0 software. A $P<0.05$ was considered significant. All data points are presented as means±SE (standard error).

2 Results

2.1 Efficient expression of the HIV-1 *gag*, *Pol*, and *env* genes in 293 cells generates HIV-1 VLPs

To generate a stable cell line expressing our genes of interest, we optimized the ratio of vector D-GPEi and pcDNA3.1 and determined a 3∶1 ratio was optimal. We changed the selective media (G418) after 48 h and selected for positive colonies that express the HIV-1 gag, pol and env structural proteins, as determined by Western blot assays (data not shown). To test for secreted protein, cell supernatant was collected by 30% sucrose density centrifugation and pellets resuspended in PBS and assayed by SDS-PAGE and Western blot (Figure 1).

2.2 Stability of cellular expression

We investigated the expression stability in the stable cell line. Screened cell lines were maintained continuously in medium with G418. Expression of HIV-1 gagpol, and env protein were examined in cell lysate one month and 12 months after transfection, respectively. Efficient expression of the target protein could still be detected in the cell lines after 12 months. HIV-1 VLPs could also be detected in cellular supernatants of stable cell lines (Figure 2).

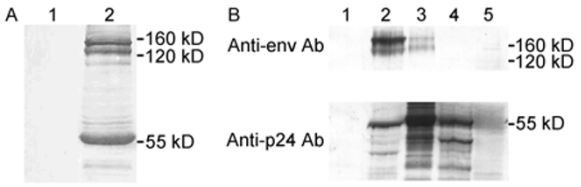

Figure 1 VLPs were prepared and analyzed by immunoblotting as described in Materials and methods A, Immunoblot of purified VLPs using HIV-1-positive human serum. Lane 1, Mock supernatant; lane 2, purified VLPs. B, Lane 1, Mock; lanes 2–4, different positive cells and purified VLPs analyzed by immunoblotting with anti-p24 monoclonal antibody or polyclonal sheep anti-gp120 antibody.

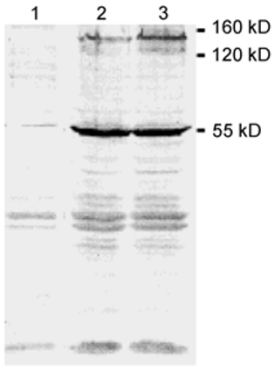

Figure 2 Immunoblot analysis of positive cell lines after 12 months. Lane 1, Cell lysis of mock transfected cells; lane 2, cell lysis after one month; lane 3, cell lysis after 12 months.

2.3 Assembly of HIV-1 VLPs

To investigate the synthesis and assembly of HIV-1 gagpol and env protein, we collected supernatant from positive cells by centrifuging at 26000 r min^{-1} at 4°C, for 4 h through a 10%–40% sucrose gradient. We found that the gagpol and env proteins passed through the lower density fractions and migrated to the 40% and 30% density layers. Western blot analysis indicated that protein could assemble into VLPs that included gagpol and env (Figure 3).

2.4 VLPs morphology

The MW of VLPs is too large to be analyzed by non-denaturing PAGE or by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry. To confirm that Gag and Env were assembled into VLPs, we applied transmission electron microscopy (TEM) to su-

pernatants obtained by ultracentrifugation through a 30% sucrose gradient cushion. This analysis showed that the proteins formed virus-like particle about 100 nm in diameter, and that the shape and the size were similar to that of a natural virus (Figure 4).

2.5 HIV-1-specific humoral responses

The sera specificity was evaluated by immunoblotting. The results presented in Figure 5 demonstrate that antibodies were induced in response to vaccination. New epitopes were not produced and no changes in immunodominance were detected. HIV-1-Gag-specific antibody responses from all VLPs treatments (2.5–25 μg) were higher than that obtained for 100 μg DNA (Figure 5A). In contrast, the HIV-1-Env-specific antibody response from 0.5–25 μg VLPs was about the same as that observed with 100 μg DNA (Figure 5B).

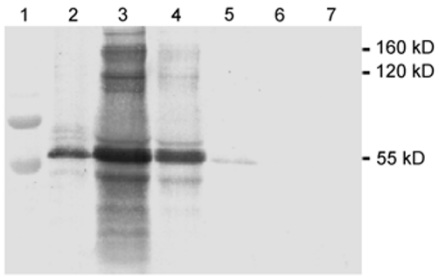

Figure 3 Gradient analysis of VLPs. Supernatant of positive cells was placed onto 30%–40% sucrose density gradients. Centrifugation was carried out in a SW28 rotor at 4 and 100000×*g* for 4 h. Fractions were collected from the bottom to the top (left to right) and analyzed by SDS-PAGE followed by Western blotting using anti-HIV-1 human serum. Lane 1, Marker; lane 2, fraction of bottom; lanes 3–6, 40%–10% gradient; lane7, supernatant layer.

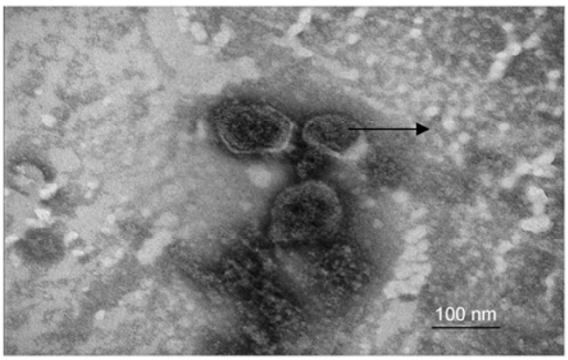

Figure 4 TEM image of VLPs.

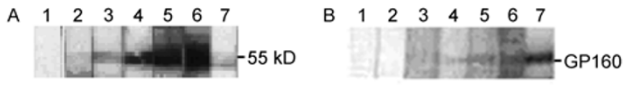

Figure 5 Western blot analysis of mouse serum antibody in response to HIV-1 Gag and Env protein. Mice sera collected after the third injection were tested at 1∶20 dilution (pools of six). A, Lane 1, PBS group; lanes 2–5, VLP 0.5, 2.5, 12.5, 25 μg; lane 6, positive control; lane 7, DNA 100 μg. B, Lane 1, PBS group; lane 2, DNA 100 μg; lanes 3–6, VLP 0.5, 2.5, 12.5, 25 μg; lane 7, positive control.

2.6 HIV-1-specific CTL responses

VLPs are processed by antigen presenting cells (APCs) for MHC class I presentation to $CD8^+$ cytotoxic T lymphocytes (CTL) [14]. To quantify the T-cell response, mice were injected with VLP (0.5, 2.5, 12.5, and 25 μg) and DNA (100 μg) using a 3-dose regimen. One week after the last injection, HIV-1-specific $CD8^+$ T cells were measured *in vitro* by assessing the cell death of P815/BALB target cells, sensitized with an H-2^d-restricted CTL epitope. The CTL response was low in the 0.5 μg and DNA group, but increased in a dose-dependent manner. The highest CTL response was observed at 12.5 μg (Figure 6), indicating that the CTL epitope is contained primarily in the NC domain of GAG (p7).

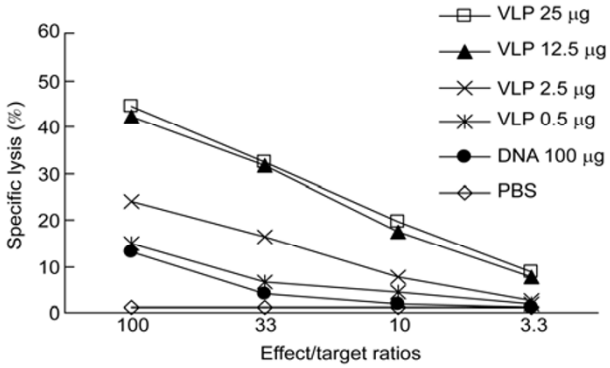

Figure 6 Analysis of splenic mononuclear cells cytolytic activity harvested from immunized mice. P815 mastocytoma cells (pool of six) pulsed with epitope were used as targets. Percent specific lysis was determined by a 4 h LDH-release assay.

2.7 Vaccination enhances HIV-1 specific CTL as indicated by IFN-γ ELISPOT

During natural infection, HIV-specific Th1 cells, CTLs, and associated antiviral cytokines (IFN-γ, TNF-α, IL-2) play key roles in virus recognition [24]. HIV-1-specific $CD8^+$ T cells were measured *in vitro* by determining splenic IFN-γ production in response to brief re-stimulation with CTL epitopes, as measured by ELISPOT. The results indicate that the IFN-γ response to VLPs immunization at 25 μg was the same as that for 12.5 and 0.5 μg, and 2.5 μg was also lower than 12.5 μg, indicating that 12.5 μg was needed to induce a cellular immune response in mouse (Figure 7).

2.8 VLPs induce neutralizing antibody and alter antibody levels

The plasmid pNL4-3.Luc.R–E contains luciferase and the full HIV-1 genome of except for a frameshift mutation in the Env

gene. The 293 T cells expressed constitutive Gag and Env following cotransfection of plasmids pNL4-3.Luc.R–E and pcDNA 3.1 EnvB/C, which contained the Env gene. These two proteins could be packaged into virus particles wherein the particle also contained luciferase. Such a particle is referred to as a pseudotype virus since it cannot replicate, but can infect HOS-CD4-CCR5 cells and the infection level could be detected by quantifying luciferase activity. The neutralization level could be evaluated by co-incubating blood samples and cotransfected supernatants and observing the change in luciferase. Our results showed that 0.5 μg of VLPs could induce neutralizing antibody and that antibody levels increased as the immunizing dose increased. These antibodies could also counteract the infectivity of the pseudotype virus. The epitopes of the neutralizing antibodies resided mainly in Pr55gag and gp120. Neutralization activity was highest in VLPs samples 12.5 μg, and 25 μg, as indicated by the low luciferase activity. Interestingly, 100 μg plasmid DNA could only elicit a lower level neutralization activity than all VLPs amounts tested. It is also conceivable that 12.5 ug is the optimal dose. The neutralization level in different dose groups tended to be similar at 1∶1, 1∶2 and 1∶4 dilution (Figure 8).

3 Discussion

The major advantage of a VLPs approach compared to live-attenuated virus is that VLPs express multiple viral epitopes that stimulate a diverse set of immune responses, without many of the deleterious effects of a live-attenuated virus. VLPs have the potential for activating both endogenous and exogenous antigen processing pathways, leading to presentation of viral peptides by MHC class I and class II molecules. These multi-epitope vaccines are more likely than their single component counterparts to generate a broad-based immune response capable of clearing HIV-1 immune evasion mutants. VLPs may be more cost efficient than co-inoculating multiple single gene vaccines for future Phase I clinical trials. Another advantage of VLPs compared to single recombinant protein vaccines is the ability of VLPs to bind and enter cells expressing appropriate receptors. HIV-1 VLPs are able to bind to CD4 and chemokine receptors via gp120, and to enter into professional antigen presenting cells such as macrophages and dendritic cells (both cell types express CD4 and CCR5). After infection, viral proteins can be processed and presented on MHC class I molecules, therefore promoting presentation to T cells by

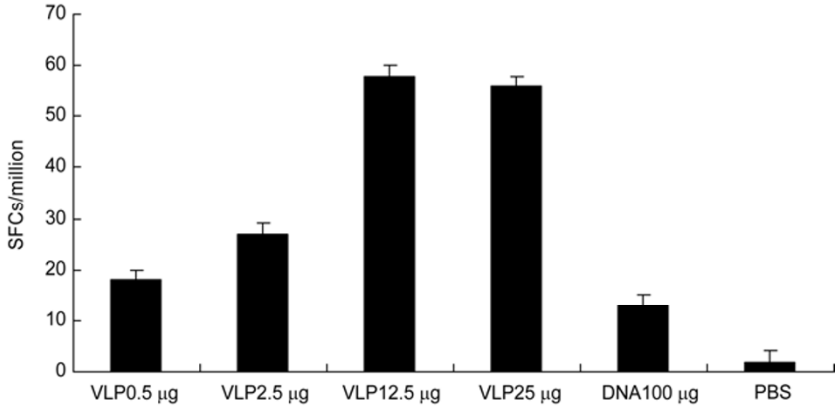

Figure 7 HIV-1 Ag-specific IFN-γ producing splenocytes are generated by VLP and DNA intramuscular vaccination. Each bar represents the average number of cells secreting IFN-γ per 10^6 cells.

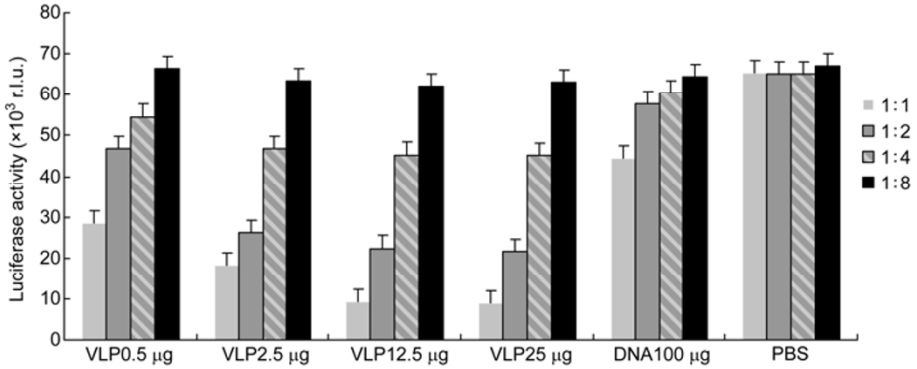

Figure 8 Detection of luciferase endpoints after pseudotype virus infection of HOS-CD4-CCR5 cells. Infected cells were co-incubated with blood samples and cotransfected supernatants. The expression of intracellular luciferase was measured as indicated. The values shown represent the means±standard deviation of three replicate cultures.

APCs. In addition, cell-free VLPs bound with antibodies can be taken up by phagocytic cells via Fc receptors, thus increasing MHC class II presentation.

VLPs have been expressed in Baculovirus expression systems [25] and *Saccharomyces cerevisiae* [26,27]. These systems produce large amounts of particles from eukaryotic cells. However, they are usually limited to expressing only one gene, and therefore are not easily used to produce multigene VLPs. In addition, in order for particles to bud from yeast, the outer membrane needs to be removed.

To overcome the above disadvantages, we first cotransfected two plasmids, and used neomycin as stable screen marker. We then screened mammalian cell lines that could express the gagpol and env proteins with high efficiency. The gag protein readily packs into non-enveloped VLPs and the presence of the env protein can provide more antigenic determinants [22]. We took advantage of gag self-assembly and co-expressed gag and env to produce VLPs. Our approach demonstrated that this cell line could not only produce stable VLPs, but also secrete significant quantities of VLPs into the supernatant. The VLPs produced by our method without viral nucleic acid are not only safer, but also induce a strong humoral and cellular immune response, and provide a foundation for improving HIV vaccination. Because the recombinant Gagpol and Env proteins have similar structures to that of the wild HIV-1, additional work is required to fully understand how this approach could be used for advanced clinical applications.

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