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Reproducing SIV nef vaccine correlates of protection: trimeric gp41 antibody concentrated at mucosal front lines

James E. Voss^a, Matthew S. Macauley^b, Kenneth A. Rogers^c, Francois Villinger^c, Lijie Duan^d, Liang Shang^d, Elizabeth A. Fink^e, Raiees Andrabi^a, Arnaud D. Colantonio^f, James E. Robinson^g, R. Paul Johnson^{h,i}, Dennis R. Burton^{a,j}, and Ashley T. Haase^d

^aDepartment of Immunology and Microbial Science, IAVI Neutralizing Antibody Center, and Center for HIV/AIDS Vaccine Immunology and Immunogen Discovery, The Scripps Research Institute, La Jolla, CA 92037

^bDepartment of Chemical Physiology, The Scripps Research Institute, La Jolla, CA 92037

^cDepartment of Pathology and Laboratory Medicine and Emory Vaccine Center, Emory University, Atlanta, GA 30329; Current Address: New Iberia Research Center, University of Louisiana at Lafayette, New Iberia, LA 70560

^dDepartment of Microbiology and Immunology, Medical School, University of Minnesota, Minneapolis, MN 55455

^eProtein Expression and Proteomics Core of the Center for AIDS Research, University of California San Diego, La Jolla, CA 92093-0716

^fNew England Primate Research Center, Harvard Medical School, Southborough, MA, 01772

^gDepartment of Pediatrics, Tulane University Health Sciences Center, New Orleans, LA 70112

^hInfectious Disease Unit, Department of Medicine, Massachusetts General Hospital, Boston, MA 02115

ⁱYerkes National Primate Research Center, Emory University, Atlanta, GA 30329

^jRagon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard, Cambridge, MA 02139

Abstract

Vaccination with SIV_{mac239} nef provides robust protection against subsequent challenge with wild type SIV, but safety issues have precluded designing an HIV-1 vaccine based on a live attenuated virus concept. Safe immunogens and adjuvants that could reproduce identified immune correlates of SIV_{mac239} nef protection therefore offer an alternative path for development of an HIV vaccine. Here we describe SIV envelope trimeric gp41 (gp41t) immunogens based on a protective correlate of antibodies to gp41t concentrated on the path of virus entry by the neonatal Fc receptor (FcRn) in cervical vaginal epithelium. We developed a gp41t immunogen-MPLA

Correspondence to Ashley T. Haase, M.D., Department of Microbiology and Immunology, University of Minnesota, 689-23rd Avenue S.E., Campus Delivery Code 2821A, Minneapolis, MN 55455-1507, USA. Tel: +1 612-624-4442; fax: +1 612-626-0623; haase001@umn.edu.

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adjuvant liposomal nanoparticle for intra-muscular immunization and a gp41t-Fc immunogen for intranasal immunization for pilot studies in mice, rabbits, and rhesus macaques. Repeated immunizations to mimic persistent antigen exposure in infection elicited gp41t antibodies in rhesus macaques that were detectable in FcRn+ cervical vaginal epithelium, thus recapitulating one key feature of SIV_{mac239} nef vaccinated and protected animals. While this strategy did not reproduce the system of local production of antibody in SIV_{mac239} nef-vaccinated animals, passive immunization experiments supported the concept that sufficiently high levels of antibody can be concentrated by the FcRn at mucosal frontlines, thus setting the stage for assessing protection against vaginal challenge by gp41t immunization.

Keywords

SIV Vaccine; trimeric gp41 antibodies; mucosal concentration; SIV_{mac239} nef

Introduction

In the quest for an effective HIV-1 vaccine, particularly to prevent transmission to the young women who bear the brunt of infection in the pandemic's epicenter in Africa [1,2], we have been seeking design principles to guide vaccine development by identifying correlates of the robust protection afforded by the live attenuated SIV_{mac239} nef vaccine in the rhesus macaque model of HIV transmission to women [3–8]. With those principles in hand, we could then design alternatives to circumvent the safety issues associated with SIV_{mac239} nef vaccination [9, 10].

We recently found that persistent SIV_{mac239} nef infection induced collections of plasma cells producing antibodies (Abs) to trimeric gp41 (gp41t) positioned beneath FcRn - expressing cervical and vaginal epithelium. This organized mucosal epithelial immune system concentrates Abs on the path of virus entry on subsequent vaginal challenge with wild type SIV, and thus could thereby account for the observed constraints on establishment of founder populations of infected cells at the portal of entry [8]. Here we describe the design of immunogens and vaccine strategies that mimic this protective correlate of gp41t-Abs concentrated at mucosal frontlines without the immune response to local viral replication, thus potentially providing a safer approach to live attenuated vaccination.

Materials and methods

Trimeric gp41 (gp41t) liposomal nanoparticle immunogen

The anti-gp41t Abs induced by SIV_{mac239} nef vaccination are likely elicited by conformational, immunodominant cluster I and II epitopes presented on gp41 trimer stumps [11–13]. We generated an immunogen that mimicked these epitopes that could be displayed at high density on liposomes into which the lipophilic TLR-based adjuvant, MPLA, could be incorporated [14, 15]. The recombinant gp41 ectodomain shown schematically in Fig. 1 was expressed in 293F cells from a pHCMV plasmid (Genlantis) that included gp160 residues 554–676 (SIV_{mac239} numbering), flanked N-terminally by an IgK signal sequence to target the protein to the ER for glycosylation, disulfide bond formation, and secretion; and C-

terminally, by a strep tag for affinity purification from Freestyle 293 media (Gibco) using StrepTactin resin (IBA). Avidin was added to the filtered, concentrated media to block biotin before loading onto the column. The column was washed with PBS, and protein eluted with 2.5mM desthiobiotin in PBS. The eluent was concentrated and loaded on a Superdex200 column (GE healthcare) to purify a 66KDa protein from higher molecular weight aggregates (approx. 1mg from 2L). The predicted 16 KDa recombinant purified protein migrated at 22 KDa by SDS-PAGE under reducing conditions (Fig. 1C), which is consistent with 3 predicted sites of glycosylation (2KDa per glycan), and eluted at 66KDa by size exclusion chromatography (Fig. 1C). This size is consistent with a trimeric quaternary structure, likely the 6-helix bundle as previously solved by X-ray crystallography and NMR [16] shown in Fig. 1B.

Stealth liposomes displaying gp41t were prepared as described previously [17] by first linking the gp41t trimer to pegylated DSPE using maleimide chemistry (Fig. 2A). The trimeric protein was reacted with 2.5 molar equivalents of *N*-succinimidy 3-(pyridyldithio)-propionate (SDPD; Pierce) and the disulfide bonds in the gp41t trimeric structure were preserved by deprotecting the 2-pyridyldithio group with DTT at pH 5.5 in sodium acetate. On average, 1.25 molar equivalents of thiol were introduced per trimer, therefore, on average each trimer had only one attached lipid. Following coupling of the thiol-modified gp41t with Mal-PEG₂₀₀₀-DSPE (NOF America) and column chromatography to remove any unreacted protein, lipid-modified gp41t was used to hydrate dried lipids to produce a final mol ratio of 60:35:4.9:0.1 of distearoyl phosphatidylcholine (DSPC) (Avanti Polar Lipids), cholesterol (Sigma-Aldrich), PEG₂₀₀₀-DSPE, and gp41t-PEG-DSPE (Fig. 2B). When MPLA was included, 5 mol% DSPC was replaced by 5 mol% MPLA (Avanti). Lipids were hydrated in PBS containing the gp41t-PEG-DSPE to achieve a total lipid concentration of 5 mM. Following rigorous sonication, liposomes were extruded using a hand extruder (Avanti) through an 800, 200, and finally 100 nm filters.

Trimeric gp41-Fc fusion protein [18]

Three copies of the gp41t described above were linked in tandem and fused at the C-terminus to the Fc domain of the rhesus macaque IgG1 K322A mutant, flanked N-terminally by an IgK signal sequence to target the protein to the ER for glycosylation, disulfide bond formation, and secretion; and C-terminally, by a strep tag. The protein was purified in the same way as gp41t using StrepTactin affinity and size exclusion chromatography (Fig. 1B, C).

Animals and vaccination

In the initial assessment of the liposomal gp41t-MPLA immunogen/adjuvant, four 8-week old female BALB/C mice were primed and boosted at 3 weeks with 10 µg of gp41t incorporated into liposomes with or without 22.5µg MPLA in 200µl PBS by intravenous administration via the tail vein. Ocular bleeds were taken before immunization and 1-week after boosting. Four female 12-week old New Zealand White Rabbits were primed and boosted with 100 µg of gp41t liposomes with 225 µg of MPLA in 600 µl PBS (300 µl intramuscular injection into each hind leg). Blood was drawn before immunization and 1 week after. A boost was given 4 weeks post prime and blood was drawn one week later. All

procedures were performed in anesthetized animals. The animals were cared for in conformance to the guidelines of the Committee on the Care and Use of Laboratory Animals ((U.S.), 1996). All experimental protocols and procedures were reviewed and approved by The Scripps Research Institute Department of Animal Resources.

For the pilot experiment to evaluate the immunogens and adjuvants in the rhesus macaque animal model of HIV-1 transmission to women, four adult female rhesus monkeys of Indian origin (*Macaca mulatta*) were obtained from the Yerkes National Primate Center colony and housed in pairs. Monkeys were immunized under anesthesia following 2 distinct protocols: Two monkeys (RTe8 and ROz6) received 100 µg soluble gp41 trimers presented by liposomes (sgp41t-stealth liposomes) containing MPLA, along with 500 µg CpG ODNs (TCGTCGTTTTGTCGTTTTGCTGTT, Trilink Biotechnologies, San Diego, CA) in 1 ml and 1 ml Emulsigen-D (MVP Laboratories, Omaha NE) divided equally between the right and left leg quadriceps (i.m.) on day 0, and months 1, 3 and 6 (Rte8), at which point RTe8 had to be euthanized because of chronic diarrhea and associated weight loss that exceeded IACUC approved limits. Necropsy findings were normal and the diarrhea was attributed to stress and not to immunization. ROz6 received additional immunizations at months 9 and 12. As described under results, we also generated a gp41t-macaque IgG1 Fc fusion protein as an immunogen for intranasal administration to target the female reproductive tract (FRT) as described for HSV-2 studies [18]. The other two monkeys, RBq-6 and RDj-11, initially received 100 µg sgp41t-Fc with 3 mg MPLA and 500 µg CpG ODNs in 400 µl, dispensed drop-wise into both nares on day 0 and months 1, 3, 6, 9 and 12. Because of low titers in response to intranasal immunization alone, these 2 monkeys additionally received the same i.m. immunizations as ROz-6 and RTe-8 on months 3, 6, 9 and 13. Serum and plasma cells were collected over the course of the experiment and cervical vaginal swabs were also taken. Plasma was heat inactivated for 1 hour at 56 degrees and filtered for ELISA and SIV western blot analysis. The animals were cared for in conformance to the guidelines of the Committee on the Care and Use of Laboratory Animals ((U.S.), 1996). All experimental protocols and procedures were reviewed and approved by the Emory Institutional Animal Care and Use Committee.

Monoclonal antibody 4.9C production and passive immunization

Rhesus MAb 4.9C was originally produced by a Rhesus B cell line derived from jejunal lamina propria lymphocytes of a monkey infected by low dose, intra-colonic administration of SIV 17E-Fr [19], as previously described [20]. MAb 4.9C was found to react identically with oligomeric gp41 in Western blots to antibodies to gp41 elicited by SIV_{mac239} nef vaccination [8]. Variable heavy chain and kappa chain genes of MAb 4.9C were RT-PCR amplified from RNA extracted from the 4.9C B cell line and cloned into expression vectors for human IgG1. In order to produce authentic rhesus IgG1, we replaced the human heavy chain constant region with the 4.9C rhesus IgG1 constant region by first generating a rhesus constant region DNA fragment from 4.9C cellular RNA. Using forward and reverse primers to create 15–18 nt terminal homologies with the ends of rhesus IgG1 constant region DNA fragment and amplify all of the 4.9C human heavy chain plasmid except for the human IgG1 constant region, the product was inserted into the linearized vector with the use of the In-Fusion reaction as described [21]. To prepare large quantities of MAb 4.9C needed for

passive infusion experiments, 4.9C heavy and light chain expression plasmids were transfected into 293T cells. MAb was purified from culture fluids by protein A chromatography and MAb was concentrated by ultrafiltration. To remove endotoxin from the final product, MAb 4.9C was captured on protein A sepharose columns that were then washed with PBS containing 1 mg/ml polymyxin B (Sigma Cat #P4932) and then 1% sodium deoxycholate (Sigma cat # D5670) and endotoxin free PBS to remove residual polymyxin and sodium deoxycholate. A second batch of MAb 4.9C was prepared at Mass Biologics by cloning the DNA coding for the heavy and light chains of the rhesus IgG1 into a proprietary expression vector and electroporated into Chinese hamster ovary (CHO) cells. A high expressing, oligoclonal pool of transduced CHO was selected and used to express 4.9C in serum-free, chemically defined medium. Antibody was purified by protein A affinity chromatography and formulated in sodium citrate buffer, pH 6. Endotoxin level was <0.1 EU/mg. MAb 4.9C at 25 or 50 mg/kg was administered iv and sera and cervical vaginal lavage (CVL) fluids were collected 1, 6 and 24 hours post injection. Mab 4.9C concentrations were assessed by ELISA and SIV western blot by the same methods described for the active immunization experiment.

Tissue collection and processing

Animals were euthanized 14 days after the last boost. At the time of euthanasia, tissues were collected and fixed in 4% paraformaldehyde or SafeFix II and embedded in paraffin for later sectioning and analysis, as previously described [8].

ELISA assays

Total IgG (IgGT) and sgp41t specific IgG titers were assessed in serum and cervical vaginal lavage fluid (CVF) by ELISA. 50 µl/well of 1 µg/ml unconjugated anti-mouse/rabbit/human IgG F(ab')₂ (Jackson ImmunoResearch) or sgp41t in PBS was added to 96-well high bind micro-titer plates (Corning) and incubated overnight at 4°C. (Anti-human antibodies cross react well with those of rhesus macaque). Plates were washed (3× 100 µl PBS-0.01% tween20) and blocked for 1 hour at room temperature in 100 µl/well of 3%BSA in PBS. Plates were washed and 50 µl/well of sample dilutions added. In general, starting dilutions for serum were around 50× or 5000× for sgp41t specific or IgG total ELISAs respectively. For CVL, starting dilutions were 5 and 50× for sgp41t-specific and IgGT-ELISAs respectively with some further optimization required. Samples were diluted with PBS-0.01% tween20 + 1% BSA. Titration curves were created by diluting duplicate samples 6× down each column of the 96-well plate. Plates were incubated 2 hours at room temperature, and then washed. For urea washes to remove low affinity antibodies, 50 µl/well of 6M urea in water was added for 10 minutes before washing the plates. HRP-conjugated anti-mouse/rabbit/or human IgG fc fragment specific antibody (Jackson ImmunoResearch) was diluted 1:15000 in PBS-0.01% tween20 + 1%BSA and 50 µl/well was added to plates. Plates were incubated for 1 hour at room temperature, washed and developed in 50 µl/well of TMB substrate (Thermo Scientific Pierce). The reaction was stopped with 50 µl/well of 1M sulfuric acid, and the absorbance at 450nm was recorded for each well. Standard IgG curves were made for macaque samples using Protein A/G purified IgG from rhesus macaque serum. EC₅₀ values were calculated from non-linear regression analysis of the absorbance values using Prism. Antigen capture ELISAs were attempted in order to exclude the

possibility that the above direct capture format obscures a detectable ELISA correlate between early (unprotected) and later (protected) time points as is observed by western blot, as described under results. First, 50µl of 2µg/ml anti-strep tag monoclonal antibody (2-1507-001 IBA) in PBS was added to wells and incubated O/N at 4°C. Liquid was removed and wells were blocked with 100l 3% BSA or 5% milk for 1 hour. Wells were washed 3× with PBS-T before adding 50µl of the strep-tagged sgp41t at 2µg/ml in PBS-T and incubated for 2 hours at room temperature. Wells were washed with PBS-T 3× before proceeding with the serum dilution incubations as described above. This ELISA gave a poor signal to background ratio and so a similar ELISA was attempted using an anti-HIS tag mAb (MA1-21315 Thermo Fisher) and a HIS-tagged version of the sgp41t protein where 8× HIS replaced the strep tag. As observed with direct coating, antigen-capture offered no observable titer correlate between protected and unprotected serums. Purification of the His-tagged sgp41 was similar to the streptactin purification protocol except Ni-NTA was used as purification resin. 10mM imidazole (pH 7.5) was added to supernatant and loaded on column. The column was washed with PBS+20mM imidazole and protein was eluted with 300mM imidazole pH7.5 100mM NaCl.

Neutralization assays

Neutralization assays were performed as previously described [8, 22, 23]. In brief, protease inhibitors and the non-denaturing detergents in the tissue extracts were removed by ultrafiltration and replaced by RPMI 1640 containing 10% normal rhesus sera. Tissue extracts and sera were serially diluted (10-fold) in RPMI 1640 containing 10% normal rhesus sera, incubated with TCLA-SIV_{mac251}, SIV_{mac251 32H} or SIV_{mac251} vaginal challenge stock before the addition of C8166-45-SEAP reporter cells (M.O.I.=0.05) and subsequent measurement of Secreted Alkaline Phosphatase (SEAP) activities.

Isolation of antibodies from tissues and cervical vaginal fluids and Western Blot analysis

Isolation of antibodies from tissues and cervical vaginal fluid, and Western blot analyses followed previously published protocols [8].

Reverse immunohistochemistry (RIHC)

Reverse immunohistochemistry staining to detect gp41-specific antibody in cells was performed as previously described [8].

Results

Gp41t-immunogen design

We previously showed that Abs to gp41t concentrated by the FcRn in cervical reserve and vaginal epithelium was one correlate of protection conferred by SIV_{mac239} nef vaccination [8]. We sought to reproduce this correlate with immunogens and adjuvants rather than live attenuated SIV infection, and chose a recombinant soluble gp41trimer (sgp41t) as the immunogen for several reasons. First, sgp41t had been previously shown to specifically bind to antibodies directed against gp41t that were produced in response to the SIV_{mac239} nef vaccine [8] and thus might be expected to induce antibodies similar to those elicited by vaccination. Second, gp160 residues 554–676 (SIV_{mac239} numbering; Figs. 1A, B) represent

broadly conserved epitopes in SIV, and in HIV clades as well, and therefore the potential for heterologous binding of vaccine-induced antibodies across virus clades. Third, expressing the gp41 ectodomain as a secreted protein product, lacking the fusion peptide and truncated just before the MPER with 9 N-linked glycosylated sites per trimer (Fig. 1B), should enhance solubility, and the exclusion of the MPER should, in principle, enable us to test the role of non-neutralizing antibodies in protection. Fourth, the predicted structure of our sgp41t, shown in Figure 1B, is a 6-helix bundle that should present trimeric conformationally relevant cluster I and II epitopes that are critical for mimicking the immunodominant epitopes on viral gp41 stumps. In particular, because cluster I loops are juxtaposed so closely in the trimer, the quaternary structure of the protein favors interactions with residues on more than one cluster I peptide to elicit conformational antibodies with reactivity with oligomeric gp41 observed in Western blots [8, 25, 26]. For intra-nasal immunization, we also generated a gp41t macaque IgG1 Fc-fusion protein for transport to antigen presenting cells (APCs) (Fig. 1B) with a K322A mutation to disrupt C1q binding and inflammatory responses, based on the rationale described for HSV-2 to elicit protective antibody responses in the murine FRT [18].

The sgp41t reagent was expressed and purified as described in the methods section and displayed on the surface of 80–100 nm liposomes for optimal antigen presentation and immune activation [14, 15]. MPLA was incorporated into the liposomes to further enhance immune cell activation. Liposomes displaying sgp41t as unilaminar liposomal nanoparticles (Fig. 2B) were prepared to generate an immunogen with 5–10 nm between antigens, optimal spacing for crosslinking of the B cell receptor to induce robust B cell activation [27]. We estimated that 0.1 mol% sgp41t would incorporate about 80 molecules of sgp41t per liposome into liposomes to approximate this optimal spacing. Following extrusion, the average diameter of liposomes displaying sgp41t was measured at 90 $\pm$ 28 nm, moderately larger than control sham liposomes that did not contain sgp41t (Fig. 2C). Monophosphoryl Lipid A (MPLA) was stably incorporated into the lipid bilayer to serve as a safe TLR4-based adjuvant [28].

Antibodies elicited by sgp41t-MPLA-liposomes in mice and rabbits

The gp41t-liposomes alone were potent immunogens in mice and rabbits, and inclusion of MPLA further increased binding Ab titers in mice by about 10-fold (Fig. 3A). The elicited Abs also stained the prominent gp160 oligomeric gp41t band in WBs in a similar pattern to that previously observed [8] in SIV_{mac239} nef vaccinated animals (Fig. 3B).

Gp41t Abs concentrated in the rhesus macaque FRT

With these encouraging results in hand, we undertook a pilot experiment in rhesus macaques to determine if we could reproduce concentration of anti-gp41t antibodies at the mucosal interface. We focused the strategy and assessment of the immunogens and adjuvants on showing that anti-gp41t antibodies could be induced and sustained at sufficiently high levels to be concentrated in the female reproductive tract (FRT) in a way that mimicked SIV_{mac239} nef vaccination. The principal components of our strategy (Fig. 4A) were: 1) prime and repeatedly boost animals over an extended period to mimic the persistent antigenic stimulus in animals infected with SIV_{mac239} nef; 2) combine conventional

intramuscular (i.m.) inoculations with intra-nasal (i.n.) immunization with the gp41t-Fc construct that could potentially induce antibodies localized to the FRT; use a TLR9 agonist and CpG adjuvant, with the rationale that TLR9 agonists have been shown to induce strong B cell responses in other settings [29] and have been used safely in rhesus macaques [30].

The combination of gp41t liposomes with MPLA and CpG adjuvants stimulated production of Abs in high titers in serum in rhesus macaques that recognized gp41t (Fig. 4B). The i.n. immunization with gp41t-Fc initially failed to induce detectable Abs reactive to gp41t, but later boosts, which included i.m. administered gp41 liposomes along with i.n. gp41-Fc, induced high titers in serum. Titers plateaued after the second boost with the i.m. immunization route (Fig. 4B), and, in one animal euthanized after the third boost for stress-related chronic diarrhea and associated weight loss, high titers of binding Abs achieved after the third boost were equivalent to titers in the remaining three animals after two more boosts. None of the sera neutralized either TCLA or challenge virus above background inhibition observed in baseline sera (Supplemental Fig. 1).

The serum titers assessed 14 days post boost 5 were comparable to SIV_{mac239} nef-vaccinated animals at 20 weeks after vaccination. At this time point, animals are significantly protected against challenge in contrast to 5 weeks when they are not protected, reflecting the maturation of protection [8, 31]. However, we found that the gp41t ELISA assays of sera did not reproduce this maturation of protection, since the SIV_{mac239} nef-immunized 20-week and 5-week sera were indistinguishable (Fig. 4B). By contrast, Abs reacting with previously identified [8] oligomeric forms of gp41 (running at 160 and 80 KDa on western blotting strips) were detectable after the third boost and continued to increase with boosts four and five, and paralleled the increases at 20 weeks compared to 5 weeks in SIV_{mac239} nef-vaccinated animals (Fig. 4B). We investigated a variety of conditions for the ELISA assay (see Methods and Supplemental Figure 2) that did not affect the discrepancy between the assays and the important conclusion from this analysis is that it is important to assess the potential efficacy of vaccination from the Western blots and not ELISA titers.

Even more encouraging, Abs to oligomeric gp41t were detectable in WBs of proteins extracted from cervical vaginal tissues collected at necropsy (Fig. 4B, far right panel), and by RIHC, the three animals that received a prime and five boosts had comparable staining of FcRn⁺-cervical reserve epithelium to animals vaccinated with SIV_{mac239} nef at the 20-week time point (Fig. 4B, representative image compared with SIV-negative control).

We also examined CVL and colorectal lavage (CRL) collected with wicks, but the assays were confounded by significant difficulties in the variability of collection and contamination with blood in menses. While gp41 was consistently detected in gp41 vaccinated CVL and colorectal washes by ELISA, the actual titers were widely variable as were total IgG levels (not shown).

Differences between immunization and SIV_{mac239} nef vaccination

The concentration of gp41t antibodies particularly in the vagina in SIV_{mac239} nef vaccinated animals has been shown to be associated with a system of local production by plasma cells and ectopic follicles, presumably reflecting persistent systemic infection and

low levels of antigen at mucosal sites [8]. While we tried to mimic persistent antigen exposure with repeated boosts with gp41t-liposomes, we did not directly expose FRT tissues to antigen. It is thus perhaps not surprising that in the immunized animals we did not see induction of ectopic follicles or vaginal epithelial CXCL10 expression to recruit CXCR3+ plasma cells to the vaginal mucosa (not shown) as we had previously documented in SIV_{mac239} nef vaccinated animals despite immunization of the nasal mucosa with our gp41-Fc construct.

Passive immunization pilot and systemic delivery hypothesis

While immunization did not reproduce the system of local antibody production in SIV_{mac239} nef vaccinated animals, it nonetheless did achieve high levels of antibodies in the FRT tissues and concentrated in FcRn+ cervical vaginal epithelium by RIHC (Fig. 4C). This result is consistent with the idea that sustained high levels of antibody in the circulation can be concentrated in the epithelium by the FcRn. There were only sufficient resources in this pilot study to conduct a preliminary test of this hypothesis by passively immunizing with either a dose of 25 mg/kg of a rhesus monoclonal antibody (4.9C), which in Western blots reacts identically with oligomeric gp41 to sera from SIV_{mac239} nef vaccinated animals [8]; or a 2-fold higher dose of 50 mg/kg. The levels of anti-gp41t IgG levels in the circulation were comparable in ELISA and WB assays for the 25 and 50mg/kg doses (Fig. 5A), but levels in the CVL were slightly higher at the higher dose, and reactivity with oligomeric gp41t in WB was only detected in cervical vaginal tissue extracts at 50mg/kg, albeit at about 10-fold lower levels than at 20 weeks after SIV_{mac239} nef vaccination and animals immunized with gp41t-liposomes (Fig. 5B, right panels). Importantly, the cervical reserve epithelium was only detectably stained by RIHC for gp41t antibodies at the 50mg/kg dose (Fig. 5C).

Discussion

Desrosiers and colleagues first showed that i.v. inoculation of SIV_{mac239} nef SIV resulted in attenuated infection that subsequently provided protection against WT viral challenge [3]. The superior protection against WT virus challenge by parenteral and mucosal routes in subsequent studies compared to other approaches was impressive [4–7], but the safety issues documented soon thereafter with this approach [9, 10] precluded advancing this concept to development of an effective HIV vaccine.

Nonetheless, protection conferred by live attenuated SIV vaccines clearly provided a rationale to identify correlates of protection to guide HIV-1 vaccine design. We recently discovered one correlate of the maturation of significant protection manifest at 15–20 weeks post-vaccination but not at 5 weeks, in the local production of antibodies to gp41t that are concentrated on the path of virus entry by the neonatal Fc receptor in cervical reserve epithelium and basal vaginal epithelium [8]. Here we show in a pilot experiment that repetitive immunization with liposomal nanoparticles bearing gp41t and adjuvants can generate sufficiently high levels of antibodies to be detectable in FRT by WB assays of cervical vaginal tissue extracts and RIHC-staining for gp41t-antibodies in FcRn+ cervical epithelium.

Reproducing key features of live attenuated vaccination correlates of protection with immunogens and adjuvants by a strategy analogous to persistent infection and antigen exposure is encouraging. However, this strategy did not result in recruitment of plasma cells and induction of ectopic follicles that create the system of local antibody production previously documented in SIV_{mac239} *nef* vaccinated animals [8]. The passive immunization experiment is consistent with the hypothesis that sufficient IgG must be delivered to the FRT to result in detectable antibody in FcRn+ epithelium, presumably reflecting saturation of the receptor for detection by RIHC of antibodies in the cells. Immunization with gp41t-liposomal nanoparticles did generate antibody levels in FRT tissues higher than passive immunization with 50 mg/kg of a gp41t antibody and comparable to SIV_{mac239} *nef* vaccinated animals at 20 weeks, where about half of the animals have sterilizing immunity against high dose vaginal challenge. Future studies must now be carried out to determine if these levels of anti-gp41 antibodies correlate with protection. If not, we speculate that a next logical step in exploring ways to reproduce the protection afforded by SIV_{mac239} *nef* vaccination would be a systemic immunization strategy, combined with a “pull” with local antigen exposure to try to generate a local antibody production system that would provide protection, as has been shown for T cell protection against vaginal HSV-2 challenge [32].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Production and characterization of the gp41t and gp41 Fc immunogen
(A) Linear diagram showing features of the recombinant sgp41t construct. The amino acid sequence of the immunogen is directly below. The region of SIVmac239 gp41 not included in the construct is faded out. The numbering corresponds to SIVmac239. This sequence was aligned to SIVsmE660 as well as other HIV sequences, including consensus sequences obtained from the Los Alamos database for 9 different clades of HIV to show the high level of sequence conservation in this region, especially for the immunodominant epitopes (boxed in red). Predicted N-linked glycosylation sites are highlighted in light blue. **(B)** On the left is

a pymol rendering of the mean structure of the ectodomain of the trimeric SIV gp41 6-helix bundle (RCSB:1QCE) solved by NMR. This structure should approximate the structure of the secreted immunogen, and shows the importance of the quaternary structure in forming the conformational cluster I epitope. The protein includes the HR1 through to the end of the HR2. The coloring shown is the same as for the linear diagram in (A). Sites predicted to be glycosylated are labeled. To the right is a cartoon showing the tertiary structure of the immunogen as a monomer. Colors correspond to the linear diagram in (A). Residues making up the cluster I and II epitopes are outlined in red. On the far right is a depiction of the two immunogens used in this study; the soluble gp41 trimer (sgp41t) and the sgp41t-Fc, which is made up of three tandem sgp41t sequences followed by the Fc sequence of rhesus macaque IgG1. Both are shown as a monomer and how they naturally exist as noncovalent trimers or as covalent dimers respectively. C) S200 10/300 size exclusion profiles of StrepTactin affinity purified sgp41t (left) or sgp41t-Fc (right). The trimer and dimer fractions are shown respectively along with reducing and non-reducing SDS-PAGE gels. SDS and heat alone does not completely disrupt the 6-helix bundle showing three diffuse bands representing monomer dimer and trimer, which stain weakly with Coomassie blue. The gp41-Fc dimer is disrupted only upon reduction of the disulfide bridges in the Fc portion of the protein. Sizes are consistent with 3 N-linked glycans/sgp41t.

Figure 2. Design, production and characterization of gp41t liposomes

(A) sgp41t was first linked to PEG-DSPE via a surface lysine residue using the heterobifunctional crosslinker SPDP using conditions that gave approximately 1 lipid per trimer. The disulfide bonds in the gp41t trimeric structure were preserved by deprotecting the 2-pyridyldithio group with DTT at acidic pH. (B) A solution of lipid-modified gp41t was used to hydrate a mixture of lipids that included DSPC, cholesterol, PEG-DSPE, and MPLA. Sonication and extrusion of the solution produced unilaminar liposomal nanoparticles that multivalently display gp41t. (C) Characterization of liposomes displaying no protein (sham)

or gp41t by transmission electron microscopy. The relative size distribution was determined by measuring the size of > 20 liposomes from each condition.

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Figure 3. Antibodies elicited by sgp41t-MPLA-liposomes

A) Immunogenicity of sgp41t liposomes with or without MPLA adjuvant was tested in 4 BALB/c mice by assessing sgp41t specific IgG titers in the serum by sgp41t capture ELISA 1 week after boost. MPLA adjuvant increased titers about 10-fold ($p=0.029$ by unpaired nonparametric Mann-Whitney t test). New Zealand white rabbits were also immunized with the gp41 liposomes+MPLA and their sgp41t specific serum IgG titers assessed 1 week post boost by ELISA **B)** The sera from liposome+MPLA boosted mice and rabbits were assessed for IgG binding reactivity to SIVmac239 gp41 from viral lysates on western blotting strips. Staining of the oligomeric gp41 band gp160 (boxed) was observed and faint staining could be detected in one rabbit and one mouse to the gp80 gp41 band (arrows).

Figure 4. Antibodies elicited in rhesus macaques react with oligomeric gp41 and detection of gp41t antibodies in cervical reserve epithelium

A) Overview of the pilot immunization study in 4 rhesus macaques. Animals 1 (RBq6) and 2 (RDj11) received the intranasal sgp41-Fc as a prime and first boost while all subsequent boosts included these as well as the intramuscular liposome immunization. Animals 3 (ROz6) and 4 (RTe8) received only the intramuscular liposome immunization at all the same prime and boosting time points; animal 4 (RTe8) was euthanized for stress-related diarrhea 14 days after the third boost. The time intervals between immunizations are shown in months. The animal numbers, prime boost schedule and time intervals carry through in

Figure 3B. **B)** ELISA assays and Western blots (WB). Total IgG (in blue) and sgp41t specific IgG titers (red and pink bars-latter showing change in titer following a 6M urea wash) in plasma (EC_{50} s on left Y-axis) at the times shown on the X-axis. For comparison, gp41 specific IgG titers in nef-vaccinated macaques at 5 or 20 weeks is shown on the far right. Letters indicate individual animals. Western blots were done using serum samples from the same time points (indicated below the strips.) The numbers above the strips correspond to the animal numbers in Figure 4A. To the right are WBs of samples from unprotected (5 week) and protected (20 week) nef-immunized macaques illustrating that similar or stronger staining of gp41 compared to the protected monkey samples is achieved after the last boost. WBs of cervical tissue extracts at the far right. Both immunogens and routes of immunization elicited antibodies in proteins extracted from cervix to oligomeric gp41t in WBs-bands at gp160 and 80-comparable to SIV_{mac239} nef vaccination the 20-week (20w) time point when animals are protected against high dose vaginal challenge. There are also antibodies against gp41 monomer and truncated forms of gp41 in the animals vaccinated with the immunogens and adjuvants that are barely detectable in the SIV_{mac239} nef vaccinated animals, which have antibodies reactive with viral capsid antigens p27 and p17. The black boxes and double headed arrow show the lack of correlation between ELISA titer and staining intensity of oligomeric gp41t bands. The red box shows the lack of correlation between ELISA titers and WBs. Only the WBs correlate with increased protection at 20 weeks. **C)** RIHC. SIV-negative animal versus brown-stained gp41t-Ab+ cervical reserve epithelium (arrows). Representative staining for the three animals examined after the fifth boost.

Figure 5. Passive immunization with a gp41t-antibody
A ELISA assays of sera and CVF at the time points shown. Total IgG and 4.9C titers from two rhesus macaques passively administered either 25 or 50mg/kg of 4.9C monoclonal antibody. Concentration of 4.9C with 95% confidence intervals in the serum is given in the table below obtained from a 4.9C standard curve B), WB analyses and comparisons of antibodies reactive with oligomeric gp41t (gp160 and gp80 bands) in sera and vaginal and cervical tissue extracts following infusion of 25 or 50 mg/kg of a rhesus monoclonal antibody 4.9C, which reacts in WB with oligomeric gp41t indistinguishably from

SIV_{mac239} nef vaccinated animals at 20 weeks. Sample time points correspond to those shown in A. The vaginal and cervical extracts obtained at 24 hours are compared with cervical extracts at this time in the gp41t-immunized animals and a representative animal at 20 weeks post SIV_{mac239} nef vaccination. The lower panel in B quantifies and compares the prominent gp160 band in WB as the ratio of band intensity to the positive serum control. C) RIHC-staining of gp41t antibodies in cervical reserve epithelium in animals infused with 4.9C. Upper panels show successively higher magnifications of red-stained cervical reserve epithelium with gp41t antibody (black arrow and label, far right panel) of the animal that received 50mg/kg of 4.9C. Lower panels, animal infused with 25 mg/kg. No staining of cervical reserve epithelium is evident.