

# Immunization with HIV-1 gp41 Subunit Virosomes Induces Mucosal Antibodies Protecting Nonhuman Primates against Vaginal SHIV Challenges

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## SUMMARY

Human immunodeficiency virus (HIV)-1 is mainly transmitted mucosally during sexual intercourse. We therefore evaluated the protective efficacy of a vaccine active at mucosal sites. *Macaca mulatta* monkeys were immunized via both the intramuscular and intranasal routes with an HIV-1 vaccine made of gp41-subunit antigens grafted on virosomes, a safe delivery carrier approved in humans with self-adjuvant properties. Six months after 13 vaginal challenges with simian-HIV (SHIV)-SF162P3, four out of five vaccinated animals remained virus-negative, and the fifth was only transiently infected. None of the five animals seroconverted to p27<sub>gag</sub>-SIV. In contrast, all 6 placebo-vaccinated animals became infected and seroconverted. All protected animals showed gp41-specific vaginal IgAs with HIV-1 transcytosis-blocking properties and vaginal IgGs with neutralizing and/or antibody-dependent cellular-cytotoxicity activities. In contrast, plasma IgGs totally lacked virus-neutralizing activity. The protection observed challenges the paradigm whereby circulating antiviral antibodies are required for protection against HIV-1 infection and may serve in designing a human vaccine against HIV-1-AIDS.

## INTRODUCTION

Human immunodeficiency virus (HIV)-1 is mainly acquired at the mucosal site during sexual intercourse. Previous vaccine strate-

gies against HIV-1 were aimed at inducing circulating neutralizing IgG antibodies or cytotoxic T cells (CTLs). Both strategies have repeatedly failed to elicit protection against HIV-1 infection in vivo (Tatsis et al., 2009; Rerks-Ngarm et al., 2009; Buchbinder et al., 2008). An alternative strategy could be the development of a vaccine that elicits a mucosal immune response and blocks the entry of the virus at mucosal sites before primary infection takes place locally in the lamina propria.

The advantage of mucosal antibodies as a potential protection mechanism is supported by studies on individuals who are HIV-1-exposed but remain persistently seronegative (HEPS). In these individuals of both genders, one correlate of protection is the presence of gp41-specific IgAs in the blood and genital secretions, which display HIV-1-neutralizing activity and HIV-1 transcytosis-blocking activity (Devito et al., 2000a; Miyazawa et al., 2009; Tudor et al., 2009). Transcytosis is one of the mechanisms of entry of the virus into mucosal tissues (Bomsel 1997; Bomsel et al., 1998). Gp41-specific IgAs also block in vitro infection of mucosal target cells (Devito et al., 2000b; Miyazawa et al., 2009; Tudor et al., 2009), thereby preventing entry into and transcytosis across epithelial cells (Devito et al., 2000a; Bomsel et al., 1998; Alfsen et al., 2001) and blocking access of the virus to the lamina propria (Hladik and McElrath, 2008). Although IgGs may interfere with viral infection in tissues underlying mucosal epithelia and secondary lymphoid tissues, mucosal IgAs, which have a known compartmentalized distribution and repertoire, are thought to best protect mucosal surfaces.

Gp41 is the most conserved envelope subunit of HIV-1 with very few potential glycosylation sites as opposed to the surface envelope subunit gp120, whose neutralizing epitopes are widely masked by glycans. Gp41 and especially its hydrophobic membrane proximal external region (MPER), whose structure is strictly dependent on the lipidic environment provided by the viral membrane (Coutant et al., 2008; Sun et al., 2008), is

targeted by the broadly neutralizing IgGs 2F5 and 4E10. These IgGs provide sterilizing immunity against mucosal HIV-1 challenge, as shown by passive transfer studies, even at a low or undetectable concentration of serum antibodies at the time of protection (Hessell et al., 2010). Mucosal IgAs in HEPS individuals also target the extended MPER and exhibit both transcytosis-blocking and infection-neutralizing activity (Devito et al., 2000a, 2000b; Miyazawa et al., 2009; Tudor et al., 2009).

As reviewed by Montero et al. (2008), several immunogens based on peptides and proteins containing the MPER were evaluated in various animal models by monitoring blood IgGs and CTL. HIV-1-neutralizing IgG antibodies could not be elicited in vivo and specific CTL responses, despite an in vitro activity, lacked protective activity in vivo. In these studies, the immunogen design did not properly take into account the 3D structure of viral antigens that most likely differs greatly from its native structure on the virus. Furthermore, the route of immunization was not adapted to induce a humoral mucosal response, either in terms of compartmentalization or epitope specificity (Brandtzaeg 2009).

In this study, we used market-approved virosomes (Moser et al., 2007; Herzog et al., 2009) as a carrier—in use for humans for more than 10 years—to deliver two distinct HIV-1 gp41 antigens: a recombinant, truncated, trimeric gp41 (rgp41) antigen (Delcroix-Genête et al., 2006) and the P1 peptide (Alfsen and Bomsel, 2002; Magérus-Chatinet et al., 2007; Yu et al., 2008; Coutant et al., 2008). P1 corresponds to the extended MPER region of gp41 and includes the binding site to the mucosal receptor galactosyl-ceramide that is present on epithelial and dendritic cells (Alfsen and Bomsel, 2002; Magérus-Chatinet et al., 2007; Yu et al., 2008; Coutant et al., 2008). We designed the antigens by removing from gp41 the immuno-dominant epitopes (Delcroix-Genête et al., 2006) that are known to be nonneutralizing. Therefore, the current design of the gp41-derived antigens allows for focusing the immune response on protective HIV-1 envelope epitopes. Immunization of nonhuman primate females by both the intramuscular (i.m.) and intranasal (i.n.) routes with both gp41-derived virosome-bound antigens elicited full protection against repeated SHIV-SF162P3 vaginal challenges, whereas immunization by the i.m. route alone elicited protection in just 50% of the animals. The protected animals showed gp41-specific cervicovaginal IgAs and IgGs with transcytosis-blocking and antiviral activities, but showed no neutralizing IgG activity in their serum.

## RESULTS

### Antigen Design and Vaccine Strategy

The HIV-1 vaccine strategy of this study was designed to focus on the mucosal humoral immune responses against conserved gp41-derived antigens, the P1 peptide (Alfsen and Bomsel, 2002), and a newly engineered trimeric recombinant gp41 (rgp41) (Delcroix-Genête et al., 2006) from the X4 tropic, clade B HXB2 isolate of HIV-1. This new rgp41 (residues 540–664) is a stable trimer, produced in *E. coli*, that has several conserved key epitopes of gp41 (Serres, 2001; Delcroix-Genête et al., 2006), a deletion of the conserved immunodominant cluster I region, and additional point mutations introduced to break up homology with interleukin-2 (IL-2) and other human proteins. These later deletions were introduced to prevent a potential

autoimmune response upon vaccination (Serres, 2001). In addition, rgp41 contains the caveolin binding site-1 (CBD1) shown to induce HIV-1 neutralizing antibodies (Hovanessian et al., 2004) and the QARILAV neutralizing epitope, the latter being targeted by IgAs from HEPS individuals (Miyazawa et al., 2009). The P1 peptide corresponds to the gp41 MPER and contains the galactosyl ceramide-binding site and the 2F5 and 4E10 epitopes. Both the rgp41 antigen and the P1 peptide were linked by the C terminus to phosphatidylethanolamine, allowing their easy insertion into the lipid membrane of virosomes. In this lipidic context, HIV-1 antigens can fold as in situ in the viral membrane (Coutant et al., 2008; Sun et al., 2008).

The virosome vector is a nonreplicative virus-like particle derived from influenza that is formed by a lipid bilayered vesicle into which molecules of the influenza virus hemagglutinin (HA) and neuraminidase (NA) are inserted, thereby allowing efficient targeting of antigen-presenting cells. Virosomes have intrinsic adjuvant properties, elicit blood and mucosal antibodies (data not shown and Moser et al., 2007), and are licensed for use in human vaccines (Moser et al., 2007). Importantly, pre-existing antibodies against the influenza hemagglutinin (HA) were not reported to prevent revaccination with virosomes in human, in contrast to the situation with some viral vectors (Tatsis et al., 2009).

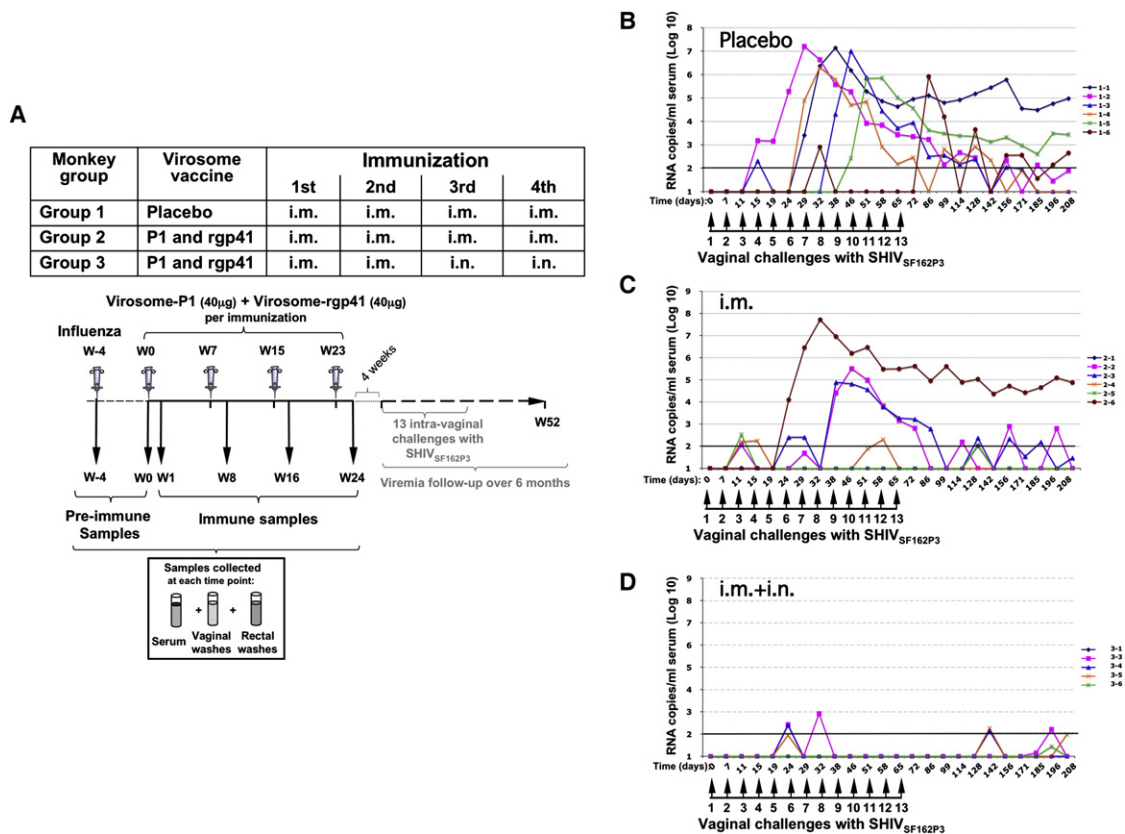
Female *Macaca mulatta* of Chinese origin were used to evaluate the in vivo protective efficacy of the gp41-based virosome vaccine against a virulent SHIV vaginal challenge. The mucosal antibody response is highly compartmentalized, at the level of both the induction (immunization route) and the effector sites, in terms of antibody repertoire and specificity (reviewed in Brandtzaeg, 2009). Therefore, the animals were vaccinated by either the intramuscular (i.m.) route of immunization alone, or by a combined intramuscular plus intranasal route (i.m.+i.n.).

Three groups of Chinese rhesus monkeys received four administrations of vaccine at weeks 0, 7, 15, and 23 (Figure 1A). Six animals (control group 1) received the virosome carrier alone. Six animals (group 2) were vaccinated four times by the i.m. route only, whereas six animals (group 3) received two injections by the i.m. route followed by two booster injections by the i.n. route. Each vaccine dose contained 40 µg of P1 and 40 µg of rgp41. One of the animals in group 3 unfortunately died of an unrelated cause before immunization was completed.

### Gp41 Virosome Immunization Protects Female Monkeys from Repeated SHIV-SF162P3 Vaginal Challenges

One month after the last vaccination, all animals were challenged intravaginally thirteen separate times, biweekly for the first four weeks and then once a week for the remaining five weeks, with a low dose (20–30 TCID<sub>50</sub>) of the heterologous SHIV-SF162P3 (clade B, R5 tropism).

Viremia was investigated blindly for up to 208 days after the initial challenge (Figures 1B–1D). Starting after the ninth challenge, the six animals in control group 1 all became infected (Figure 1B), with viral loads peaking at 10<sup>6</sup> to 10<sup>7</sup> copies/mL, as expected (Harouse et al., 2001). In group 2 (i.m. immunization), three of the animals were infected with peaks of viremia at 10<sup>5</sup> to 10<sup>7</sup> copies/mL, whereas two animals showed only transient peaks of viremia of ≤250 copies/mL and one consistently had no detectable viremia (Figure 1C). In contrast, only one of the animals in Group 3 (i.m.+i.n.) showed a low transient viral load



**Figure 1. Immunization and Challenge**

As experimental schedule (A), Female *Macacca mulatta* were immunized four times at week (W) 0, 7, 15, and 23 with a mixture of two distinct virosome formulations corresponding to virosome-P1 and virosome-rgp41 in a total volume of 100  $\mu$ l per dose. Group 1 received the empty virosome carrier as placebo, whereas groups 2 and 3 received 40  $\mu$ g of P1 and 40  $\mu$ g of rgp41 grafted onto virosomes. All animals from groups 1, 2, and 3 were immunized by the i.m. route at W0 and W7. Afterward, groups 1 and 2 continued to receive i.m. immunizations at week 15 and 23, whereas animals from group 3 received two i.n. immunizations with 50  $\mu$ l of the vaccine in each nostril administered with a specific spray device (Accuspray, BD). Blood, vaginal, and rectal washes were collected from each animal at week -4 and 0 for preimmune samples and 1 week after each vaccination event for immune samples (weeks 1, 8, 16, and 24). One month after the last immunization, animals from groups 1 (B), 2 (C), and 3 (D) were challenged 13 times with 20–30 TCID<sub>50</sub> of SHIV-SF162P3 intravaginally (indicated by black arrows). Blood samples were drawn over 6 months and analyzed at indicated time points for plasma viral load determination. The horizontal black line indicates the lower detection limit of RNA copies/mL.

of ~800 copies/mL after the seventh challenge that returned to undetectable levels a week later, and all the other animals in the group had very low or undetectable amounts of viremia (Figure 1D).

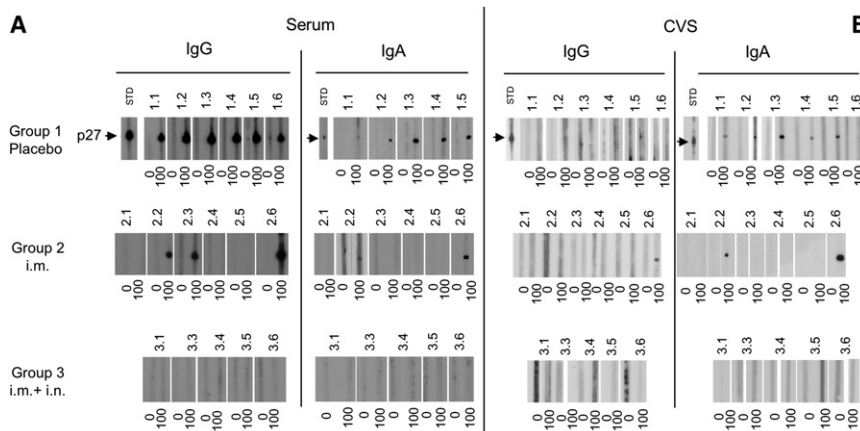
For confirming the absence of infection in the viremia-negative animals, the presence of serum IgG against SIV p27<sub>gag</sub> antigen was investigated at day 100. As expected, the protected group 3 (i.m.+i.n.) animals and the viremia negative animals in group 2 (i.m. only) did not develop p27<sub>gag</sub>-specific serum IgGs. In contrast, all the infected animals from group 1 and group 2 showed a strong IgG response against p27<sub>gag</sub> SIV (Figure 2A). Furthermore, p27-specific IgAs were detected in the vaginal compartment of almost all viremia-positive animals, whereas the signal for specific mucosal IgG was very weak (Figure 2B). These results were confirmed with serum samples taken 3 months later (not shown).

The absence of detectable p27-specific blood IgG and mucosal IgA in animals from groups 2 and 3 with undetectable

viremia suggests that in these animals, local exposure to the p27 antigens during the 13 consecutive challenges with SHIV-SF162P3 was not sufficient to elicit the induction of gag-specific antibodies in detectable quantities. Furthermore, the fact that animal 3.3 from the i.m.+i.n.-vaccinated group did not seroconvert in spite of its transient viremia suggests that if infected, this animal rapidly resolved its infection.

### Protection Is Not Associated with a Specific MHC Class I Haplotype

Specific MHC class I antigens (including Mamu-A\*01, -A\*02, -B\*08, and -B\*17) have been associated with SIV control (Lofredo et al., 2009). In the present study, all animals were negative for Mamu-A\*01 and Mamu-A\*02 (data not shown). There were two animals in group 2 (2.4 and 2.6) that were Mamu-B\*08 positive, and one animal per group (1.1, 2.1, and 3.3) was positive for Mamu-B\*17. These genetic traits did not apparently affect the results of the study.



**Figure 2. p27 Serology at Day 100 after the First Challenge with Heterologous SHIV-SF162P3**

Sera (A), and cervico-vaginal secretions (CVS, B) from preimmune animals (day 0) and at day 100 after the first challenge (100) were analyzed for p27gag SIV-specific antibody content by immunoblotting.

### Gp41 Virosome Vaccination Induces Both Plasma and Mucosal Antibody Responses

Because the virosomes used in the present study are not designed for triggering a CTL response (Amacker et al., 2005; Wilschut 2009), we limited our investigation to the humoral response. In the serum at week 24, the rgp41- and P1-specific IgG and IgA responses were observed in almost all animals in group 2 (i.m. only), whereas in group 3 (i.m.+i.n.), the response was not always detectable (Table 1). At the mucosal level at week 24, gp41- and P1-specific IgAs were detected in cervico-vaginal secretions (CVSs) and rectal secretions (RSs) of most immunized and protected animals. Regarding mucosal IgGs, only rgp41-specific IgGs, but not P1-specific IgGs, could be detected (not shown). A sizeable immune response required four immunizations as indicated by analyses of the samples at weeks 8 and 16 (Tables S1 and S2 available online). Overall, the mucosal immune response detected here may have been underestimated because detection of mucosal antibodies is always limited by the minute quantity of fluid that can be collected at each sampling time and by the dilution factor. Furthermore, the ELISA test used here only measured antibodies specific for linear and lipid-independent epitopes as we have shown earlier (Coutant et al., 2008).

Taken together, these results indicate that the vaccine formulation was immunogenic, inducing both gp41-specific IgAs and IgGs in the systemic and mucosal compartments.

### HIV-1-Blocking Antibodies Develop in the Mucosal Compartment, but Not in Serum

The functional inhibitory potential of the serum and mucosal antibodies in the vaccinated animals was investigated in an HIV-1 epithelial cell transcytosis assay (Bomsel 1997; Alfsen et al., 2001). HIV-1-neutralizing activity was also studied with CD4<sup>+</sup> T cells as target. The potential protective role of IgGs using not only their variable regions—that recognize the antigen—but also their Fc region was also evaluated in antibody-dependent cell cytotoxicity (ADCC) assay.

None of the sera of the vaccinated and protected animals at a dilution of 1/250 exhibited measurable neutralizing activity in any of the classical neutralization tests (data not shown), including inhibition of HIV-1 infection with TZM-bl cells and various recombinant viruses expressing primary envelopes,

namely SF162, qh0692, and du172, SOS assays or CD4<sup>+</sup> T cell assays with clade B R5-tropic HIV-1 JR-CSF (data not shown). Furthermore, ADCC mediated by serum could not be detected with P1 or recombinant gp160-coated CD4<sup>+</sup> T cell targets (data not shown).

In contrast to serum, cervico-vaginal secretions (CVSs) exhibited various highly marked HIV-1 neutralization activities (Table 2, Figure 3). Table 2 shows that mucosal antibodies from CVS from the five animals in group 3 at a 6-fold dilution (corresponding to 0.2–15 µg of total antibodies/mL) could block HIV-1 transcytosis of a primary clade B, R5-tropic virus by 85%–100%. No blocking activity was detected in CVS samples from the placebo group. When tested against a primary clade C virus strain (Table 2), four out of five CVS samples could block transcytosis by more than 75%. These results demonstrate that the i.m.+i.n. immunization strategy could induce mucosal antibodies with in vitro cross-clade transcytosis-inhibition activity.

Transcytosis-blocking activity in CVS samples from group 2 animals (i.m. only) was >90% for clade B and >60% for clade C viruses in two of the three uninfected animals but negligible in the infected animals. Pairwise group comparisons showed a significantly higher transcytosis-blocking activity in CVS from group 3, as compared to group 1 (placebo) for both clade B ( $p = 0.0054$ ) (Figure 3A) and clade C ( $p = 0.0097$ ) viruses (Figure 3B). Statistically significant differences, although lower, were also detectable between groups 2 (i.m.) and 1 (placebo) for both viral clades ( $p = 0.016$  and  $0.04$ , respectively).

To determine which antibody isotype contained in CVS was actually involved in the prevention of HIV-1 transcytosis (Bomsel 1997), we depleted samples of IgA content and retested them. Figure 4 clearly demonstrates that no inhibition of transcytosis could be observed in the CVS samples depleted of IgAs. In contrast, IgG-depletion of the same samples had no effect on their transcytosis-blocking capacity. These results strongly suggest that the IgA fraction harbored the inhibitory activity, whereas the IgGs had no substantial role in preventing HIV-1 transcytosis in this model.

As opposed to sera that contain very high, specific antibody concentrations upon vaccination, mucosal secretions generally contain a 100- to 1000-fold lower concentration of antibodies, which make their evaluation difficult in neutralization assays. CVS from group 3 generated limited neutralization activity for three out of the five monkeys when tested for neutralizing of human CD4<sup>+</sup> T cell infection by HIV-1, a standard neutralization assay (Table 2, Figure 3E), although statistically significantly



## Immunity

### Gp41 Vaccine Protects from Mucosal SHIV Infection

**Table 1. Evaluation of Antibodies toward the P1 and rgp41 Antigens in Each Animal**

Sample Monkey Group	Serum			CVS			RS		
	1:CT	2:i.m.	3:i.m.+i.n.	1:CT	2:i.m.	3:i.m.+i.n.	1:CT	2:i.m.	3:i.m.+i.n.
<b>P1</b>									
IgA	0/6	5/6	4/5	0/6	5/6	5/5	0/6	5/6	2/5
IgG	0/6	6/6	3/5	0/6	6/6	2/5	0/6	0/6	0/5
<b>rgp41</b>									
IgA	0/6	6/6	5/5	0/6	6/6	4/5	0/6	5/6	4/5
IgG	0/6	6/6	3/5	0/6	6/6	5/5	0/6	0/6	0/5

Systemic and mucosal responses to P1 and rgp41 in female *Macaca mulatta* monkeys after completion of the vaccination procedure with virosome-rgp41 and virosome-P1 (week 24 compared to week 0) in sera and CVS were analyzed by antigen-specific ELISA. For mucosal samples, results were calculated as the percent specific IgA or IgG of the total IgA or IgG in each sample, respectively, so as to account for the variation of total Ig between each mucosal sample. Samples showing a specific signal at least twice above the background were considered positive. Shown is the number of animals with specific IgAs or IgGs over the total number of animals in each group.

different from group 1 (placebo) ( $p = 0.0097$ , Figure 3E). In contrast, no biologically relevant neutralizing activity was found in CVS samples from groups 1 and 2.

Table 2 further shows that CVS IgGs from all group 3 animals exhibited substantial ADCC activity, regardless of the IgGs specificity for either P1 or rgp41, although P1-specific ADCC was always stronger. An ADCC activity was also detected for CVS IgGs from group 2 animals that were viremia negative, although with an overall lower ADCC titer. In contrast, IgGs from the placebo group (group 1) totally lacked ADCC activity. Pairwise group comparisons showed clearly a significantly higher P1-specific ADCC in CVS from group 3 (i.m.+i.n.) as compared to group 1 (placebo) ( $p = 0.0055$ ) (Figure 3C), whereas the difference for rgp41-specific ADCC was less substantial ( $p = 0.028$ ) (Figure 3D).

These sets of results were confirmed when comparing either viremia positive (including animals with low viremia blips fewer than 300 RNA copies/ml) versus viremia negative animals (Figures S1A–S1D) or when comparing persistently infected versus protected animals (Figures S2A–S2D).

Finally, for all animals taken together, a strong highly significant inverse correlation was observed between clade B and clade C HIV-1 transcytosis-blocking activity in CVS and peak acute viremia (Figures 5A and 5B) ( $r = -0.832$ ;  $p < 0.0001$  and  $r = -0.850$ ;  $p < 0.0001$ , respectively). P1-specific ADCC also inversely correlated significantly with peak acute viremia ( $r = -0.682$ ;  $p = 0.0036$ ) (Figure 5C), but no correlation was found between viral loads and gp41-specific ADCC (Figure 5D) or neutralization activities (Figure 5E).

In summary, these experiments showed altogether that i.m.+i.n. immunization with P1- and rgp41-coupled virosomes fully protected female rhesus macaque from 13 repeated vaginal challenges with SHIV-SF162P3, whereas placebo-vaccinated animal all become viremic and seroconverted to p27. The protected animals showed gp41-specific cervicovaginal IgAs and IgGs with antiviral and transcytosis-blocking activities but showed no neutralizing IgG activity in their serum.

## DISCUSSION

Because HIV-1 is mainly a sexually transmitted infection, we designed a vaccine strategy to protect the initial sites of viral entry,

especially the rectum and female genital tract, by inducing a mucosal humoral immune response that hopefully would be able to prevent the establishment of early viral infection at these mucosal sites.

Although there is recurrent interest in gp41, and especially its MPER moiety, to serve as a vaccine antigen, little information exists on the ability of gp41-specific antibodies to act as protective antiviral antibodies in vivo. However, intravenously administered MPER-specific monoclonal IgGs obtained from HIV<sup>+</sup> patients were recently shown to protect male rhesus monkeys from a single high-dose rectal SHIV challenge (Hessell et al., 2010), supporting MPER as a potent vaccine candidate. Yet, in that study neither the mucosal titers nor the in vitro functions of the gp41-specific antibodies were evaluated. The best evidence for an antiviral role at mucosal sites of gp41-specific HIV-1 blocking IgAs, including MPER-specific ones, is offered by HEPS individuals for which such mucosal IgA antibodies are one of the correlates of protection (Miyazawa et al., 2009; Tudor et al., 2009).

How to raise such HIV-1 gp41 antibodies with antiviral activities by vaccination remains an open question (Montero et al., 2008). The gp41 antigen described here was split into a rgp41 (Delcroix-Genête et al., 2006) and peptide P1 (Alfsen and Bomsel, 2002) engineered to adopt a 3D structure close to the structure adopted on the virus in close proximity to the lipid bilayer (Coutant et al., 2008), provided here by the virosome vaccine vector. Immunodominant, although nonneutralizing, gp41 epitopes were deleted so that inducing a useless humoral response was avoided, thereby refocusing the humoral response on the less immunogenic key epitopes of the antigen.

Studies comparing humoral immunity at mucosal surfaces with serum immune responses have repeatedly shown a separation between secretory and systemic immune responses. Immunization at mucous membranes resulted in high titers of protective antibodies at mucosal effector sites, whereas the serum humoral response showed a lower neutralization titer (reviewed in Macpherson et al., 2008). Specific combinations of chemokine receptors and adhesion molecules mediate B cell homing to mucosal and peripheral tissues. Accordingly, nasal vaccination leads to development of a humoral response locally but also in the female genital tract and in the large intestine (Macpherson et al., 2008). In contrast, i.m. immunization induces both

**Table 2. Transcytosis-Blocking, ADCC, and HIV-1 Neutralization Activities in Cervico-Vaginal Secretions, after the Last Immunization with P1- and rgp41-Virosomes**

	Monkey	Transcytosis Inhibition (%)		ADCC Titer		Neutralization IC50 (CVS Dilution)
		Clade B	Clade C	Specific for P1	Specific for gp41	
Placebo						
	1.1	0	0	0	0	0
	1.3	5	0	0	0	0
	1.4	0	0	0	0	0
	1.5	0	0	0	0	0
	1.6	0	12	0	0	0
i.m.						
	2.1	28.2	65	27	9	6
	2.2	3.1	4.5	3	3	6
	2.3	2.0	0.75	3	3	6
	2.4	89.8	12	3	0	6
	2.5	96.0	71	27	3	0
i.m.+i.n.						
	3.1	96	99	27	9	6
	3.3	94	73	3	3	0
	3.4	96	85	3	0	6
	3.5	100	42	27	9	0
	3.6	85	78	27	9	18
Positive Controls						
	2F5 IgG	98	N.A.	800 pg/ml	N.A.	15 µg/mL
	98.6 IgG	N.A.	N.A.	N.A.	32 pg/ml	N.A.

For transcytosis blockade, preimmune (week 0) and immune (after the fourth immunization) (week 24) CVS at 1:6 dilutions from group 1 (placebo), group 2 (i.m.), and group 3 (i.m.+i.n.) animals were tested as described in [Experimental Procedures](#) and in the legend of [Figure 3](#). For ADCC, preimmune and immune (after the fourth immunization) CVS at serial dilutions from 1:3 to 1:27 from groups 1, 2, and 3 were tested comparatively for P1- or rgp41-specific ADCC activity as indicated in [Experimental Procedures](#). Results, obtained from at least two independent experiments, are expressed as ADCC titer as defined in [Experimental Procedures](#). For neutralization, preimmune and immune (after the fourth immunization) CVS (1:6 dilution) from groups 1 and 3 were compared for their neutralizing activity against infection of CD4<sup>+</sup> T cells with JR-CSF R5 tropic HIV-1 as indicated in [Experimental Procedures](#). Results are expressed as the highest inhibitory CVS dilution resulting in 50% specific neutralization (IC<sub>50</sub>). Results were obtained from at least two independent experiments. Specific neutralization is defined by the neutralization observed in presence of immune CVS related to that observed in presence of preimmune CVS (week 24 % week 0).

a systemic and a vaginal humoral response ([Vajdy et al., 2004; Vajdy 2006; Barnett et al., 2008](#)). The combination of i.m. and i.n. immunizations in the rhesus monkey model results in substantially enhanced anti-HIV-1 plasma and vaginal antibodies ([Vajdy et al., 2004; Vajdy 2006; Barnett et al., 2008](#)).

Accordingly, we compared in this study the gp41-specific antibody response elicited with the sole i.m. route or the combined i.m.+i.n. routes of vaccination. The two procedures induced a clear-cut differential degree of antibody antiviral activ-

ities as measured both in vitro and upon virulent mucosal heterologous challenge in vivo. Hence, after the mucosal challenge, we observed a full protection in the i.m.+i.n.-immunized group only, as compared to 50% protection in the i.m.-immunized group. In the combined i.m.+i.n. immunization procedure, the i.m. step might have primed the serum antibody response (IgG), whereas the i.n. step would have boosted the mucosal responses with antiviral activities ([Zhou et al., 2009](#)).

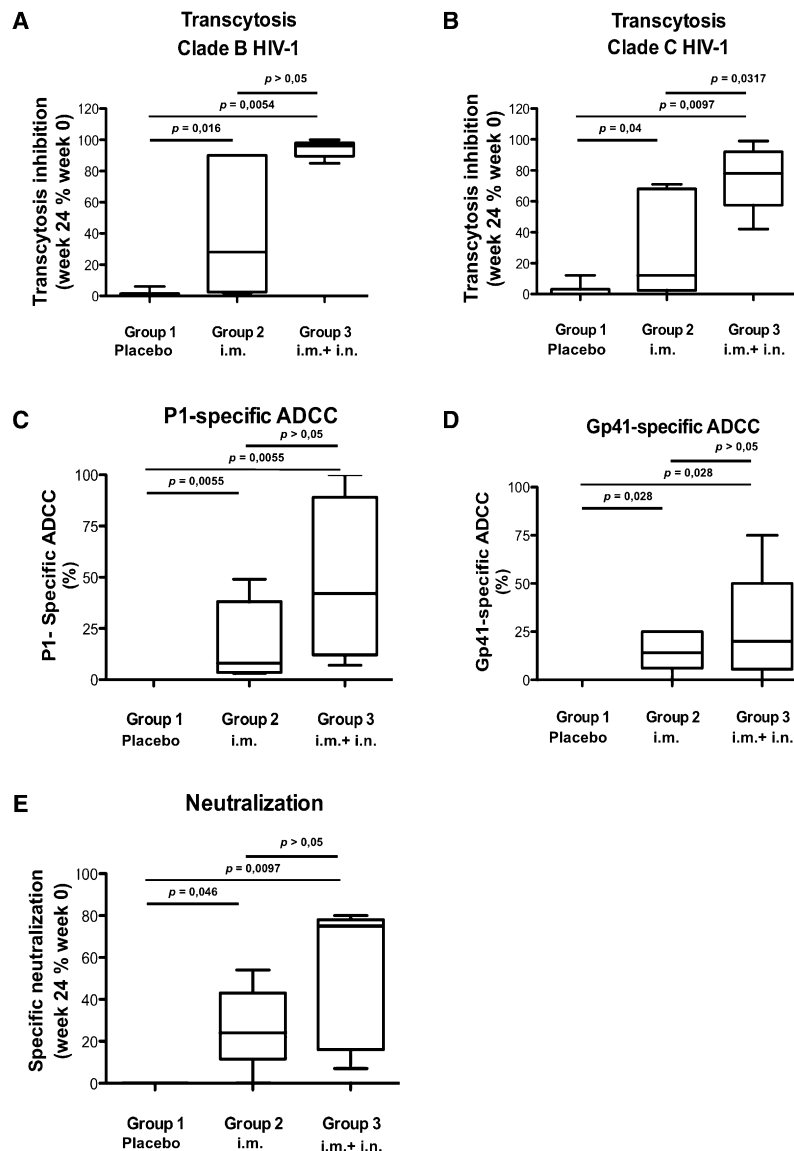
The six-month vaccination period allowed for affinity maturation of the mucosal antibodies (data not shown) that characterizes neutralizing antibodies ([Wu et al., 2010](#)). Such maturation affinity was also observed in our initial vaccination experiments with the virosome-P1 alone.

In vivo protection was investigated by repeated heterologous challenges by the vaginal route with physiological doses (20 and 30 TCID<sub>50</sub>) of infectious SHIV-SF162P3. This strategy was designed to be close to natural HIV-1 transmission, using the genital route of transmission and a dose of infectious virus close to that present in unprotected intercourse, even though this viral dose is 10 times greater than the dose found in semen in seropositive men ([Hessell et al., 2009](#)). The antigens in the vaccine were derived from HXB2, an X4, clade B, HIV-1 strain, whereas challenge of the vaccinated animals was done with the R5, clade B, SHIV-SF162P3 virus strain. Most previous studies only tested homologous challenges (reviewed in [Demberg and Robert-Guroff, 2009](#)).

SHIV, although slightly attenuated as compared to SIV, was chosen as the challenge virus for the following reasons: the HIV-1 envelope glycoprotein is essential in the initial steps of HIV-1 infection, but SIV and HIV-1 envelopes differ largely in amino acid sequence, length, and structure; as a result, antibody specific for HIV-1 gp41 cannot recognize the corresponding region on the SIV envelope and SIV is not sensitive to an immune response targeting HIV-1 envelope. Therefore, for mimicking the situation in the human and evaluating the initial steps of HIV-1 infection that are exclusively dependent on HIV-envelope-mediated fusion with target cells, one needs to use a virus expressing an HIV-1 envelope. Rather, SIV is appropriate to evaluate the development of the infection and its prevention. Given that the vaccine described here targeted specifically HIV-1 envelope subunit gp41, the use of SIV was prohibited.

Our results indicate that combining the i.m.+i.n. routes (group 3) offers the best protection to the animals, with undetectable viral load for six months in five out of five animals—except for a transient and low peak of viremia that lasted 1 week in one of the monkeys—as compared to 50% protection for animals vaccinated by the i.m. route only (group 2). Because of the low sensitivity of the viremia assay (threshold at 200 copies/mL), it remains possible that the protected animals were actually infected to a very low degree. However, none of the i.m.+i.n. vaccinated animals had detectable blood or mucosal antibodies against SIV p27gag antigen at 3 and 6 months after challenge, confirming the absence of an established infection. The same was true for the three protected monkeys in group 2.

The most likely correlate of protection in the vaccinated animals appears to be contributed by the antiviral activity of mucosal gp41-specific IgGs and IgAs. Circulating antibodies were devoid of anti-HIV activity, whether in neutralization tests, in transcytosis-inhibition tests, or in ADCC tests. Besides,



**Figure 3. HIV-1 Transcytosis-Blocking, ADCC, and Neutralization Activities in Cervico-Vaginal Secretions after Immunization with P1 and rgp41 Virosomes**

For transcytosis-blockade, preimmune (week 0) and immune (after the fourth immunization: week 24) CVS at 1:6 dilution from group 1 (placebo), group 2 (i.m.), and group 3 (i.m.+i.n.) animals were preincubated with PBMCs infected with primary HIV-1 clade B 93BR029 (A) or clade C 92BR025 (B) prior to addition to the luminal side of polarized epithelial cell monolayers. Transcytosis inhibition is defined by the ratio of neutralization observed in presence of immune CVS related to that observed in presence of preimmune CVS (week 24 % week 0). Values are the mean ( $\pm$ SEM) of at least two independent experiments. For ADCC, preimmune and immune (after the fourth immunization: week 24) CVS at serial dilutions from 1:3 to 1:27 were tested comparatively for P1- (C) and rgp41- (D) specific ADCC activity as described in [Experimental Procedures](#). Results are expressed as ADCC-specific lysis. Values are the mean ( $\pm$ SEM) of at least two independent experiments. For neutralization, preimmune and immune (after the fourth immunization: week 24) CVS (1:6 dilution) from groups 1, 2, and 3 were compared for their neutralizing activity against infection of human CD4<sup>+</sup> T cells by JR-CSF R5 tropic HIV-1 as described in [Experimental Procedures](#) (E). Results are expressed as specific neutralization defined as the ratio of neutralization observed in presence of immune CVS related to that observed in presence of preimmune CVS (week 24 % week 0). Values are the mean ( $\pm$ SEM) of at least two independent experiments. For all functional tests, statistical analyses were performed first by the Kruskal-Wallis test; pairwise comparisons were performed by the Mann-Whitney U-test, the p values are presented in each panel. KW statistic = 11.212,  $p = 0.0037$  (A); KW statistic = 10.562,  $p = 0.0051$  (B); KW statistic = 11.677,  $p = 0.0029$  (C), KW statistic = 6.881,  $p = 0.0320$  (D); KW statistic = 8.856,  $p = 0.0119$  (E).

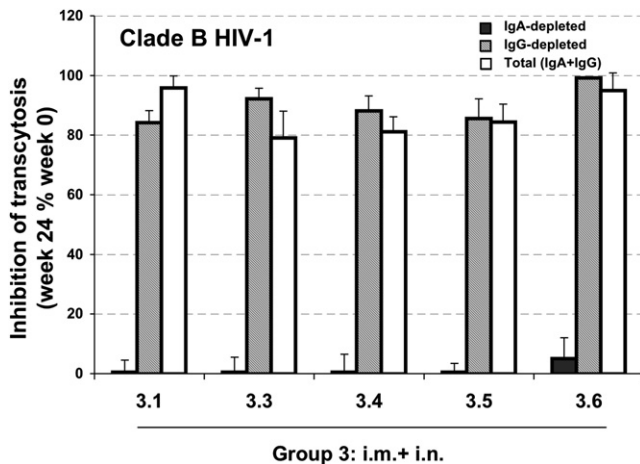
response has recently been suggested to provide robust control of mucosal infection after a repeated mucosal virulent SIV challenge in 4 out of 12 rhesus monkeys vaccinated with

because of the vaccine design with antigens grafted onto the virosome surface, a role for vaccine-induced CTLs is unlikely ([Amacker et al., 2005](#); [Kammer et al., 2007](#)) ([Wilschut 2009](#)), although it remains a possibility that a (weak) CTL response was induced. We and others have previously shown that CTL responses can be triggered by the virosome strategy when specific CTL epitopes from the membrane bound antigens are encapsulated inside the virosomes ([Amacker et al., 2005](#); [Kammer et al., 2007](#)) ([Moser et al., 2007](#)) ([Wilschut 2009](#)). However, this was not the case in the present study. Therefore, possible cell-mediated immune responses were not investigated in the present study.

It should be noted that no CTL epitopes have been described in the P1 subunit used in the present study and only two potential—but weak ones ([Currier et al., 2002](#); [Novitsky et al., 2002](#))—are present on the rgp41 subunit (see the LANL HIV immunology database), with the most potent one being deleted by our immuno-refocusing strategy. Interestingly, a protective CTL

a SIV protein-encoding replicative vaccine ([Hansen et al., 2009](#)). In the protected (although infected) animals, the protective CTL response observed was directed to Rev, Nef, and Tat. Yet no response to Env—including gp41—was reported.

Antibodies contained in the cervico-vaginal secretions of the i.m.+i.n.-vaccinated animals were capable of inhibiting by >80% the *in vitro* transcytosis of primary HIV-1 viruses from clades B and C, suggesting cross-clade recognition. This is particularly interesting, given that the tested HIV-1 clade C virus does not have the 2F5 and 4E10 motifs in its gp41 sequence. This suggests that other functional epitopes and/or 3D epitope (s) might be shared between the clade B and C gp41 molecules, despite the absence of linear epitope identity. HIV-1-blocking IgAs specific for such cross-clade epitopes in HIV-1 envelope ([Devito et al., 2002](#)) and more specifically in P1 are detected in HEPS individuals ([Tudor et al., 2009](#)) and were also induced in monkeys upon intramuscular or mucosal immunization with virosome-P1 alone.



**Figure 4. HIV-1 Transcytosis-Blocking Activities in Cervico-Vaginal Secretions from Group 3 after IgA or IgG Depletion**

CVS from group 3 (white bars) were IgA- (black bars) or IgG- (gray bars) immuno-depleted prior to incubation with HIV-1-infected cells. After 2 hr contact with the mucosal barrier at 37°C, transcytosis was evaluated in the basal compartment by measuring the amount of translocated p24 gag protein and expressed as percent inhibition of transcytosis in presence of immune CVS (at week 24) related to percentage of transcytosis in the presence of preimmune CVS (at week 0). Error bars represent the means ( $\pm$ SEM) of at least two independent experiments.

Furthermore, the transcytosis-blocking activity observed here at the mucosal level in the CVS of the i.m.+i.n.-vaccinated animals relied almost entirely on IgAs because IgA depletion, but not IgG ones, totally abolished transcytosis blockade in vitro. These findings are in agreement with previous studies showing that gp41-dimeric IgAs and secretory IgAs could either block HIV-1 intracellularly during transcytosis and redirect it to the mucosal surface (Bomsel et al., 1998) or impair its mucosal entry (Devito et al., 2000a; Alfsen et al., 2001; Broliden et al., 2001; Miyazawa et al., 2009). IgAs have also been shown to retro-translocate pathogens from the lamina propria into the lumen (Corthésy 2007).

Taken together, our results strongly suggest that vaccine-induced mucosal gp41-specific IgAs efficiently prevented HIV-1 binding to the mucosal receptor galactosyl ceramide (Alfsen and Bomsel, 2002) or to other gp41 epitopes such as the caveolin binding motif (Hovanessian et al., 2004), thus blocking initial virus entry in mucosal tissues. This conclusion will have to be strengthened and confirmed in further studies.

IgGs constitute an important fraction of vaginal antibodies, whereas in other mucosal secretions IgAs predominate. P1- and/or rgp41-specific ADCC activity mediated by mucosal IgGs could also be detected in protected animals from group 3 and animals from group 2 with undetectable or transient viremia  $\leq 250$  viral RNA copies/mL. In contrast, CVS from the infected animals in group 2 showed low IgG-dependent ADCC activity and lacked transcytosis-blocking activity. Therefore, it is possible that transcytosis inhibition must be combined with ADCC to block the initiation of HIV-1 infection at mucosal sites. Indeed, previous studies have associated mucosal ADCC activity with lower blood viremia in HIV-1-positive women (Nag et al., 2004), and disappearance of ADCC seems to correlate

with lower CD4<sup>+</sup> T cell counts and progression to AIDS in men and in the nonhuman primate model (Xiao et al., 2010 and reviewed in Forthal and Moog, 2009). The discrepancy between serum and mucosal IgG activities confirms the presence of locally produced IgGs (Ogra and Ogra, 1973; Brandtzaeg 2009; Bouvet et al., 2002) and the compartmentalization of the mucosal immune response (Bouvet and Fischetti, 1999; Macpherson et al., 2008).

Knowing that the mucosal immune response is relatively short lived, compared with blood antibodies, it will be important to determine in future studies the duration of the protective immune response observed here.

The most remarkable finding in this study was that mucosal protection against a heterologous SHIV challenge in rhesus macaques occurred in the absence of detectable neutralizing antibodies in the serum. This is reminiscent of data on the protection induced by injection of a panel of HIV-1-envelope-specific IgGs (Hessell et al., 2009; 2010) and clearly challenges the paradigm that mucosal protection requires the presence in serum of high titers of IgGs with virus-neutralizing capacity.

Altogether, this study demonstrates that a vaccine that stimulates the production of HIV-1 envelope gp41-specific antibody in the vaginal tissue was sufficient to protect monkey from vaginal exposure to virulent virus. This may serve toward the development of a human vaccine against HIV-1 and acquired immunodeficiency syndrome and help to explain why a few individuals who lack HIV-1-specific antibodies in the blood are able to resist infection, even when they are repeatedly exposed to HIV-1.

## EXPERIMENTAL PROCEDURES

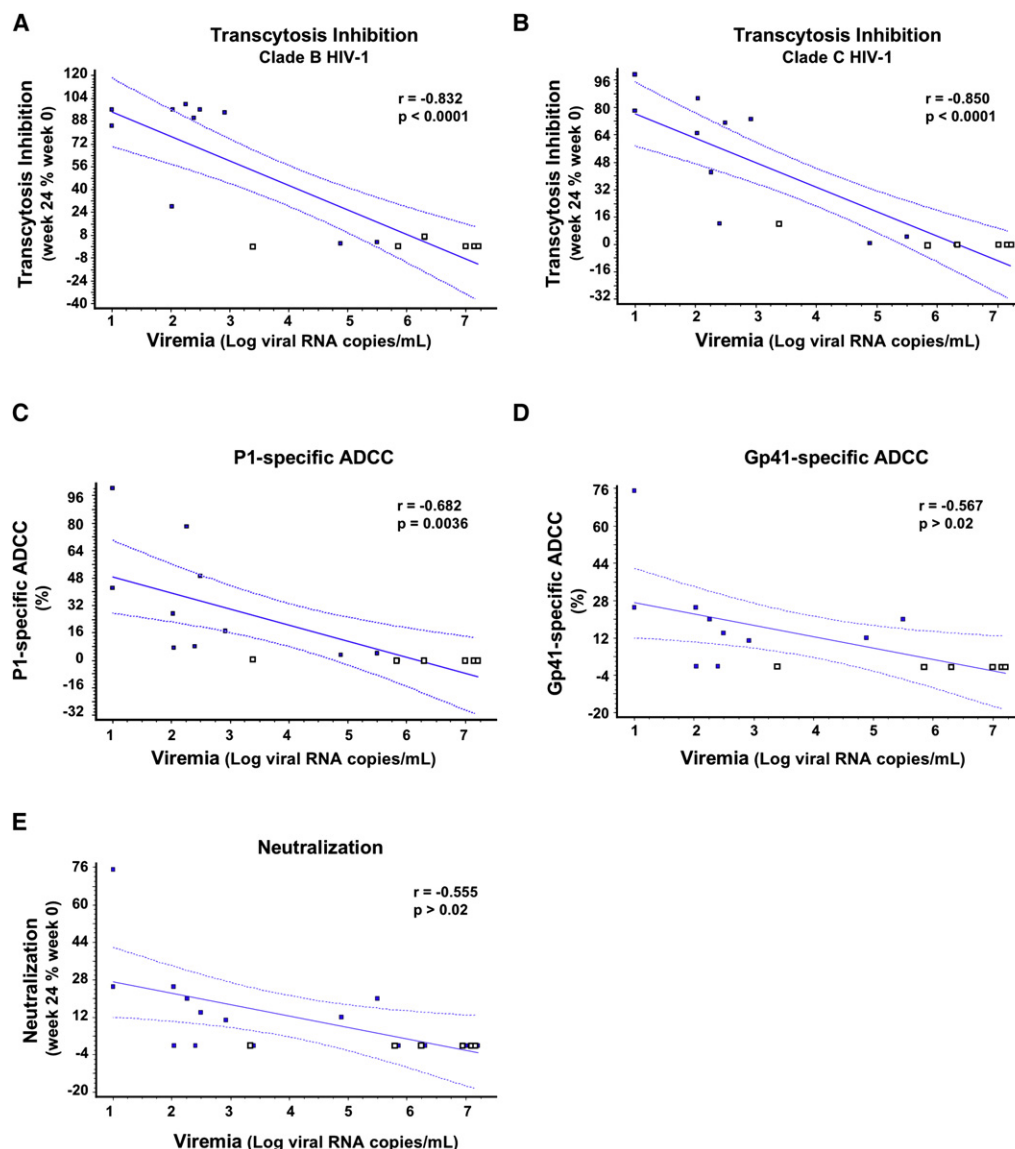
### Antigens

The P1 sequence (649–683) (originally described in Alfsen and Bomsel, 2002) derived from the HXB2 clade B HIV-1 strain and was modified to a lipid-linked synthetic peptide having 38 amino acids with the following sequence, SQTQQEKNEQELLELDKWASLWNWFDITNWLWYIKLSC, linked to phosphatidyl-ethanolamine (PE) via its C-terminal cysteine. The rgp41 protein was derived from the amino acid sequence 540–663 of HXB2 clade B HIV-1 strain and was produced in *E. coli* with the following sequence, MQARQLLSG IVQQNNLLRAIEAQHLLQLTVWGKQLQARILAVERYLKDQQLSGGRGSS LEQIWNHTTWMEWDREINNYTSLIHSLEESQNNQKEKNEQELLEHHHC, (italic letters correspond to the overlap region with the P1 peptide). The molecule was covalently linked via a bifunctional succinate linker to PE. This rgp41 is lacking the binding motifs for the mucosal receptor galactosyl ceramide (Alfsen and Bomsel, 2002) and the 2F5 and 4E10 motifs (Muster et al., 1993; Stiegler et al., 2001). A deletion of 25 amino acids (593–618) was introduced into the molecule to keep it soluble and trimeric, as well as point mutations to greatly reduce 3D homology with the human IL-2, as previously published (Delcroix-Genête et al., 2006). The rgp41 was purified through several steps of chromatography as described (Delcroix-Genête et al., 2006), resulting in a stable trimeric protein of 39.9 kDa.

### Viroosomes

Influenza virus strain A/Singapore/6/86 (H1N1) was cultivated in embryonated hen eggs and inactivated with  $\beta$ -propiolactone before solubilization with 100 mM octaethylene glycol monododecyl ether in phosphate-buffered saline-NaCl (OEG-PBS). The solubilized viral hemagglutinin (HA) and neuraminidase (NA) were mixed with egg phosphatidylcholine (PC) and synthetic phosphatidylethanolamine and the antigens of interest, P1 or rgp41, previously conjugated to PE. Detergent was removed on polystyrene beads on two subsequent batch chromatography steps, and homogenous influenza viroosomes, that spontaneously reconstituted, were filtered for sterilization without further purification (Amacker et al., 2005) (Kammer et al., 2007).





**Figure 5. Correlation between Peak Acute Viremia and HIV-1 Transcytosis-Blocking, ADCC, and Neutralizing Activities in Cervico-Vaginal Secretions for All Animals**

Inverse correlations (straight line) for all animals between peak acute viremia and (1) transcytosis-blockade of HIV-1 clade B (A), or HIV-1 clade C (B); (2) P1- (C) or rgp41- (D) specific ADCC; and (3) virus neutralization (E) activities in CVS. Dotted lines represent the 95% confidence intervals. Open symbols represent placebo-virosome-vaccinated macaques (group 1); filled symbols represent P1- and rgp41-virosome-immunized macaques. The correlation coefficients ( $r$ ) and  $p$  values are from Spearman rank analysis.

### Immunization

Four weeks prior to vaccination, female *Macaca mulatta* monkeys of Chinese origin were preimmunized with inactivated influenza (A/Singapore/6/86) strain, propagated in the allantoic cavity of embryonated eggs obtained from Berna Biotech AG and purified as described previously (Amacker et al., 2005) to mimic the situation in human usually immune to influenza. Monkeys were then subdivided into three groups of six animals each. Each animal received four vaccine injections, at weeks 0, 7, 15, and 23. The placebo control group (group 1) received intramuscular injections of IRIV (virosome alone; no HIV-1 antigen). Groups 2 and 3 received vaccine doses containing each 40  $\mu$ g of P1 peptide and 40  $\mu$ g of rgp41. Group 2 received only intramuscular (i.m.) injections, whereas group 3 received two i.m. injections at weeks 0 and 7 and then two intranasal (i.n.) injections at weeks 15 and 23. For intramuscular

injections, each dose (100  $\mu$ l volume) was administered in one site (upper leg), whereas intranasal administrations were equally distributed between the two nostrils (~50  $\mu$ l per nostril) with a specific spray device (BD Medical Devices).

Animals were maintained under guidelines established by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals, with protocols approved by the local ethical committee.

### Samples Harvesting

Blood, vaginal, and rectal washes were performed on each animal at week -4 and 0 for preimmune samples and 1 week after each vaccination event for immune samples (weeks 1, 8, 16, and 24). Cervico-vaginal and rectal secretions were obtained by gentle lavages at indicated times with 3 ml of cold saline buffer, then added with antibiotics and protease inhibitors and

centrifuged. Resulting supernatants were immediately aliquoted and snap frozen in liquid nitrogen before storing at  $-80^{\circ}\text{C}$  (Tudor et al., 2009).

### ELISA

Total antibodies were measured by standard sandwich ELISA, whereas P1- or rgp41-specific antibodies were evaluated by P1- or rgp41-coated direct ELISA (Coutant et al., 2008; Tudor et al., 2009) with monkey specific-IgAs or IgGs coupled to HRP (Rockland) as secondary antibodies.

### Challenges

SHIV-SF162P3 (R5 clade B) was kindly provided by N. Miller, NIAID (NIH AIDS Research and reference reagent program). The genome of this chimeric simian-human immunodeficiency virus contains the *env* (gp120 + gp41), *tat*, *rev*, and *vpu* genes from HIV-1 SF<sub>162</sub> (Harouse et al., 2001) inserted into the genome of the pathogenic SIV<sub>mac239</sub>. One month after vaccination, all animals were challenged intravaginally 13 times with 2 ml of a phosphate-buffer viral solution containing 20 TCID<sub>50</sub> of the heterologous SHIV-SF162P3 for the first 7 challenges and 30 TCID<sub>50</sub> for the 6 last challenges. Challenges were done every 4–7 days.

### Viremia

Blood samples were drawn over 6 months, twice a week for the first 2 weeks, once a week for the subsequent weeks, and then once every 2 weeks, and analyzed for plasma viral loads (viremia) with the QIAGEN QuantiTect SYBR-Green RT-PCR kit with specific SIV gag probes (sensitivity 1000 copies/mL blood), then repeated blindly as described (Li et al., 2009) with a more sensitive technique. In brief, viral RNA from 0.2 ml of EDTA-anti-coagulated cell-free plasma was directly extracted by a MagNa Pure LC robotic workstation (Roche Molecular Biochemicals). A one-step reverse transcriptase PCR (RT-PCR) method using the TaqMan EZ RT-PCR CORE REAGENT kit was performed for quantification of SHIV viral RNA. Standard curves were prepared with a series of six 10-fold dilutions of viral RNA of known concentration. The sensitivity of the assay theoretically was 100 RNA equivalents per mL, but because of volume issues, it was fixed at 200 copies/mL. Samples were analyzed in triplicate and the number of RNA equivalents was calculated per milliliter of plasma.

### P27 Antibody Detection

Western blots with serum (diluted 1/100 for IgA, 1/200 for IgG) and cervico-vaginal secretions (diluted 1/6) were performed in accordance with the manufacturer's instruction (New Lav Blot I from Bio Rad) as described (Tudor et al., 2009) but with a monkey-specific secondary antibody coupled to HRP (Rockland).

### Functional In Vitro Assays

Inhibition of HIV-1 transcytosis (Alfsen et al., 2001; Tudor et al., 2009) and TZM-bl (Pastori et al., 2008) (Bomsel et al., 2007), SOS (Pastori et al., 2008) (Bomsel et al., 2007), and CD4<sup>+</sup> T cell neutralization assays (Alfsen et al., 2001; Tudor et al., 2009) were performed as previously described. For the CD4<sup>+</sup> T cell neutralization assays using the JR-CSF, R5 tropic HIV-1 molecular clone, specific neutralization was expressed as follows:

Specific neutralization =  $100 - 100 \times (\text{Infection in presence of secretion collected at week 24} / \text{Infection in presence of secretion collected at week 0})$

For the transcytosis assays, clade B (93BR029) and C (92BR025) HIV-1-infected PBMCs were used to inoculated HEC-1 endometrial cell lines cultured in a polarized manner in a two-chamber system as described (Alfsen et al., 2001; Tudor et al., 2009). CVS were tested at dilutions 1:6 and 1:12 and the observed transcytosis-blockade was directly dependent on CVS concentration (not shown). 2F5 IgA (1  $\mu\text{g/mL}$ ) (Shen et al., 2010) was used as positive control and inhibited transcytosis by 95%.

Where indicated, cervico-vaginal secretions were depleted of IgAs or IgGs by immunodepletion. In brief, biotinylated-anti-human IgA or biotinylated-anti-human IgG (Caltag) were bound to Streptavidin-Agarose (Pierce), 10  $\mu\text{g}$ /30  $\mu\text{L}$  of beads. Beads coupled with anti-human IgA or IgG antibody were washed 3 times to removed unbound biotinylated anti-IgA or anti-IgG, after

which 30  $\mu\text{L}$  of anti-IgA- or anti-IgG-bound beads were incubated overnight at  $4^{\circ}\text{C}$  on a rotating wheel with CVS (1/6 dilution). The mixture was centrifuged for 10 min at 10 000 g at  $4^{\circ}\text{C}$  and the resulting supernatant represented the IgA- or IgG-depleted CVS fraction. Transcytosis activity is expressed as follows: transcytosis inhibition =  $100 - 100 \times (\text{transcytosis in presence of secretion collected at week 24} / \text{transcytosis in presence of secretion collected at week 0})$ .

For the TZM-bl assays (Tudor et al., 2009), three different HIV-1 strains were tested: SF162, the primary clade B QH0692, and the primary clade C DU172. Virus neutralization was evaluated with the following positive controls: 2F5 IgG, which gave a 50% inhibitory concentration (IC<sub>50</sub>) of 3  $\mu\text{g/mL}$  (range: 5–2  $\mu\text{g/mL}$ ), and an IC90 of 50  $\mu\text{g/mL}$  (range 67–34  $\mu\text{g/mL}$ ); the TRIMAB gave an IC<sub>50</sub> of 0.9  $\mu\text{g/mL}$  (range: 0.7–1.7  $\mu\text{g/mL}$ ) and an IC90 of 5  $\mu\text{g/mL}$  (range: 4–6  $\mu\text{g/mL}$ ).

For the SOS assays (Bomsel et al., 2007) evaluated on U87.CCR5.CD4 target cells, the positive controls were 2F5 IgG with an IC<sub>50</sub> of 2.2  $\mu\text{g/mL}$  (range 3.5–1.7  $\mu\text{g/mL}$ ) and IC90 of 21  $\mu\text{g/mL}$  (range: 18–32  $\mu\text{g/mL}$ ).

Of note, SF162 and JR-CSF envelopes have 88% sequence homology, with the differences located in the variable sequences of gp120 but not in gp41; therefore, they are very similar in terms of gp41 sequence.

ADCC assay was performed as described (Xiao et al., 2010), with THP1 monocytic cells as effector cells and CCR5<sup>+</sup>-NK resistant CEM lymphoid cells (NIH AIDS reagents program) coated with either P1 or recombinant X4 tropic gp160 as target cells. In brief,  $1 \times 10^6$  CEM-NKR-CCR5 target cells were incubated with 0.2  $\mu\text{M}$  of gp160 (MN/LAI Pateur-Merieux) or 5  $\mu\text{M}$  of P1 peptide for 1 hr at room temperature in a total volume of 300  $\mu\text{L}$  of RPMI. Coated cells were dually labeled with the membrane dye PKH-26 (Sigma-Aldrich) and the vital dye CFSE (Molecular Probes, Invitrogen), then incubated for 30 min at room temperature with the samples (3-fold dilutions) or the control antibodies, 2F5 IgG for P1-specific ADCC and 98.6 IgG for rgp41-specific ADCC, respectively, in a 96-well microtiter plate. Effector THP1 cells were then added at a relatively low effector/target ratio of 10:1 (Xiao et al., 2010). The reaction mixture was incubated for 4 hr at  $37^{\circ}\text{C}$  in 5% CO<sub>2</sub> after which the cells were immediately analyzed with a Becton Dickinson FACSCalibur flow cytometer. Data analysis was performed with Cytomix RXP software. We determined the percentage of ADCC cell killing by back-gating on the PKH-26<sup>high</sup> population of target cells that lost the CFSE viability dye. For each gp41 subunit, a specific ADCC was expressed as percent ADCC in the presence of sample collected at week 24 subtracted by the percentage of ADCC in the presence of preimmune sample. ADCC titers were defined as the reciprocal dilution at which the percent ADCC killing was greater than the mean percent killing of the negative controls plus three standard deviations. Data represent the mean of at least two independent experiments. The positive control for P1-specific ADCC, 2F5 IgG at 1  $\mu\text{g/mL}$  resulted in 40% specific cell lysis, and that for rgp41-specific ADCC, 98.6 IgG at 100 ng/mL resulted in 60% specific cell lysis. Both signals were entirely reversed by a 10-fold excess of non-specific IgG (not shown), indicating that the ADCC measured was IgG-dependent. Furthermore, effector cells did not bind IgA or expressed the CD89 Fc $\alpha$  receptor (not shown).

### Antibody Purification

Antibodies contained in preimmune and immune cervico-vaginal secretions were purified on a protein G (IgG) or anion-exchange (IgA) column with an automatic HPLC system. Eluted fractions in PBS were evaluated by ELISA using goat anti-human IgG, IgA, or IgM as described (Donadoni et al., 2010).

### MHC Genotyping

It was performed by sequence-specific PCR as described (Loffredo et al., 2009).

### Statistical Analysis

For comparing functional activities of cervico-vaginal secretions among monkey groups, the nonparametric Kruskal-Wallis test was used. Pairwise comparisons were performed by the nonparametric Mann-Whitney U test. The Spearman rank correlation test was used for assessing the relationship between viral load and transcytosis-blockade, ADCC, and neutralization, respectively. Statistical analyses were carried out with Instat GraphPad Software, version 5.0.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2011.01.015.

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