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Immunogenicity and efficacy of immunodeficiency virus-like particles pseudotyped with the G protein of vesicular stomatitis virus

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Abstract

Vaccination with exogenous antigens such as recombinant viral proteins, immunodeficiency virus-derived whole inactivated virus particles, or virus-like particles (VLP) has generally failed to provide sufficient protection in animal models for AIDS. Pseudotyping VLPs with the vesicular stomatitis virus G protein (VSV-G), which is known to mediate entry into dendritic cells, might allow more efficient stimulation of immune responses. Therefore, we pseudotyped noninfectious immunodeficiency virus-like particles with VSV-G and carried out a preliminary screen of their immunogenicity and vaccination efficacy. Incorporation of VSV-G into HIV-1 VLPs led to hundred-fold higher antibody titers to HIV-1 Gag and enhancement of T cell responses in mice. Repeated vaccination of rhesus monkeys for 65 weeks with VSV-G pseudotyped simian immunodeficiency virus (SIV)-like particles (VLP[G]) provided initial evidence for efficient suppression of viral load after mucosal challenge with the SIVmac239 virus. Challenge of monkeys after a 28 week vaccination regimen with VLP[G] led to a reduction in peak viremia, but persistent suppression of viral load was not achieved. Due to limitations in the number of animals available for this study, improved efficacy of VSV-G pseudotyped VLPs in nonhuman primates could not be demonstrated. However, mouse experiments revealed that pseudotyping of VLPs with fusion-competent VSV-G clearly improves their immunogenicity. Additional strategies, particularly adjuvants, should be considered to provide greater protection against a challenge with pathogenic immunodeficiency virus.

Keywords: SIV; VLP; Vaccine; Immunization; HIV

Introduction

With HIV spreading worldwide, the need for a preventive or therapeutic vaccine is more urgent than ever before. Efficacy studies in humans require large cohorts, and only two phase-III trials using recombinant gp120 surface protein have been performed that gave no evidence of protection. Therefore, most of our knowledge on the efficacy of HIV vaccines comes from animal models, particularly the infection of macaques with simian immunodeficiency viruses (SIV). Work on live attenuated immunodeficiency viruses in nonhuman primate models has shown that a vaccine can provide protection from progression to AIDS even in the absence of a sterilizing immunity (Daniel et al., 1992; Marthas et al., 1990). A number of effector mechanisms, including neutralizing antibodies and

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CD8+ cytotoxic T lymphocytes, are likely to contribute to protection (reviewed in Johnson, 2002). In addition to the live-attenuated vaccines, which for safety reasons are unlikely to be applicable in humans, a number of nonreplicating or exogenous vaccine approaches have been studied in the SIV macaque model. However, vaccination with recombinant Env proteins does not provide sufficient protection against pathogenic SIV (Giavedoni et al., 1993; Lu et al., 1998) consistent with results from the human phase III trials (Flynn et al., 2005). Similarly, whole inactivated SIV vaccines and virus-like particles do not provide significant protection if the challenge virus is grown in monkey cells (Le Grand et al., 1992; Notka et al., 1999; Stott, 1991). A common feature of vaccination with recombinant viral proteins and whole inactivated viruses is injection of exogenous antigens, which predominantly leads to MHC-II-restricted cellular immune responses and production of antibodies. In contrast, some virus-like particles of HIV or SIV also induced CTL responses (Deml et al., 1997; Paliard et al., 2000).

We and others have previously generated single cycle immunodeficiency viruses (SCIVs) that can undergo only a single round of replication leading to the production of noninfectious virus-like particles in the vaccinees (Evans et al., 2004; Kuate et al., 2003). After booster immunizations with SCIVs, SIV-specific humoral and cellular immune responses were observed and peak viremia during primary infection with the SIVmac239 challenge virus was significantly reduced (Evans et al., 2005; Kuate et al., 2003). To increase in vivo expression levels, we pseudotyped the single cycle immunodeficiency virus (SCIV) vaccines with VSV-G. VSV-G mediates efficient entry into a broad spectrum of cells (Burns et al., 1993) including dendritic cells (Granelli-Piperno et al., 2000) and is therefore frequently used in gene transfer experiments with lentiviral and retroviral vectors. VSV-G has also been shown to enhance the immunogenicity of an HIV-*gag* DNA vaccine if coexpressed with the antigen (Marsac et al., 2002). We now analyzed the influence of VSV-G on the immunogenicity of virus-like particles and compared VSV-G pseudotyped, non-replicating VLPs (VLP[G]) of SIV to SCIV vaccines to determine whether expression of viral antigens in the vaccinee improves immunogenicity and protective efficacy. We provide initial evidence that in vivo expression of viral antigens by SCIVs is not required for the protective properties observed and show that incorporation of VSV-G into noninfectious VLPs enhances humoral and cellular immune responses.

Results

In previous experiments, monkeys immunized with VSV-G pseudotyped single cycle immunodeficiency virus vaccines (SCIV) showed SIV-specific humoral and cellular immune responses and peak viremia during primary infection with the SIVmac239 challenge virus was significantly reduced (Kuate et al., 2003). Restriction of SCIVs to a single round of in vivo expression of viral antigen was achieved by mutating the primer binding site of SIV and complementation of these mutations by cotransfection of an expression plasmid encoding a matched artificial tRNA during vaccine production (Kuate et al., 2003). Omitting the tRNA expression plasmid during vaccine production results in noninfectious VLPs that do not differ in protein composition from the SCIVs (Kuate et al., 2003). Since it remained unclear, whether in vivo expression of viral antigens by SCIVs is indeed beneficial, we now performed a comparative analysis of VSV-G pseudotyped SIV particles in an infectious (SCIV) and noninfectious virus-like particle (VLP [G]) form. SCIVs were produced as described previously (Kuate et al., 2003) by cotransfection of SIV genomic DNA with mutations in the primer binding site and deletions in accessory genes with a VSV-G expression plasmid and the tRNA expression plasmid. For production of VLP[G], the same mutated SIV-DNA and VSV-G expression plasmid were cotransfected, but the tRNA expression plasmid was omitted. Omitting the tRNA expression plasmid does not affect the protein composition of the particles, but reduces the infectivity of such particles below the level of detection (Kuate et al., 2003).

Monkeys were immunized by seven subcutaneous injections of 10^7 infectious units of SCIV at week 0, 4, 8, 29, 33, 37, and 65. Since the ratio of infectious units to the amount of viral proteins can vary between different preparations, the amount of reverse transcriptase in the SCIV preparations was used as a measure of viral antigen content. The dose of the VLP[G] was always adjusted by its content of reverse transcriptase. Thus, both groups received approximately equal amounts of exogenous viral antigens, but only the SCIV-injected monkeys should also express the viral antigens themselves. An additional group of two monkeys did not receive any exogenous viral antigens, but was immunized with a DNA vaccine encoding SIV structural proteins and Tat and Rev. To increase in vivo expression levels of the DNA vaccine, it was complexed with cationic lipids. Monkeys of a fourth group served as nonvaccinated controls.

During immunization, SCIV and VLP[G] immunized monkeys developed similar titers of antibodies to Gag, while the Env antibody response seemed to be stronger in SCIV immunized monkeys (Figs. 1B, D). However, the antibodies to Env induced by vaccination did not neutralize homologous SIVmac239 even at a 1:10 dilution of serum (Fig. 2B). In contrast, neutralization of the closely related SIVmac251 virus was readily detectable at a 1:100 dilution of serum (Fig. 2D). Neutralizing antibodies against VSV-G were also detectable (Fig. 2A), but they did not seem to prevent the booster effect of repeated injections of VLP[G] or SCIV on humoral immune responses. The lipid–DNA-immunized monkeys and the naive control monkeys showed no antibody response at all (Figs. 1 A, C). Results of an IFN-γ ELISPOT assay after stimulation with an overlapping SIV Gag peptide pool are shown in Figs. 1E and F. Two weeks after the last booster immunization, a strong ELISPOT response was seen in the SCIV and VLP[G]-immunized monkeys, but not in the lipid–DNA immunized monkeys. Therefore, VSV-G-pseudotyped SIV particles in an infectious (although only a single cycle) and noninfectious form are immunogenic without additional adjuvants. However, repeated immunizations seemed to be necessary for the induction of detectable cellular immune reactions, since the

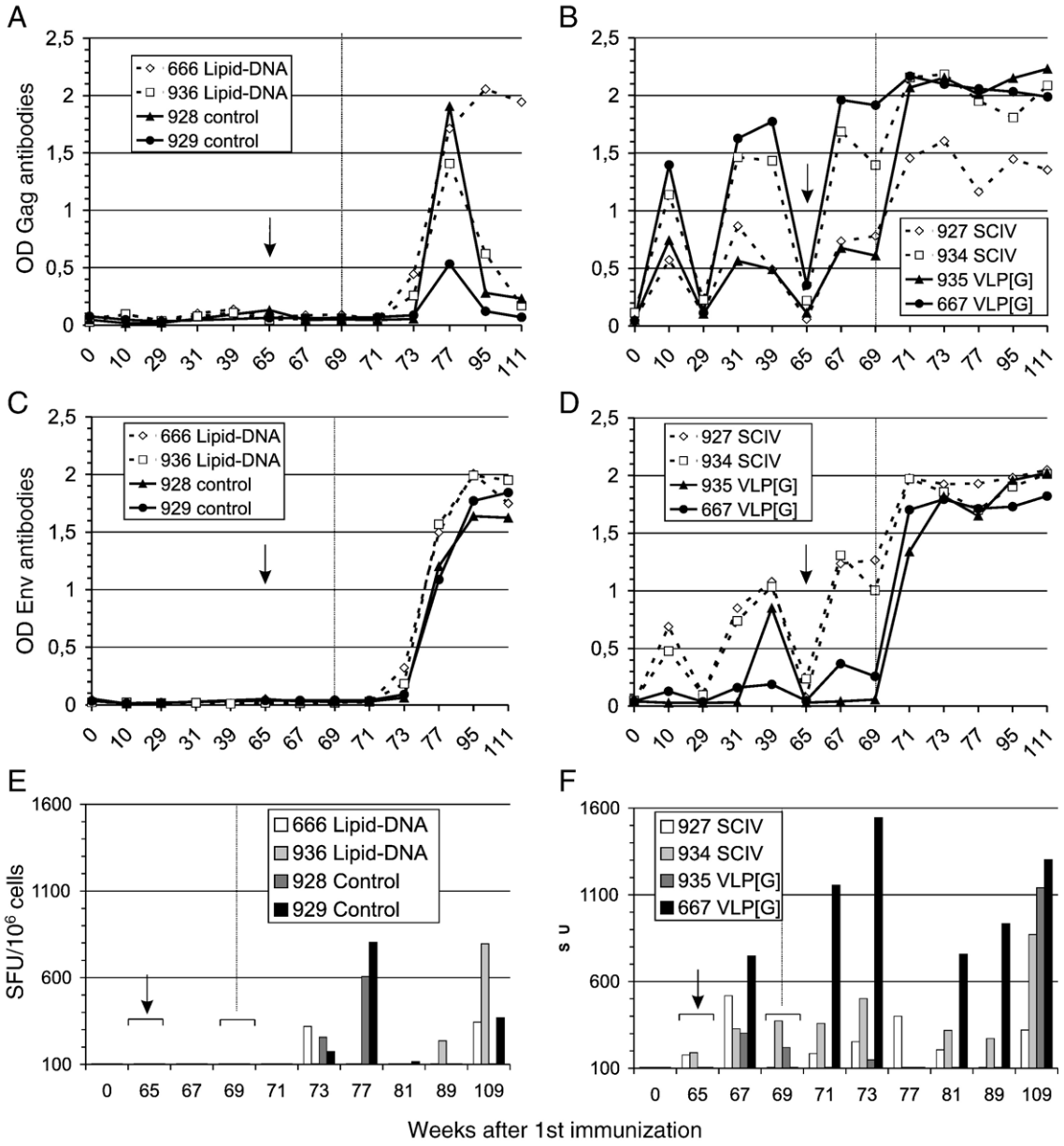

Fig. 1. Monkeys were immunized on weeks 0, 4, 8, 29, 33, 37, and 65 with SCIV or VLP[G] preparations normalized for RT activity. A third group received a lipid–DNA vaccine while a forth group was left untreated (control). Antibody titers to SIV Gag (A, B), SIV Env (C, D), and IFN-gamma ELISPOT response after stimulation with an overlapping SIV Ca peptide pool (E, F) were determined at the indicated time points after the 1st immunization. The arrow marks the time point of the last booster immunization, the dotted line separates the data obtained before and after challenge at 69 weeks after the first immunization. The three-digital numbers indicate the individual macaques.

ELISPOT responses in SCIV and VLP[G]-immunized monkeys at earlier time points were generally weak and rather variable (data not shown).

Four weeks after the last immunization, monkeys were challenged with SIVmac239 by the tonsillar route, thus providing a mucosal challenge with a stringent form of SIV. All eight animals became infected, although peak viremia was ~20-fold lower in the SCIV and VLP[G]-immunized monkeys (Fig. 3). Importantly, the viremia rapidly dropped below the level of detection in SCIV and VLP immunized monkeys (two animals each) and remained low except, for one animal (macaque 927 SCIV), up to 95–117 weeks after infection (Figs. 3A, B). In contrast, lipid–DNA immunized monkeys and the control monkeys maintained high viral loads throughout the observation period. A similar pattern was observed in the cell-associated viral load. However, the last time points analyzed indicate a progressive increase of virus load in both SCIV- and one of the VLP[G]-immunized monkeys (Fig. 3D). The decline in the percentage of CD4+ T cells (Fig. 3E) in the control group indicated immune dysfunction. One control monkey (macaque 928 control) progressed to AIDS and had to be euthanized 62 weeks after infection due to severe *Pneumocystis carinii* pneumonia and a pharyngeal lymphoma. Three of the four monkeys

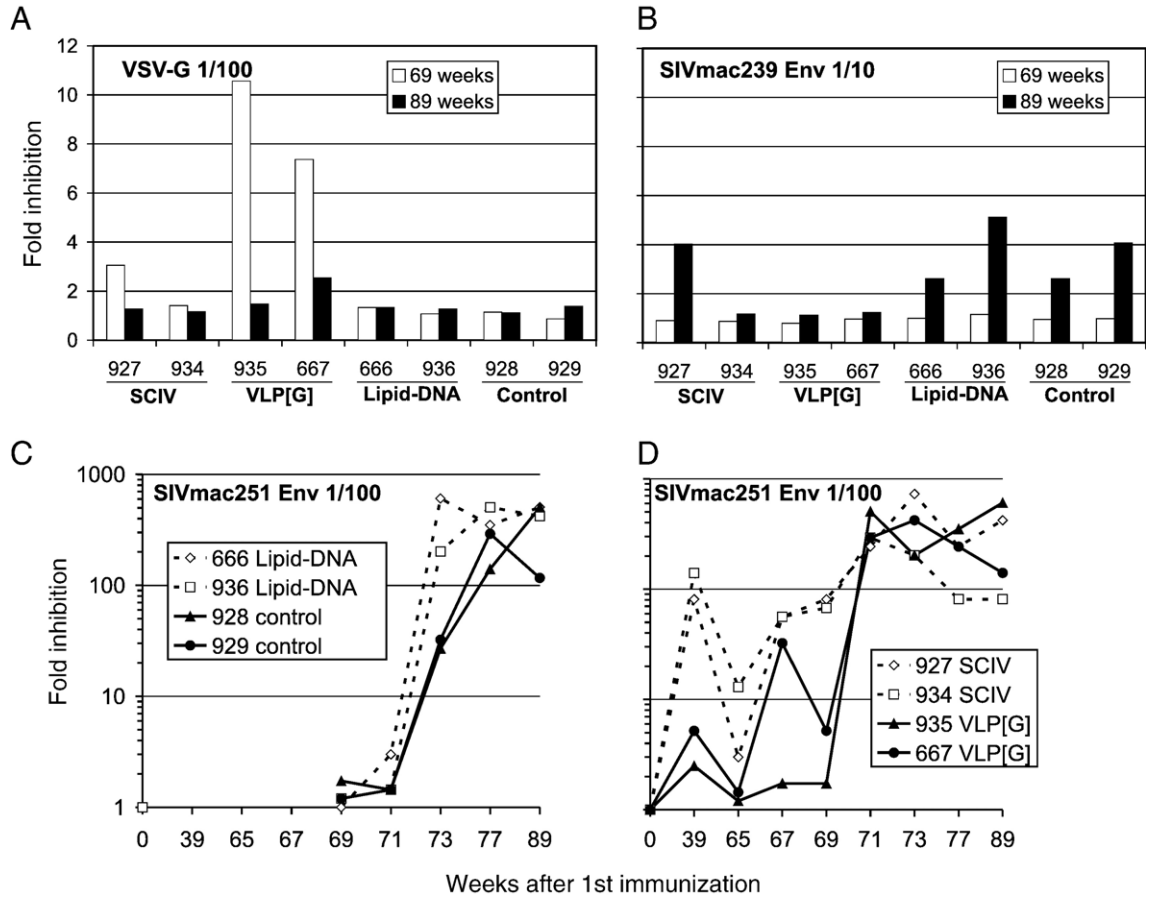

Fig. 2. Neutralizing antibodies against VSV-G (A), SIVmac239 Env (B), and SIVmac251 Env (C, D) at a 1/100 (A, C, D) or a 1/10 dilution (B) of sera obtained from the monkeys at the indicated time points after the first immunization with week 69 being the time of challenge.

vaccinated with VLPs or SCIV had normal CD4 counts (Fig. 3F) and remained healthy up to 120 weeks after infection. One of the SCIV-immunized monkeys (927 SCIV) showed a progressive decline in the percentage of CD4+ T cells, consistent with increasing viral load levels in this monkey.

The control of SIVmac239 replication in monkeys vaccinated with VSV-G pseudotyped VLPs was striking given the prior reports of inefficacy of whole inactivated viruses and conventional VLPs lacking VSV-G (Le Grand et al., 1992; Lu et al., 1998; Stott, 1991), including our own experience (Notka et al., 1999). To determine if incorporation of VSV-G into VLPs enhances antiviral immune responses, mice were immunized with HIV-1 VLPs, which allowed monitoring of cellular immune responses with immunodominant HIV-1 peptides. VSV-G pseudotyped HIV-VLPs ($VLP^H[G]$) or nonpseudotyped VLPs (VLP^H) were prepared by transient tranfection and pelleting through a 20% sucrose cushion. In addition, VLPs were prepared containing a fusion-defective mutant of VSV-G ($VLP^H[G^{mut}]$). Western blot analyses with antibodies to HIV-1 Gag or Env revealed similar amounts of both proteins for all preparations (Fig. 4A). Similar amounts of wild type and fusion-defective VSV-G were also detected in the $VLP^H[G]$ and $VLP^H[G^{mut}]$ preparations (Fig. 4A). Balb/C mice were immunized twice by subcutaneous injections. Control mice were left untreated (control). IgG, IgG1, and IgG2a antibody titers were determined in the sera of the immunized mice. IgG and IgG1 antibody titers to Gag were approximately 100-fold higher in $VLP^H[G]$ immunized mice than in mice immunized with VLPs containing fusion-defective VSV-G or lacking VSV-G (Fig. 4B). $VLP^H[G]$ immunized mice also had T cells recognizing an immunodominant peptide in Gag p24CA as demonstrated by IFN-γ ELISPOT assay (Fig. 4C). The IFN-γ ELISPOT response was lower in VLP^H-immunized mice. Interestingly, cellular immune responses induced by VLPs pseudotyped with the fusion-defective mutant of VSV-G also seemed to be reduced in comparison to those induced by $VLP^H[G]$. To further exclude nonspecific stimulation of immune responses by VSV-G leading to increased IFN-γ production, mice were also immunized with VSV-G vesicles [G]. These vesicles were prepared exactly the same way as the VLPs and contained similar amounts of VSV-G as determined by Western blot analyses (data not shown). IFN-γ ELISPOT responses to HIV antigens remained undetectable in mice immunized with VSV-G vesicles (Fig. 4C).

In the initial monkey study, animals were immunized seven times over a period of 65 weeks. Since a shorter immunization schedule would facilitate all subsequent

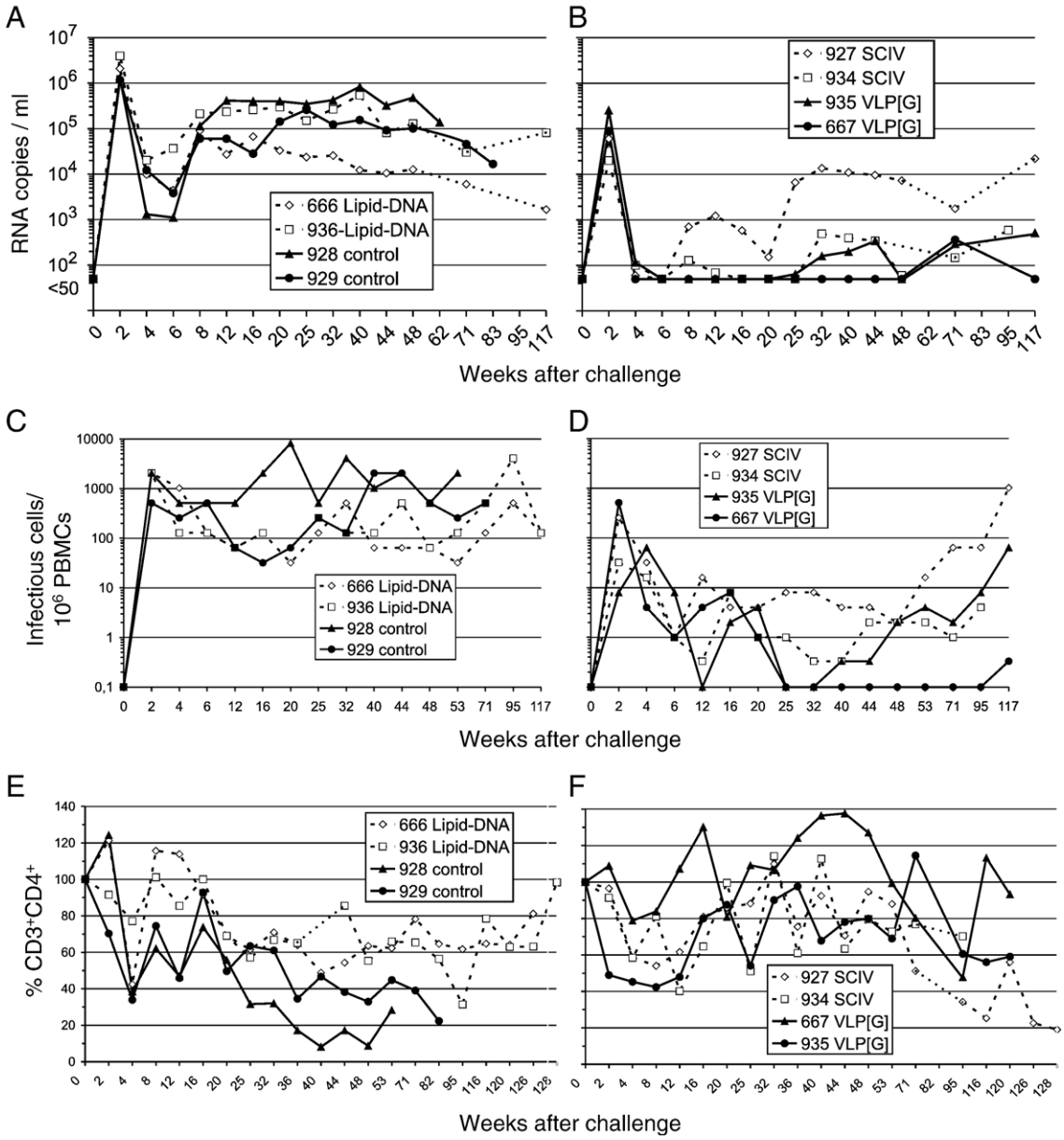

Fig. 3. Viral loads and percentage of CD4-positive cells in unvaccinated monkeys (A, C, E; control) or monkeys vaccinated with lipid–DNA (A, C, E), single cycle immunodeficiency virus vaccines (SCIV; B, D, F), or VSV-G pseudotyped VLPs (VLP[G]; B, D, F). All monkeys were challenged with SIVmac239 at 69 weeks after the first immunization. CD3+CD4+ cells are shown as percent of baseline levels ranging from 28 to 46%.

studies, a second study was performed in which a group of four monkeys was immunized with VLP[G] six times during a 28 week period. In Western blot analyses, the VLP[G] preparation displayed a similar ratio of Gag and Env proteins as previously observed for SCIV (Kuate et al., 2003) (data not shown). Two control monkeys were immunized with VSV-G in the absence of SIV antigens ([G]) after adjusting the amount of VSV-G in Western blot analyses to the VSV-G content of VLP[G] (data not shown).

Three of the four immunized animals developed antibodies to Gag and Env (Figs. 5A, C). The ELISPOT assay, however, remained negative at most of the time points analyzed (Figs. 5E, F). Four weeks after the last immunization, monkeys were challenged with SIVmac239 by the tonsillar route. The mean peak viremia was approximately five-fold lower in the immunized monkeys than in the control monkeys. After logarithmic transformation of the peak viral RNA levels, this difference was statistically significant (t test: $P = 0.048$) (Figs. 6A, B), but set point RNA levels were in the same range in both groups. The cell-associated viral load levels in the PBMCs (Figs. 6C, D) paralleled the results obtained for the viral RNA levels. Determination of cell-associated viral load levels in the lymph nodes of the monkeys 2 and 6 weeks after challenge did not reveal obvious differences between the two groups (data not shown). An anamnestic antibody response was observed in the immunized monkeys, but not in control animals (Figs. 5A, B). Positive ELISPOT

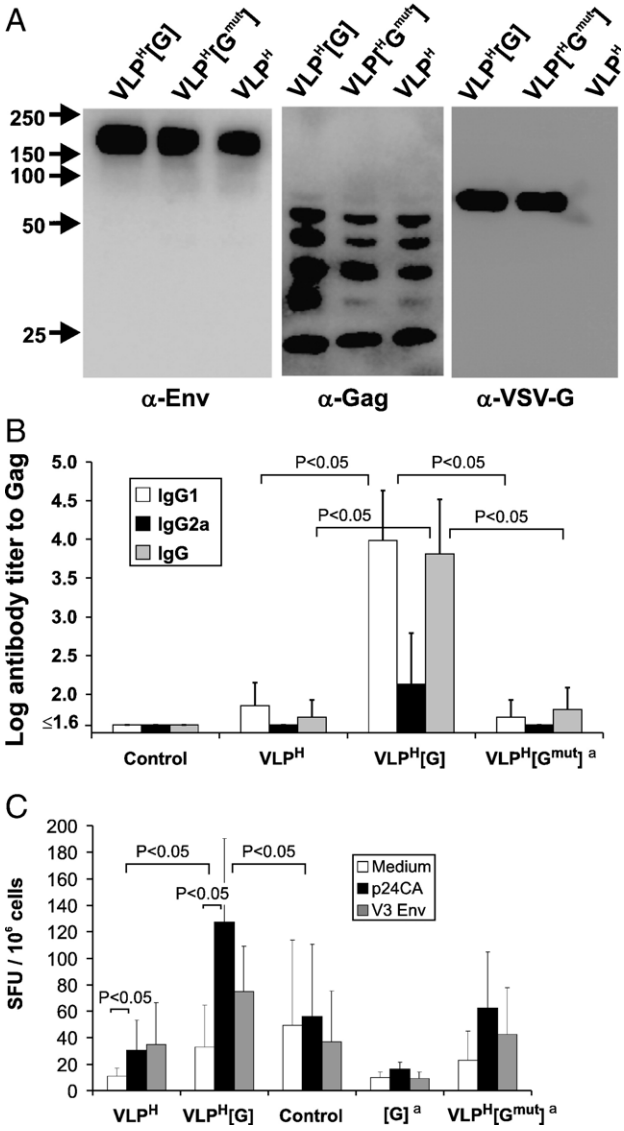

Fig. 4. Immunogenicity of VSV-G-pseudotyped VLPs in mice. VLPs were prepared by cotransfection of HIV-1 *gag-pol* and *env* expression plasmids (VLP^H), while adding a VSV-G expression plasmid led to production of VSV-G-pseudotyped VLPs ($VLP^H[G]$). $VLP^H[G^{mut}]$: VLPs pseudotyped with a fusion-defective mutant of VSV-G. [G]: VSV-G containing vesicles prepared by transfection of the VSV-G expression plasmid without HIV-1 expression plasmids. The different VLPs were pelleted through a 20% sucrose cushion and analyzed by Western blot with antibodies to HIV-1 Gag and Env and VSV-G (A). VLPs and pseudotyped VLPs were normalized for RT content and groups of mice were immunized subcutaneously twice. (B) Log antibody titers to Gag 1 week after the second immunization. The mean and standard deviation of two experiments with different VLP preparations including six mice per group in each experiment are shown. *P* values were calculated by an ANOVA test on ranks. (C) Stimulation of T cells by immundominant HIV-1 peptides 1 week after the second immunization. The IFN-γ ELISPOT assay represents the result of two independent experiments each with 5 to 6 mice per group. *P* values were calculated by an ANOVA test on ranks. SFU: IFN-γ spot forming units. Control: unvaccinated control mice. [a]Mean and standard deviation of five to six mice from one experiment.

responses were seen more frequently after challenge in both groups. During the follow-up period of 36 weeks, the percentage of CD4-positive T cells declined below 50% of the prechallenge values in one of the vaccinated monkeys and 3 of the monkeys of the control group (Figs. 6E, F).

Discussion

Our findings in mice indicate that incorporation of VSV-G into immunodeficiency virus-derived, noninfectious VLPs increases humoral and cellular immune responses to immunodeficiency virus antigens. In particular, incorporation of VSV-G into the VLPs led to 100-fold higher Gag antibody titers. Using a fusion-defective mutant of VSV-G, the stimulatory effect of VSV-G on antibody titers and IFN-ELISPOT response was reduced. Thus, fusion-active VSV-G might enhance intracytoplasmic delivery of components of the VLPs leading to improved presentation on MHC molecules as previously proposed for DNA vaccines coexpressing VSV-G and *gag* (Marsac et al., 2002). Whether VSV-G also contributes to enhanced immune responses by triggering innate immune responses remains to be determined.

Efficient induction of both humoral and cellular immune responses might explain the striking protection imparted by VSV-G pseudotyped SIV particles in the long-term rhesus monkey vaccination experiment. In the first experiment, three out of four monkeys immunized with VSV-G pseudotyped SIV particles, either in a noninfectious (VLP) or infectious (SCIV) form, were able to control replication of the SIVmac239 challenge virus for almost one year, while two naive control monkeys and two monkeys vaccinated with lipid–DNA complexes maintained high viral loads throughout the observation period (Figs. 3A to D). The comparison of viral load levels in monkeys challenged after SCIV or VLP immunization did not provide any evidence that SCIV was superior to VLP. This suggests that expression of viral antigens in the vaccinees is not required. Given the potential risk of insertional mutagenesis by integration of the SCIV proviral DNA into the genome of host cells, VSV-G pseudotyped VLPs should be safer. Use of codon-optimized expression plasmids for the production of the VLPs as done in the mouse experiments further avoids transfer of viral genomic sequences to the vaccinee and emergence of replication-competent recombinants (Wagner et al., 2000). Thus, pseudotyping VLPs with VSV-G is a strategy that could possibly be used in humans.

The control of SIVmac239 replication in monkeys vaccinated with VSV-G pseudotyped VLPs was striking in this first experiment given the prior reports of inefficacy of whole inactivated viruses and conventional VLPs lacking VSV-G (Le Grand et al., 1992; Lu et al., 1998; Stott, 1991), including our own (Notka et al., 1999). Although prepared differently, the VLP vaccine used in this study resembles the AT2-inactivated virus particle vaccine (Arthur et al., 1998) in the sense that both vaccines contain the full set of viral structural genes and maintain a fusion-competent Env protein. Despite this similarity, vaccination of rhesus monkeys with AT2-inactivated virus particle vaccines did not lead to control of replication of the SIVmac239 challenge virus (Lifson et al., 2002). Since the two vaccines differ in the VSV-G content, incorporation of VSV-G appears to contribute to efficacy. However, a short-term

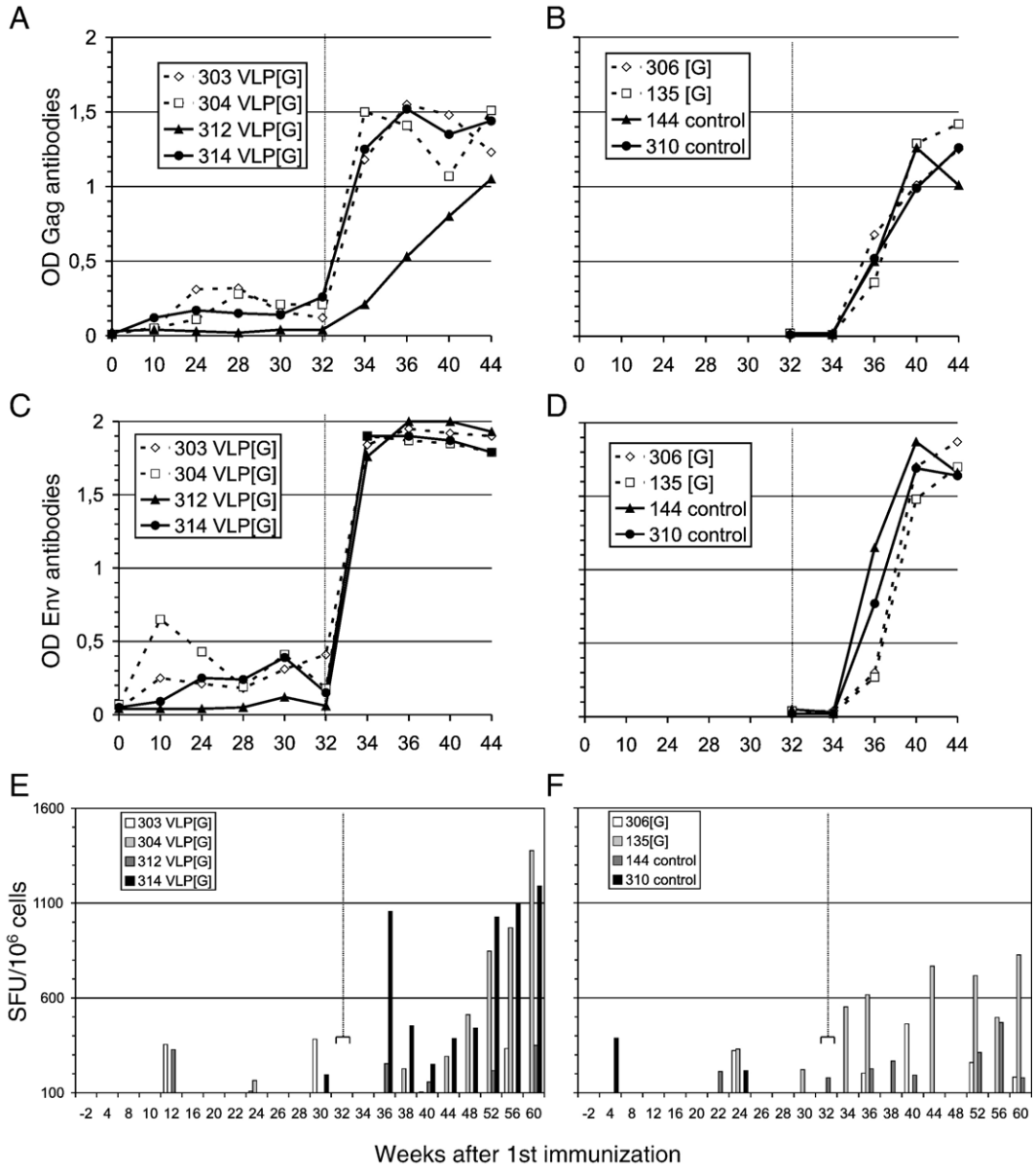

Fig. 5. Antibody titers to SIV Gag (A, B) or SIV Env (C, D) and IFN-gamma ELISPOT responses after stimulation with an overlapping SIV Ca peptide pool (E, F) in VLP[G]-vaccinated and control monkeys of the 2nd study at the indicated time points after the 1st immunization. Control monkeys either received supernatants from VSV-G-transfected cultures [G] or were left untreated (control). The dotted lines separate the data obtained before and after challenge at 32 weeks after the first immunization.

vaccination schedule did not provide persistent control of viremia. Consistently, IFN-ELISPOT responses were only seen in the long-term rhesus monkey vaccination experiment after the last booster immunization. Thus, a prolonged immunization schedule with extended resting periods between immunizations seems to be important. Consistently, the booster immunization at week 29 in the long-term vaccination experiment led to a strong increase in Gag-specific antibodies, but additional injections at week 33 and 37 had only minor effects. However, we cannot exclude that differences in the precise composition of the VLP[G] vaccine preparations or the genetic background of the monkeys are responsible for the different outcomes of the first and second vaccination experiment. Rhesus monkeys of both studies were of Chinese origin. Previous typing of 44 rhesus monkeys of Chinese origin for Mamu-A1 and B17 alleles did not reveal the Mamu-A*01 allele, while Mamu-B*17 was only rarely detected in these macaques after DNA sequence analysis of amplified PCR-products (U.S., unpublished observation). Despite extensive typing and DNA-sequence analysis, no other MHC-I alleles could be linked to disease progression in Chinese macaques. Thus, the currently established MHC-typing protocols do not allow to identify MHC class I alleles in Chinese rhesus macaques associated with better containment. However, higher set point viral load levels in the control monkeys of the first study suggest increased susceptability of these monkeys. Therefore, reduced efficacy in the second study is unlikely to be due to increased stringency of the virus host challenge system. Since the same challenge virus stock was

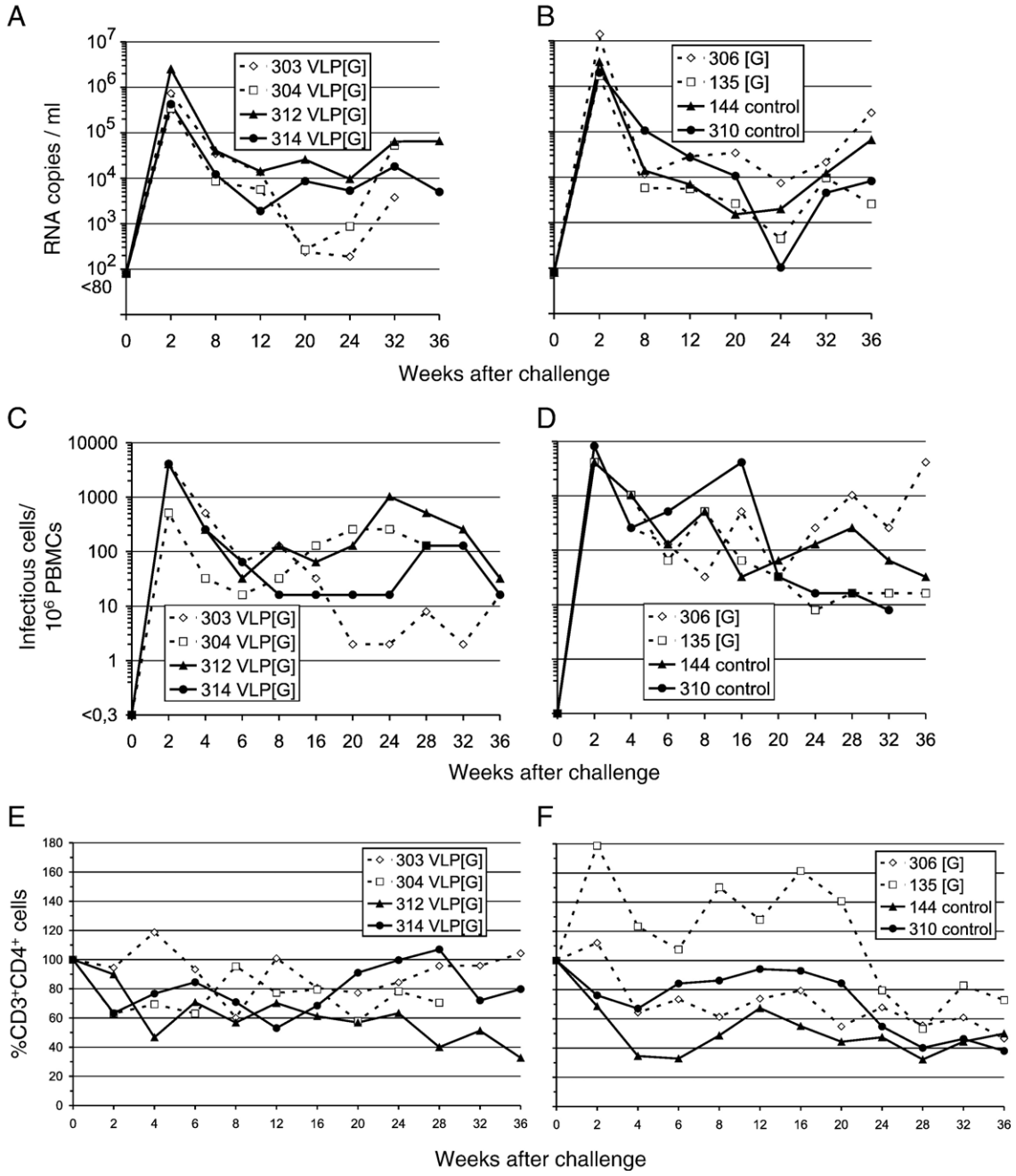

Fig. 6. Viral loads and percentage of CD4-positive cells normalized to baseline values in VLP[G]-vaccinated (A, C, E) and control (B, D, F) monkeys at the indicated time points after challenge with SIVmac239 at 32 weeks after the first immunization. Control monkeys either received supernatants from VSV-G-transfected cultures [G] or were left untreated (control).

used at the same dose in both experiments, differences in the challenge virus can be excluded.

In our studies, the VSV-G pseudotyped SIV particles were generated by transfection of the human 293T cell line and the VLPs therefore also contain human cell surface proteins. Since the challenge virus had been grown on rhesus monkey PBMCs, immune effector mechanisms directed against human proteins cannot be responsible for the protection observed. In addition, vaccinated monkeys were able to control virus replication in the absence of a sterilizing immunity. Since after one round of infection the viruses produced in the infected animals were entirely derived from the host, immune responses against nonviral heterologous proteins cannot be a relevant effector mechanism. However, human proteins could play a role during the induction phase of the antiviral immune response by for example providing costimulatory signals.

When evaluating vaccine efficacy, the stringency of the challenge system deserves attention because similar vaccines elicit different degrees of protection depending on the challenge system used. For example, monkeys primed with DNA and boosted with a MVA vaccine were able to control the SHIV89.6-challenge virus (Amara et al., 2001) but in

comparable experiments using SIVmac239 for the challenge, a reduction of viral load during primary infection was achieved, but set point RNA levels were not considerably reduced (Horton et al., 2002). Like primary isolates of HIV-1, SIVmac239 is highly neutralization-resistant and protection from this virus, while probably more relevant, seems to be more difficult to achieve than from SHIV89.6 (Desrosiers, 2004; Horton et al., 2002). Although our vaccines were based on SIVmac239, vaccine-induced neutralizing antibodies could only be detected against SIVmac251, but not against SIVmac239. This is consistent with the neutralization-resistant phenotype observed for SIVmac239 (Puffer et al., 2002).

To assess immunity at a mucosal site, we used the tonsillar route of infection (Stahl-Hennig et al., 1999). This route allows a truly atraumatic application of the challenge virus, which is difficult to confirm during rectal challenge. In contrast to rectal challenges, which often result in incomplete take of the challenge virus, tonsillar infection has been highly reproducible in our hands. So far, a total of 8 monkeys exposed to SIVmac239, 20 monkeys exposed to SIVmac251, and 28 monkeys exposed to a *nef*-deletion mutant of SIVmac239 by the tonsillar route became infected. In comparison to intravenously infected monkeys, we did not observe any evidence for an attenuated course of tonsillar infection (Stahl-Hennig et al., 1999, 2002; Tenner-Racz et al., 2004). In two ongoing experiments, an additional 10 naive monkeys, which were challenged with the same stock and dose of SIVmac239 by the tonsillar route, developed viral RNA levels comparable to the naive monkeys challenged in this study.

Despite the small number of animals in the long-term vaccination experiment, an approximately 1000-fold reduction in SIVmac239 set point RNA levels after immunization with VSV-G-pseudotyped SIV particles seems to compare favorably to the efficacy of other exogenous antigen and viral vector vaccines tested in the SIVmac239 challenge model (Horton et al., 2002; Lifson et al., 2002; Murphy et al., 2000; Wang et al., 2000). Potential enhancing effects of prime-boost regimens or adjuvants remain to be determined.

This study also suggests a note of caution to the use of VSV-G pseudotyped lentiviral or retroviral vectors for in vivo gene therapy. It is to be expected that strong immune responses are induced against the vector particle, possibly limiting the transfer efficacy and harboring the risk of hyperreactivity after repeated administrations.

In the second monkey experiment, where we hastened the vaccination schedule from 65 to 28 weeks, we observed less protection in the VLP[G]-vaccinated monkeys. The initial infection was similar in both experiments, and in both, reduced peak viral loads during acute infection were noted in the vaccines. But a reduction in set point and long-term resistance was only observed in the 65-week vaccination protocol. Although there could be variability within monkeys, we suspect that the more rapid vaccination protocol limited the induction of protective memory. Nevertheless, our observations that pseudotyping with VSV-G improves immune response in mice, and in one experiment led to substantial protection in SIV challenged vaccines, raise questions on how the VLP[G] strategy can be improved. Current understanding of the principles for strong cell-mediated immunity involves capture of vaccine by dendritic cells, migration to the T cell areas of lymphoid organs, and appropriate maturation. Therefore, it may be important to enhance the delivery of VLPs to DCs and more rigorously control the delivery of maturation stimuli with these vaccines by providing appropriate adjuvants. Although our results were raised with small numbers of animals, VSV-G-mediated enhancement of antigen uptake and/or stimulation of innate immune responses could be of broad interest in the demanding HIV vaccine field and be a more widely applicable approach to improve vaccines against other infectious diseases.

Materials and methods

Preparation of vaccines for rhesus monkey experiment

SCIVs and VSV-G pseudotyped SIV VLPs (VLP[G]) for the long-term monkey experiment were prepared side by side. 293T cells were transiently transfected with the plasmids SX2Δfrxn and pVSV-G, as previously described for SCIVs (Kuate et al., 2003). In case of SCIVs, the expression plasmid for the complementing tRNA was also included. SX2Δfrxn contains the SIVmac239 proviral genome with deletions in *vif*, *vpr*, *vpx*, and *nef* and mutations in the primer binding site. The mutated primer binding site blocks initiation of reverse transcription. Transfection of SX2Δfrxn therefore leads to noninfectious SIV-VLPs (Kuate et al., 2003). Cotransfection of an artificial tRNA expression plasmid (X2-Lys3) that matches the mutated primer binding site rescues the block (Hansen et al., 2001). This leads to the production of infectious SIV particles, that can only undergo a single cycle of replication (SCIV) because of the defective primer binding site (Kuate et al., 2003). One day after transfection, the FCS-containing medium was replaced by serum-free AIM-V medium (Gibco-Invitrogen-Corp., Karlsruhe, Germany) and supernatants were collected and filtered 24 h later. The titer of SCIVs in the supernatant of the transfected cells was determined on S-Magi cells (Chackerian et al., 1995) and the reverse transcriptase activity of the SCIV and VLP[G] preparation was determined by the RT easy kit (Roche Diagnostics, Mannheim, Germany). To control for nonspecific effects of VSV-G, supernatants of 293T cells transfected with only the VSV-G expression plasmid ([G]) were also harvested as described above. Western blot analyses with the monoclonal antibody P5D4 directed against VSV-G (Sigma-Aldrich, Taufkirchen, Germany) were used to adjust the amount of VSV-G present in the VLP[G] and [G] preparations. The lipid–DNA vaccine was prepared by complexing SX2Δfrxn with DOTAP and cholesterol as described for DC-high complexes (Nchinda et al., 2003).

Immunization and challenge of rhesus monkeys

Rhesus monkeys of Chinese origin were housed at the German Primate Center in Göttingen. Handling of the monkeys and collection of specimens were performed according to institutional guidelines as described previously (Stahl-Hennig et

al., 1990). Two young adult rhesus monkeys (*Macaca mulatta*) seronegative for SIV, D-type retroviruses, and STLV-1 were used per group. Immunizations were done at weeks 0, 4, 8, 29, 33, 37, and 65. For subcutaneous injection of SCIV, 10^7 infectious units corresponding to 9 μg reverse transcriptase (week 0, 4, 8), 1.42 μg (week 29, 33, 37) and 0.89 μg (week 65) respectively were injected in a 10 ml total volume at three different sites proxolateral of the inguinal regions. A VLP[G] preparation containing the same amount of reverse transcriptase was injected in the same way. The lipid–DNA vaccine was injected intradermally at a dose of 25 μg (week 0, 4, 8) and 80 μg (week 29, 33, 37 and 65) in a volume of 1 ml distributed to 10 sites with 100 μl each. Immunized monkeys were challenged by the tonsillar route (Stahl-Hennig et al., 1999) at week 69 along with two naive monkeys with approximately 6000 median tissue culture infectious doses ($TCID_{50}$) of the cell-free pathogenic SIVmac239 stock described previously (Gundlach et al., 1998). In the second experiment, rhesus monkeys ($n = 4$) received subcutaneous injections of VLP[G] preparations containing 4 μg RT/ml at week 0, 4, 8, 20, 24, and 28 as described above. Two of the control monkeys received injections of VSV-G containing supernatants ([G]) from transfected 293T cells, two additional control monkeys were left untreated. All monkeys of the second study were challenged at week 32 as described for the first experiment.

Monitoring the course of infection in monkeys

Viral RNA levels in the plasma were determined by real-time PCR as described (Hofmann-Lehmann et al., 2002). Antibodies to the SIV polypeptides Env and Gag were determined by a standard ELISA. Microtiter plates (Greiner) were coated overnight with recombinant SIV gp130 (repository number EVA 670, NIBSC) or recombinant SIV p27 (repository number ARP 643, NIBSC) diluted in 0.1 M carbonate buffer (pH 9.6) to 0.4 μg/ml. After blocking, a 1:200 dilution of plasma sample was added in duplicate and the plates were incubated for 3 h at room temperature. Plates were washed and incubated with anti-human IgG peroxidase conjugate (Jackson BioLab) and developed with B substrate (Sigma).

For the determination of neutralizing antibodies against VSV-G and SIVmac239 Env, 1:100 and 1:10 dilutions of sera were used, respectively. The neutralization assay was performed using VSV-G or SIV-Env-pseudotyped SIV-based vector particles as previously described (Kuate et al., 2003). The levels of neutralizing antibodies against SIVmac251 in the sera of immunized and infected macaques were measured using a yield reduction assay. Briefly, sera diluted 1:50 were incubated with serial dilutions of SIVmac251 (25 μl serum, 25 μl virus, six replicates per dilution) in U96 microtiter plates. After mixing and incubation for 1 h at 37 °C, 150 μl of a C8166 cell suspension (2000 cells) was added. After 7 days of incubation at 37 °C, the cultures were lysed and virus replication in individual wells measured by a sensitive Gag-based antigen capture ELISA. Wells giving OD values above threshold (mean of uninfected wells + 5 × SD) were scored as positive and the $TCID_{50}$ for the virus in the presence of each serum was calculated. The yield reduction for each sample was then calculated as the virus titer in the absence of serum divided by the titer in the presence of serum.

To determine SIV-specific T cell responses an IFN-γ, ELISPOT assay was employed. Ninety-six-well plates (MILLIPORE, MAIP S4510) were coated with 1 μg/well of antihuman IFN-γ monoclonal antibody (MABTECH, Sweden) overnight at 4 °C. The plates were washed with PBS and blocked with RPMI1640 containing 10% fetal calf serum (FCS) for 2 h at 37 °C. RPMI1640 medium was discarded from the plates and freshly isolated peripheral blood mononuclear cells (PBMCs) in ELISPOT medium (RPMI1640, 2 mM glutamine, 0.05 mM 2-mercaptoethanol, 10% FCS and antibiotics) were added. For antigenic stimulation, a pool of SIV Gag peptides (5 μg/ml each) comprising a set of overlapping 20mers covering the entire SIV p26 region (ARP714.1-229, Programme EVA, NIBSC) was added. 1 μg/ml SEB (Staphylococcal Enterotoxin B, SIGMA) served as positive control, medium alone as negative control. PBMCs (2×10^5) were plated in triplicate and incubated for 16–18 h at 37 °C in 5% CO_2. Cells were then removed and plates were washed with PBS-0.05% Tween-20 (PBST). Biotinylated anti-human IFN-γ detector antibody (MABTECH, Sweden) was added (0.1 μg/well) and plates were incubated for 2 h at 37 °C before being washed again with PBST. Streptavidin alkaline phosphatase conjugate (MABTECH) at 1:1000 in PBS/0.1% FCS was added and incubated for 2 h at room temperature. After washing, spots were developed with NBT/BCIP (LOEWE, Germany) solution (25 μg NBT plus 15 μg BCIP in 0.1 M Tris–HCl pH 9.5 per well) for 30 min. After washing with distilled water and air-drying, spots were counted by using a BIOSYS 2000 ELISPOT reader. The counts were normalized to 10^6 cells input. Results were calculated as the number of spots in the presence of peptides minus the spot number obtained with medium alone. To be defined as a SIV positive response, the latter number of spots had to be greater than 100 and twice that of the medium control.

For analysis of lymphocyte surface markers, PBMCs were prepared from blood by Ficoll-Paque gradient centrifugation and the percentage of $CD4^+$ T cells determined by FACS analysis in a Coulter EPICS XL flow cytometer using anti-human CD3-PE and anti-human CD4-FITC. The baseline value for each monkey was established by averaging the results of three independent analyses prior to SIVmac239 infection and all following measurements were related to the individual baseline values.

Immunization of mice

To prepare VSV-G pseudotyped HIV-1 VLPs (VLP^H[G]), a codon-optimized expression plasmid for HIV-1 *gag-pol* (Wagner et al., 2000) was cotransfected into 293T cells with an HIV-1 *env* (Helseth et al., 1990) and *tat* (Malim et al., 1988) expression plasmid and pVSV-G or a codon-optimized expression plasmid for VSV-G (GeneArt, Regenburg, Germany). VLPs lacking VSV-G (VLP^H) were prepared by omitting the VSV-G expression plasmid from the transfection

reaction. As a control, pVSV-G was transfected into 293T cells without HIV-1 expression plasmids. A fusion-defective mutant of the codon-optimized VSV-G expression plasmid was constructed by mutating the glutamine at position 117 to asparagine as previously described for wild type VSV-G (Whitt et al., 1990).

VLPs were harvested from serum-free medium as described above for SIV VLPs and pelleted through a 20% sucrose cushion by ultracentrifugation. Pellets were resuspended in PBS prior to determination of RT-activity and Western blot analyses with an antibody to VSV-G, HIV-1 Env, or an SIV antiserum (S1604) crossreacting with HIV-1 Gag. In two independent experiments, groups of five to six mice were immunized subcutaneously with VLPs containing 1 μg RT at week 0 and 5. Sera and spleen cells were harvested 1 week after the booster immunization. Gag antibodies were quantified by an endpoint dilution ELISA assay (in duplicate) on samples from individual animals essentially as described previously (Wild et al., 2004). Interferon-γ ELISPOT assays was performed as described. In the first experiment, the number of ELISPOTs was determined for six individual mice, in the second experiment, two pools of spleen cells from 2 to 3 mice each were analyzed in triplicates. The means and the standard deviations were calculated from all the 12 data points. The mouse experiments were performed blinded and were unmasked only after having measured the immune responses.

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