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Research Article

SHIV Virus-Like Particles (VLPs) Vaccination Induces Partial Protection from SHIV Challenge in a Rhesus Macaque Model

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ABSTRACT

Simian/Human immunodeficiency virus (SHIV) virus-like particles (VLPs) composed of SIV Gag, HIVsf162 gp120/gp41 envelope, and human CD40L are whole pseudovirion vaccines capable of eliciting both humoral and cellular immunity. We immunized four rhesus macaques by intranasal prime and four sub-cheek boosts with VLPs adjuvanted with conjugatable adjuvant lipid vesicles containing monophosphoryl lipid A (MPLA), and compared their immune parameters to those in five unimmunized control macaques. Increased plasma antibody titers to SIV Gag were observed in all four immunized macaques and increased sf162 gp140 titers were observed in three of the four with one macaque (10-195) maintaining sustained anti-Env antibody levels. Compared to controls, a significant increase in memory B cells and CD4+ central memory T cells was detected in the immunized group. Among these, elevated Gag-specific CD107a membrane localization in the CD8+ central memory T cells was detected in one macaque (10-195). All nine macaques were subsequently challenged intrarectally with SHIVsf162.P3. After challenge, eight of the nine macaques became infected with SHIV, while the macaque 10-195 was protected. Another immunized macaque (10-189) that got infected but consequently generated high Gag-specific IFN-γ and CD107a CD8+ T cells; and Env-specific IL-2 and CD107a CD8+ T cells controlled virus and had undetectable levels of plasma SIV copy number by the termination of the study. Our VLPs vaccine strategy represents a promising immunogenic conformationally intact HIV vaccine that may lead to possible prevention and control of HIV infection.

Keywords: SHIV, VLP vaccination, Rhesus Macaques Challenge Model

INTRODUCTION

Virus like particles (VLPs) are replication deficient sub-unit vaccines that maintain the natural antigenic conformation of the virus, but lack the proteins and nucleotides associated with virus propagation. We and others have previously shown that HIV and SHIV VLPs are effective immunogens capable of activating dendritic cells, macrophages, and B cells [1–3]. SHIV and HIV VLPs are generally composed of two immunogens: 1) the membrane bound protein HIV envelope (Env) which is expressed as a component of the plasma membrane of the host cell; 2) the structural protein SIV Gag or HIV Gag, which buds out from the host cell enveloping itself in the host cell's plasma membrane. The immunogenicity of VLPs can be further enhanced by overexpressing immune cofactors on the membrane of the host cell, which present on the surface of the VLP after it buds out from the plasma membrane. We have previously demonstrated this enhanced immunogenicity by expressing CD40L (CD154) on the surface of cells in which we produced VLPs. In mice, VLPs presenting CD40L induced an augmented immune response towards HIV Env and SIV Gag when compared to VLPs lacking CD40L on their cell surface,

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including anti-Env antibody titers and increased CD8+ T cell killing [4]. To determine the effectiveness of SHIV VLPs presenting CD40L in preventing virus transmission and controlling virus infection, we tested our VLPs in the rhesus macaque high dose mucosal virus challenge model.

The mucosal transmission of SHIVsf162.P3 has repeatedly been shown to be an effective model in mimicking HIV pathogenesis in rhesus macaques [5,6]. SHIVsf162.P3 is an R5 tropic strain composed of SIV gag and pol, and HIV env, tat, vpu, and rev genes [5,7]. In the SHIV challenge model, effective vaccines induce adaptive cellular or humoral immunity, which either delays development of simian AIDS or protects against virus acquisition [8–11].

The adaptive immunity sought in this model is broadly broken down into cellular and humoral immunity. Cellular immunity is generally directed at Gag, specifically effector memory (CD28-, CD95+) CD8+ T cells, which have been shown to be induced and maintained by vaccination and are effective in preventing or controlling SHIV after virus challenge [9,12]. On the other hand, HIV envelope is the predominant target of HIV vaccines designed to elicit humoral immunity, particularly neutralizing or non-neutralizing antibodies. HIV envelope directed antibodies require B cells to undergo hypermutation and class switching in the lymph nodes. This process is mediated by a subset of CD4+ cells expressing CXCR5 and PD-1hi (CD279) known as T follicular cells (TFH), which form and maintain the germinal center [13]. Memory B cells are formed in the germinal center and can then migrate into the periphery and can be identified by upregulation of CD27, often with co-expression of CD20 [14]. Furthermore, memory B cells lack CD38 expression, distinguishing them from plasma cells, which express CD38 in the periphery [15].

The antibodies produced by the memory B cells fall into two categories based on their interaction with HIV. Neutralizing antibodies prevent HIV from entering the target cell by blocking the CD4 or CXCR4/CCR5 binding site(s), while non-neutralizing antibodies promote other immune functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and/or antibody-dependent cell-mediated virus inhibition (ADCVI) [16-19]. In addition, central memory (CD28+, CD95+) CD4+ T cells play an immune supportive role, and have been found to be associated with prolonged survival after HIV infection and control of viremia [20,21].

In this study, rhesus macaques were immunized with our previously characterized effective vaccine route of administration, intranasal prime followed by sub-cheek boost with conjugatable adjuvant lipid vesicles containing MPLA serving as the adjuvant [22]. Each macaque received an intranasal prime and three sub-cheek boosts of SHIV VLPs presenting CD40L, and a final sub-cheek boost of HIV VLPs. Three months after the final boost, the four immunized macaques along with five control macaques were intrarectally challenged with high dose SHIVsf162.P3. The effect of VLPs on induction of humoral and cellular immunity was compared between immunized and control macaques. After challenge, CD4 count, SIV copy number, and antigen cytokine induction in CD8+ T cells were measured to evaluate if VLPs promoted virus control or complete protection.

MATERIALS AND METHODS

Animals and infections

Thirteen adult female rhesus macaques (Macaca mulatta) of Indian origin were maintained in the specific pathogen-free breeding colony at the Michael Keeling Center for Comparative Medicine and Research of The University of Texas MD Anderson Cancer Center (Bastrop, Texas). All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center and were carried out according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, and the principles of the NIH Guide for the Care and Use of Laboratory Animals, and the policies and procedures of the University of Texas MD Anderson Cancer Center. The chamber size for the animals was 44′ W × 88′ H × 160′ D. Monkeys were given water ad libitum, and fed a commercial monkey diet (Harlan). Additional enrichment was provided in the form of manipulanda, visual stimulation or auditory stimulation, and combinations thereof. Animals were monitored daily, including weekends and holidays. Anesthetics/analgesics were used to minimize any discomfort, distress, pain, and injury the animal might experience. Animals were euthanized with ketamine (11 mg/kg), followed by Beuthanasia (1 ml/10 lbs). If any animal was moribund, unresponsive to treatment, could not eat or drink, was severely sick, or had symptoms of SAIDS, it was euthanized as per guidelines. Animals were anesthetized during procedures to minimize discomfort. For infections, monkeys were fasted for a minimum of 24 h prior to exposure. Monkeys were first anesthetized with 10 mg/kg of body weight ketamine intramuscularly and 0.5 mg/kg xylazine, then placed in a sternal position with the pelvis propped at 45°. Monkeys were infected by intra-rectal inoculation of 1000 TCID50 clonal stocks of SHIVsf162.P3 (NIH AIDS Reagent Program). The infected monkey was then returned to its cage and kept tilted at 45° until full recovery from anesthesia.

Baculovirus VLP production

Production of SIV VLPs was conducted as previously described [4]. Briefly, Sf9 cells were infected with SIV Gag at a multiplicity of infection (MOI) of 1, and human CD40L and HIV sf162 gp160 at MOIs of 10. After 3 days, the supernatant was harvested, filtered through a 0.45 μm filter, and subjected to ultracentrifugation at 140,000 xg for 2 h. The supernatant was removed, and the remaining pellet, containing the VLPs, was resuspended in PBS (with Ca^{2+} and Mg^{2+}), and stored at 4°C.

Mammalian VLP production

Production of HIV VLPs was conducted as previously described [22]. Briefly, HIV-1 Gag/Env VLPs were produced from XC-18-derived cell lines engineered to express HIV-1 gag (HIVIIIB strain) and env genes (HIVBaL strain) under a tetracycline-inducible expression system (the cell lines are generous gifts from Dr. Spearman at Emory University). Production of VLPs

was induced by adding 2 µg/ml of doxycycline and 6 days after induction, media containing VLPs were collected (20 ml/T-150 flask), filtered through a 0.45 µm filter, and subjected to ultracentrifugation at 140,000 x g for 2 hours. The supernatant was removed, and the remaining pellet, containing the VLPs, was resuspended in PBS (with Ca^{2+} and Mg^{2+}), and stored at 4°C.

Immunization

Rhesus macaques were immunized four times with baculovirus derived VLPs, consisting of SIV Gag, sf162 gp120/gp41 Env, and human CD40L and one additional time with mammalian derived VLPs consisting of HIV Gag and BaL gp120/gp41 Env. For each immunization, of the liposomal adjuvant VesiVax Conjugatable Adjuvant Lipid Vesicles (CALV) containing 75 µg/dose MPLA (500 µg/ml) was mixed with 200 µg/kg (between 1 to 1.4 mg) of VLPs [CALV(MPLA)+VLPs] in a total volume of 300 µl (Table 1). All samples were mixed and incubated for approximately 1 hour at RT before immunization. The macaques were immunized by an intranasal prime (week 0), and a succession of 3 sub-cheek boosts at weeks 4, 8, and 20 (Supplementary Figure 1A). The fifth immunization (4th boost) of mammalian cell line derived VLPs, was given at week 32.

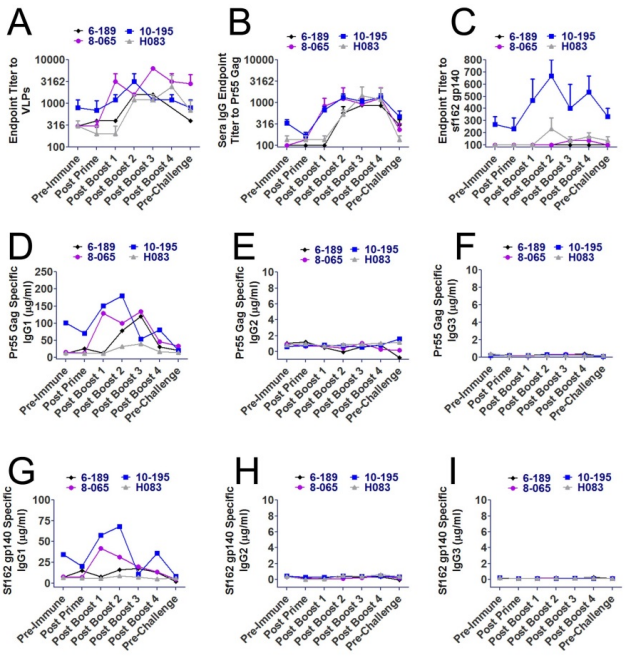

Figure 1: Immunogen-specific IgG and IgG subtype End Point Plasma Titers. Endpoint ELISAs of CALV(MPLA)+VLPs immunized macaque plasma probed for IgG titers against (A) VLPs, (B) Pr55 Gag, and (C) sf162 gp140 at the indicated time points. Quantitative ELISAs of plasma specific Pr55 GAG of (D) IgG1, (E) IgG2, and (F) Igg3 at the indicated time points. Quantitative ELISAs of plasma specific sf162 gp140 of (G) IgG1, (H) IgG2, and (I) Igg3 at the indicated time points. Assay was repeated in triplicate.

Table 1: Pre-challenge Plasma Neutralizing Antibody titers against Tier 1 and Tier 2 HIV viruses.

	Strain (CIade)	SVA-MLV	MN.3	SF162.LS	Bal.26	MW965.26	SHIV SF162P3
	Clade	Control	Clade B (Tier 1A)	Clade B (Tier 1A)	Clade B (Tier 1A)	Clade C (Tier 1A)	Clade B (Tier 2)
Sample ID Irrriun ization Grou p	Bleed Week						
10-195 CALV(MPLA)+VLPs	0	<20	<20	<20	<20	<20	<20
	26	<20	<20	<20	<20	<20	<20
	50	<20	<20	<20	<20	<20	<20
8-065 CALV (MPLA)+VPLs	0	<20	<20	<20	<20	<20	<20
	26	<20	<20	<20	<20	<20	<20
	50	<20	<20	<20	<20	<20	<20

H083 CALV(MPLA)+VLPs	0	<20	<20	<20	<20	<20	<20
	26	<20	<20	<20	<20	<20	<20
	50	<20	<20	<20	<20	<20	<20
6-189 CALV(MPLA)+VLPs	0	<20	<20	<20	<20	<20	<20
	26	<20	<20	<20	<20	<20	<20
	50	<20	<20	<20	<20	<20	<20

*Note: Bolded values are considered positive for neutralizing antibody activity in the post-immune sample based on the criterion of >3x the observed background against the SVA-MLV negative control pseudovirus.

Virus challenge

Challenge stocks of SHIVsf162.P3 were generously supplied by Dr. Nancy Miller, Division of AIDS, NIAID, through Quality Biological (QBI), under Contract No. HHSN272201100023C to the Vaccine Research Program, Division of AIDS, NIAID. A total of 13 female macaques were challenged with one time dose (1000 TCID50) SHIVsf162.P3 intra-rectal route. For the challenge, the animals are positioned in prone position and virus is inoculated approximately 4 cm into the rectum. Inoculated animals are maintained in the prone position with the perineum elevated for 20 minutes to ensure that virus does not leak out. Care is also taken to prevent any virus from contacting the vagina area and also not to abrade the mucosal surface of the rectum. All animals were infected after one week of challenges.

Viral load determination

SHIV viral loads in plasma from the blood were determined by determining viral RNA copy numbers by realtime RT-PCR analyses. These assays were performed at the NIH Core Facility by Dr. Jeff Lifson's group. The threshold sensitivity of the assay is 30 viral RNA copy-equivalents/ml of plasma, and the inter-assay variation is <25% (coefficient of variation).

Tissue collection

All samples were collected at the indicated time points just prior to immunization. Blood samples were collected in sodium heparin from the femoral vein. Before the separation of peripheral blood mononuclear cells (PBMC) from the blood samples, plasma was collected and stored immediately at−80 ◦C until analyzed. The PBMC prepared from the blood samples by the standard ficoll-hypaque density-gradient centrifugation were used for immune assay. The oral swabs samples were collected by swiping and rolling the swabs 2-3 times against the mucosa of the check pouch on both sides of the mouth and against the base of the tongue. The rectal swabs sample is collected by first manually extracting the feces from the rectum with a gauze covered finger if needed. The swabs is then swiped and rolled against the rectal mucosa 2-3 times. Immediately after the collection procedure, each swabs is placed in 2 ml of cold transport medium (RPMI 1640 medium supplemented with 5mM glutamine, amphotericin B, penicillin, streptomycin, and 10% FBS). Soon after collections, the various biological fluids were subjected to a short centrifugation, mixed with protease inhibitors and kept at −80 ◦C until assayed. At the end of study various tissues were collected in complete RPMI medium from the all monkeys at the time of necropsy for various immunological analyses.

Rectal biopsies

The macaques were fasted for a minimum of 12 hours prior to the having rectal biopsy to evacuate the stomach, duodenal, and proximal jejunal contents. They were then anesthetized with 3–5 mg/kg of Telazol, and placed in left lateral recumbency with a mouth guard to protect the endoscope. Using a human pediatric flexible endoscope with up to 7.8 mm in diameter, the gastroscopy was accomplished by advancing the endoscope through the colon and 1 to 2 mm biopsies were performed at the desired sites with up to 10–15 biopsies at each time point. In this procedure the monkey were given penicillin post biopsy for one day and 0.005 to 0.01 mg/kg Buprenorphine for 2 days. The monkey's health, appetite, and stool were monitored twice per day.

Isolation of lymphocytes from rectal biopsies

Rectal biopsy specimens were washed once with cold PBS and the tissues were incubated at 37uC in RPMI 1640 containing

5% fetal bovine serum supplemented with type IV collagenase (Sigma- Aldrich) at 300 U/ml and DNase I at 15ug/ml with gentle shaking. The released cells were filtered through a 70 mm nylon mesh screen. Lymphocytes were purified on Percoll gradients (35– 65%) at 1,500 RPM for 25 min. Lymphocytes were collected above 65% Percoll layers and washed two times before processing for staining.

Endpoint titer ELISA

To detect Env- and Gag-specific antibodies, microtiter plates were coated with the following recombinant proteins at a concentration of 1 μg/ml: HIV-1sf162P3 gp140 or HIVIIIB Pr55 Gag (Gag) protein (NIH AIDS Research and Reference Reagent Program). Protein-coated plates were incubated overnight at 4°C. After discarding the coating solution, plates were washed with PBS+0.05% Tween 20 (PBST), and blocked with PBS containing 5% bovine serum albumin (BSA). Sera were diluted 1:500 in PBS and the indicated serial dilutions were made for each recombinant protein. Plates were incubated overnight, washed with PBST, and incubated with anti-mouse IgG (Sigma-Aldrich, St. Louis, MO). After washing with PBST, TMB colorimetric substrate solution (Pierce, Rockford, IL) was added into each well. The HRP enzyme reaction was stopped with 2 N H2SO4, and the OD values were read at 450 nm (against reference at 570 nm) in a microtiter reader (EL800, Bio-Tek Instruments, Winooski, VT). Endpoint titer was determined by the mean background+10 times the standard deviation.

Quantitative ELISA

ELISA plates were coated with a standard of the respective isotype and the remaining wells were coated with 1 μg/ml HIV-1sf162 gp140. Plates were blocked with PBS containing 5% BSA. Afterwards, sera from the animals collected at the indicated time points were diluted 1:100 and added to each well. Secondary antibodies were rhesus IgG1-HRP, IgG2-HRP, IgG3-HRP (NIH Nonhuman Primate Reagent Resource). Plates were developed with TMB substrate for 5 minutes after which time 2 N H2SO4 was added to stop the reaction. Optical density was read at 450 nm (reference 570 nm) and sera IgG concentrations were calculated within the standard curve.

Neutralization assay

Neutralizing antibodies were measured as a function of reductions in luciferase (Luc) reporter gene expression after a single round of infection with molecularly cloned Env-pseudotyped virus in TZM-bl cells as described [23]. Neutralization titers are the dilution at which relative luminescence units (RLU) were reduced by 50% compared to virus control wells after subtraction of background RLUs in cell control wells. Activity against the indicated strains from samples was considered positive if the ID50 was >3x the observed background against the negative control MLV-pseudotyped virus. Additional information on the assay and all supporting protocols may be found at: http://www.hiv.lanl.gov/content/nab-reference-strains/html/home.htm.

Intracellular cytokine staining

PBMCs were seeded into 96-well plates at a density of 5 x 105 cells/well. To each well 10 μg/ml Brefeldin A (Sigma, St. Louis, MO), 10 μg/ml monensin (Sigma, St. Louis, MO), and anti-CD107a (clone: H4A3; Biolegend, San Diego, CA) antibody were added. Env or Gag peptide pool was added to each well at a concentration of 2 μg/ml. Cells were incubated for 5 hours at 37°C, 5% CO2. Afterwards, cells were washed and incubated with anti-CD3 (clone: HIT3a; BD Biosciences, San José, CA), anti-CD4 (clone: L200; BD Biosciences, San José, CA), and anti-CD8 (clone: SK1; BD Biosciences, San José, CA) antibodies. After 45 minutes, cells were washed, fixed in BD Perm/Fix (BD Biosciences, San José, CA), washed again, and resuspended in BD Perm/Wash (BD Biosciences, San José, CA). Intracellular antibodies used were interleukin (IL)-2 (clone: MQ1-17H12; BD Biosciences, San José, CA), IFN-γ (B27), and IL-21 (clone: 3A3-N2.1; BD Biosciences, San José, CA). Cytokine analysis was done on an LSR-Fortessa (BD Biosciences San José, CA). Positive cells were selected by calculating a threshold value as outlined by Donaldson et al. [24]. Briefly, samples were gated for positive cells, the percent of unstimulated cells was subtracted from the percent of peptide stimulated cells, and the threshold value was determined by postulating the non-specific signal followed a normal distribution. Specifically, the absolute value of the 97th percentile of all negative values for a specific cytokine was set as the threshold, with all values greater than the 97th percentile reported in the results, with all values less than the threshold considered background and set to zero.

Flow cytometry

Lymphocytes were resuspended in PBS containing 2% bovine serum albumin (BSA), 5 mM EDTA, and 0.03% NaN3 and added to 96-well conical-bottom plates, 1 x 106 cells/well. The T cell panel included antibodies against human CD3, CD4, CD8, CD28 (clone:HIT3a, L200, SK1, CD28.2; BD Biosciences, San José, CA), and CD95 (clone: DX2; BD Biosciences, San José, CA). The memory B cell panel included antibodies against human CD19 (clone: J3-119; Beckman Coulter, Brea, CA), CD3, CD20 (clone: 2H7; Biolegend, San Diego, CA), CD27 (clone: O323; Biolegend, San Diego, CA), IgG (clone: G18-145; BD Biosciences) and CD38 (clone: AT-1; Stem Cell Technologies, Vancouver, Canada). The TFH panel included antibodies against human CD3, CD4, CXCR5 (clone: 710D82.1; NIH Nonhuman Primate Reagent Resource), and PD-1 (clone: EH12.2H7; Biolegend, San Diego, CA). Cells were incubated with the primary antibody for approximately 45 minutes at RT. Afterwards, they were fixed for 15 minutes in BD Perm/Fix, resuspended in BD Perm/Wash, and then analyzed on an LSR-II.

Statistical analysis

Data from treated and control groups were analyzed and results presented as the arithmetic mean ± standard error mean (SEM). Statistical analyses were done with Student's unpaired t-test, 1-Way ANOVA, and Tukey post-hoc test, for comparison of multiple groups, or with 2-Way ANOVA and the Bonferroni post-hoc test for comparison of parametric data between two or

more groups. The Mann-Whitney test was used for non-parametric data. Graphpad Prism was used to calculate statistics (Graphpad Software, Inc., La Jolla, CA). A value of $p < 0.05$ was considered significant.

RESULTS

SHIV VLPs vaccination induces Env and Gag specific plasma antibodies

Plasma titers against recombinant sf162 gp140 and Pr55 Gag were measured before the initial prime and after each subsequent immunization. Against VLPs, plasma titers increased after boost 2 and remained stable for all four experimental monkeys for the duration of the vaccine period (Figure 1A). Plasma titers towards recombinant Gag followed a similar pattern peaking after boost 2 for macaques 10-195 and 8-065 with an end-point dilution of 1:1333 and peaking after boost 3 for macaques 6-189 and H083 with end-point dilutions of 1:866 and 1:1466 respectively (Figure 1B). Gag plasma titers fell during the 3 months between boost 4 and challenge. Finally, the macaques had poor responses to gp140 Env, with 10-195 having the best response peaking at 1:666 after boost 2, and falling back to 1:333 prior to challenge (Figure 1C). The other three macaques had minimal changes in end-point titers towards sf162 gp140 Env.

Quantitative ELISAs were used to determine the specific IgG subtypes elicited by the VLP vaccine. Plasma titers against Pr55 Gag were exclusively IgG1, peaking after boost 2 for 10-195 at 180 μg/ml, and after boost 3 at concentrations of 120, 135, and 40 μg/ml for 6-189, 8-065, and H083 respectively (Figure 1D). Similar to endpoint IgG dilutions, Gag titers for all four macaques fell to levels slightly above pre-immune starting concentrations prior to virus challenge. No specific IgG2 or IgG3 titers were detected against Gag (Figures 1E and 1F). Similar to Gag, plasma titers against sf162 gp140 were exclusively IgG1, with 10-195 having the most robust response peaking after boost 2 at 68 μg/ml and again after boost 4 at 36 μg/ml, before declining prior to challenge (Figure 1G). Only 8-065 had any response among the remaining 3 macaques, peaking after boost 1 at 42 μg/ml before returning to baseline. No sf162 gp140 specific IgG2 or IgG3 were detected in the four immunized macaques (Figures 1H and 1I). To determine if immunization induced neutralizing antibodies, plasma from before immunization, after boost 3 (week 26), and prior to virus challenge (week 52) from the four macaques was screened for antibodies capable of neutralizing SHIVsf162.P3; however, no TCID50 greater than 20 was detected in any of the immunized macaques (Table 1).

SHIV VLP vaccination induces mucosal neutralizing IgA

Because the macaques were challenged rectally, rectal washes were collected prior to initial prime (week -2), after boost 2 (week 8), after boost 3 (week 26), and before challenge (week 50). Analysis of rectal wash for specific antibodies towards sf162 gp140 showed minimal changes in absorbance of sf162 gp140 specific IgA in the rectal washes of the 4 immunized macaques; although, the pre-challenge titers were greater than the pre-immune titers in three macaques, H083, 6-189, and 8-065 (Figure 2). In addition, analysis of the neutralizing titers to SHIVsf162.P3 virus showed that both 8-065 and 6-189 had neutralizing antibodies present in their rectal wash prior to challenge, with TCID50s of 43 and 22 respectively (Table 2).

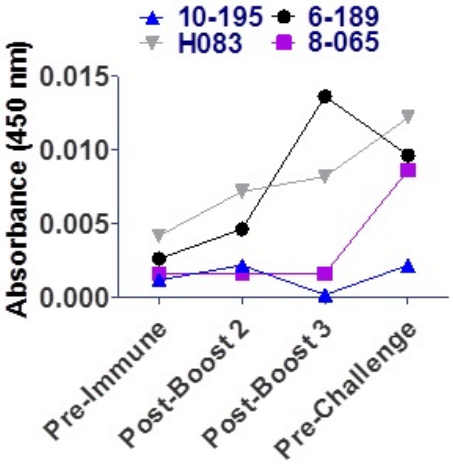

Figure 2: IgA titers against sf162 gp140 in rectal wash. IgA ELISA of rectal washes diluted 1:4 from CALV(MPLA)+VLPs immunized macaques against sf162 gp140 before immunization (week -2), after boost 2 (week 9), after boost 3 (week 21), and prior to virus challenge (week 50).

Table 2: Pre-challenge Rectal Wash Neutralizing Antibody titers against Tier 1 and Tier 2 HIV viruses.

		Strain (Clade)	SVA-MLV	MN .3	SF162.LS	Bal.26	MW965.26	SHIV SF162P3
		Clade	Control	Clade B (Tier 1A)	Clade B (Tier 1A)	Clade B (Tier 1B)	Clade C (Tier 1A)	Clade B (Tier 2)
Sample ID	Immunization Group	Bleed Week						

10-195	CALV(MPLA) +VLPs	50	<20	<20	<20	<20	<20	<20
8-065	CALV(MPLA) +VLPs	50	<20	34	<20	<20	20	43
H083	CALV(MPLA) +VLPs	50	<20	<20	<20	<20	<20	<20
6-189	CALV(MPLA) +VLPs	50	<20	<20	<20	<20	<20	22

SHIV VLP vaccination induces Memory B cells and TFH cells

Prior to challenge, five control macaques were added to the study and compared to the four immunized macaques. PBMCs were compared between the two groups for differences in T cell and B cell memory. After gating for CD3-, CD19+ B cells, the populations were characterized by CD20 and CD27 expression (Figure 3A). A significant decrease in naïve B cells, defined as CD20+, CD27-, was observed in the immunized macaques with 69.7% of B cells gated as naïve, compared to 77.9% of B cells gated as naive in control macaques (Figure 3B). Analysis of memory B cells, defined as CD20+, CD27+, IgG+, and CD38- showed a significant increase between controls and immunized macaques, with 1.46% of circulating B cells gated as memory B cells in control macaques compared to 2.33% of circulating B cells gated as memory B cells in the immunized group (Figure 3C).

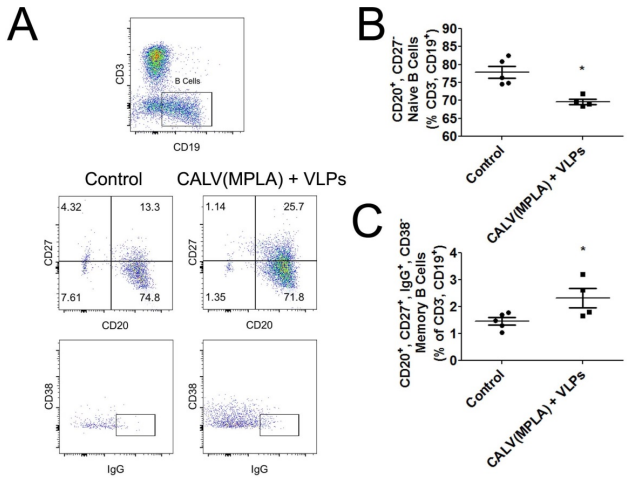

Figure 3: Pre-challenge Circulating naïve and memory B cells. (A) Representative flow cytometry of B cells (CD3-, CD19+) from control and CALV(MPLA)+VLPs immunized macaques with further gating distinguishing CD20, CD27, CD38, and IgG cell surface expression. (B) Naïve B cells as determined by CD20+, CD27-. (C) Memory B cells as determined by CD20+, CD27+, IgG+, and CD38-. * indicates $p < 0.05$ by Student's unpaired t-test.

We then sought to compare the lymph node TFH cell compartment between the control group and the CALV(MPLA) +VLPs immunized group. Prior to virus challenge, lymph nodes from each macaque were biopsied and stained for TFH cells based on surface expression of CD3+, CD4+ CXCR5+, PD-1hi (Figure 4A). Comparing the percentage of lymph node TFH cells between control and CALV(MPLA)+VLPs immunized groups, no difference in average percentage was observed (Figure 4B). Lymphocytes were then stimulated with either Gag or Env peptide pools and assayed for IL-21 expression. Two macaques showed increased TFH IL-21 expression after peptide stimulation, H083 after Gag stimulation, and 6-189 after Env stimulation (Figure 4C). No specific TFH IL-21 was detected in the other macaques.

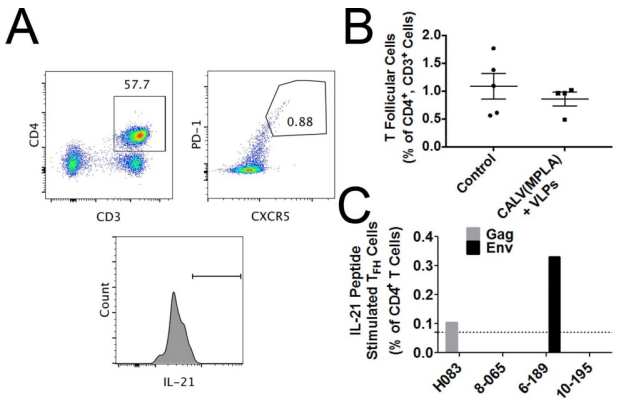

Figure 4: Pre-challenge TFH cells. (A) Representative flow Cytometry gating of pre-challenge lymph nodes for CXCR5+, PD-1hi TFH subset of CD3+, CD4+ lymphocytes. (B) Lymph node TFH cell percentages from control and CALV(MPLA)+VLPs immunized rhesus macaques. (C) IL-21 expression after stimulation with 2 μg/ml of Gag or sf162 Env peptide pools in the indicated macaques. Dotted line represents 97% positivity threshold.

SHIV VLP vaccination induces Gag and Env specific effector and central memory T cells

Circulating T cells were compared between control and CALV(MPLA)+VLPs immunized macaques for both central and effector memory populations and antigen specificity. PBMCs were gated on CD3+ cells and then divided between CD4+CD8- and CD4-CD8+ cells (Figure 5A). T cells were categorized based on their expression of CD28 and CD95 as naïve (CD28+, CD95-), effector (CD28-, CD95+), or memory (CD28+, CD95+) T cells. For CD4+ T cells, a significant decrease in naïve T cells was observed between the control (42.3%) and immunized (27.6%) macaques (Figure 5B). A corresponding significant increase was observed in the memory CD4+ T cell population between control (28.4%) and immunized (46.9%) macaques. No difference was observed between effector populations. In

contrast to CD4+ T cells, no difference was observed between the control and immunized macaques' circulating CD8+ T cells among the three populations (Figure 5C).

The functional response of central and effector memory T cells was examined by CD107a membrane localization and intracellular cytokine staining (ICC) of IFN-γ and IL-2 after Gag and sf162 Env peptide stimulation. No Gag or Env specific IFN-γ was observed for either central or effector memory CD4+ or CD8+ T cells among the immunized macaques (Supplementary Figure 2). Both H083 and 6-189 had specific central memory CD4+ T cell Gag induced IL-2, while 6-189 also had Env specific effector memory CD4+ T cell induced IL-2 (Figures 6A and 6D). Additionally, IL-2 was induced in CD8+ central memory T cells in 6-189 after Gag peptide stimulation (Figure 6B). Env peptide stimulation had no effect on CD8+ T cell IL-2 production (Figure 6E). Finally, only 10-195 had CD107a membrane localization after Gag stimulation in the CD8+ central memory population (Figures 6C). Again, no CD107a membrane localization was observed after sf162 Env peptide stimulation (Figure 6F).

Increased antibody titers in CALV(MPLA)+SHIV/HIV VLP immunized macaques compared to controls

Prior to viral challenge, plasma ELISAs antibody titers to sf162 gp140, Pr55 Gag, and VLPs were measured and compared between control macaques and the CALV(MPLA)+VLPs immunized macaques. Against Gag, macaques immunized with CALV(MPLA)+VLPs had significantly greater Gag specific IgG compared to controls with a an absorbance of 0.027 compared to 0.077 respectively (Figure 7A). Similar to Gag, the CALV(MPLA)+VLPs immunized macaques had significantly greater sf162 gp140 specific IgG when compared to controls of an absorbance of 0.033 compared to 0.009 (Figure 7B). In addition, 10-195 had the highest absorbance of all 9 macaques of 0.060. Finally, antibodies specific to VLPs were significantly greater in the CALV(MPLA)+VLPs group compared to controls, with an average absorbance of 0.108 for the immunized macaques compared to 0.019 for controls (Figure 7C).

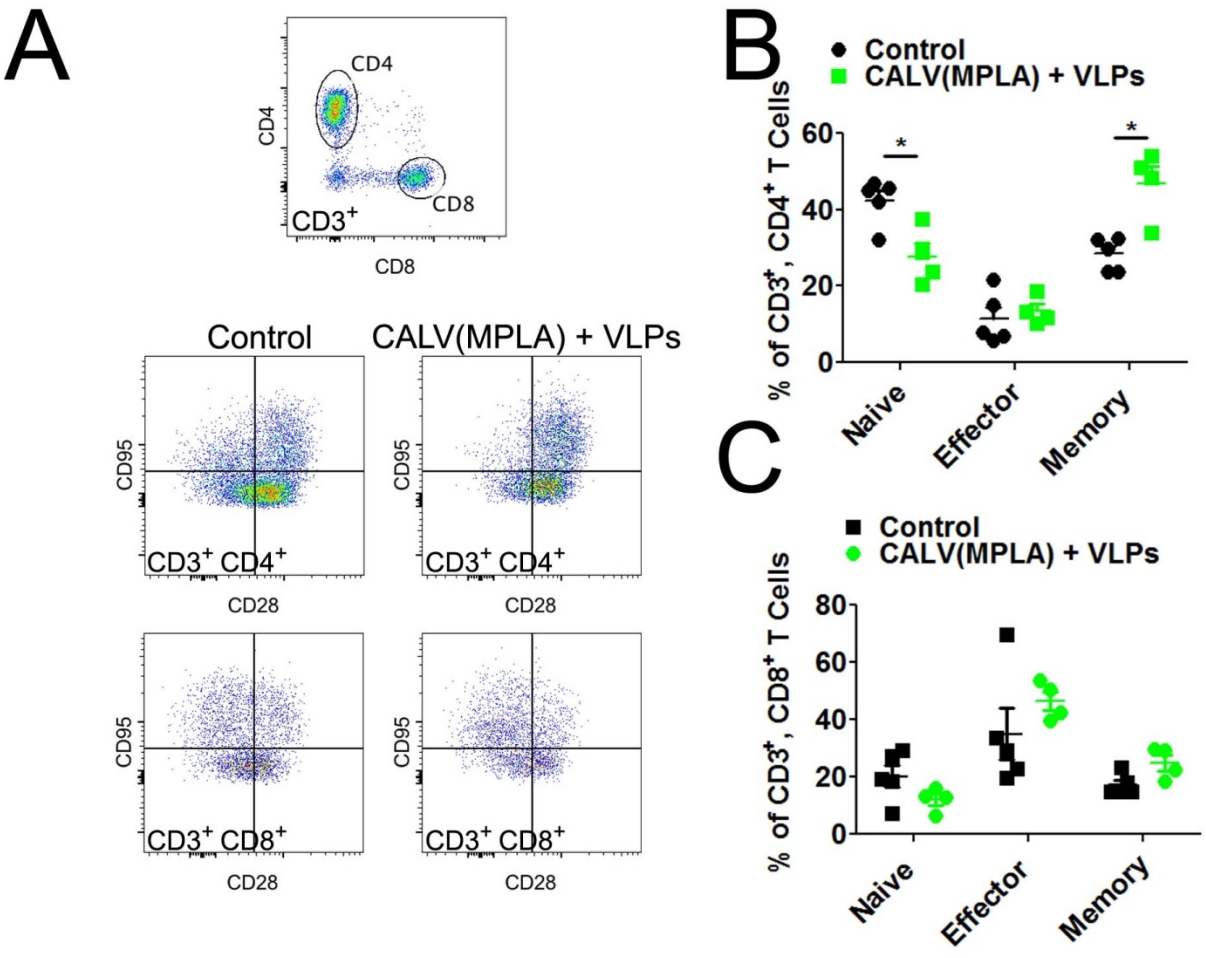

Figure 5: Circulating naïve, effector, and memory T cells. (A) Representative flow cytometry of T cells (CD3+) from control and CALV(MPLA) +VLPs immunized macaques with further gating distinguishing between CD4+ and CD8+ T cells, and naïve (CD28+, CD95-), effector (CD28-, CD95+) and memory (CD28+, CD95+) cell surface expression. (B) Naïve, effector, and memory populations as a percent of CD4+ T cells (C) Naïve, effector, and memory populations as a percent of CD8+ T cells. * indicates $p < 0.05$ by 2-Way ANOVA and Bonferroni posttest.

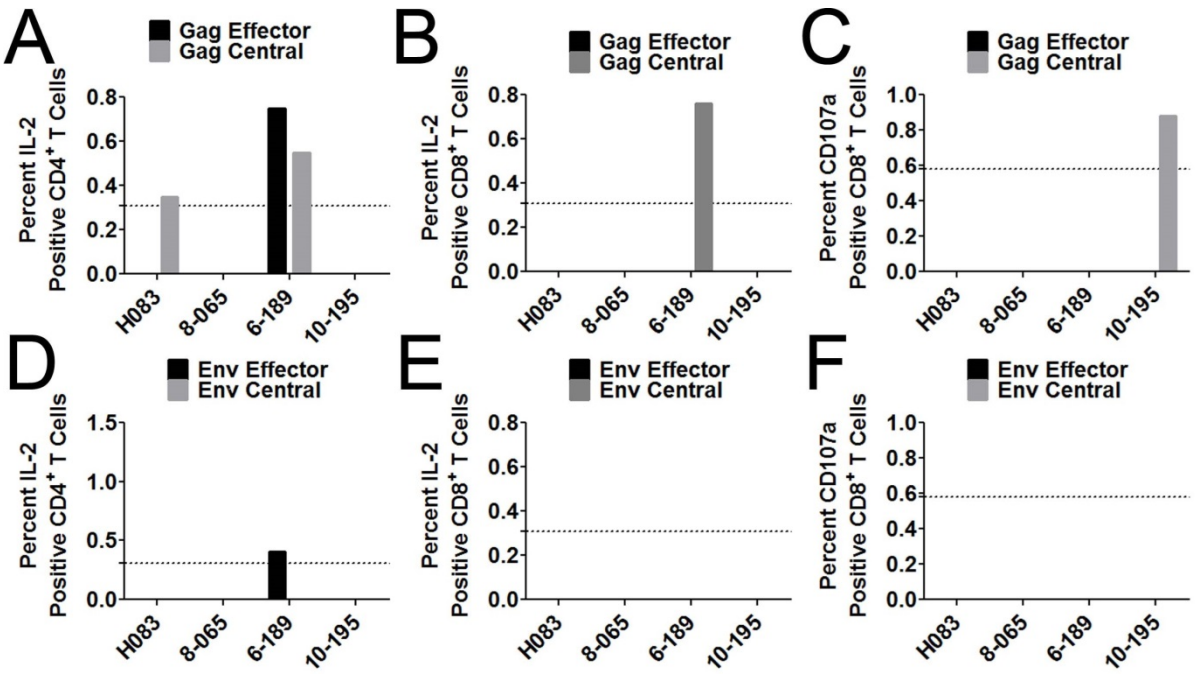

Figure 6: Pre-challenge PBMC SHIV antigen-specific T cell evaluation. PBMCs were stimulated with pooled SIV Gag peptides (2 μg/ml) or pooled HIV sf162 Env peptides (2 μg/ml) pooled. IL-2 expression or CD107a membrane localization were determined ineffector (CD28-, CD95+) and memory (CD28+, CD95+) T cells. (A) Gag-specific CD3+, CD4+ IL-2 expression; (B) Gag-specific CD3+, CD8+ IL-2 expression; (C) Gag-specific CD3+ CD8+ T cell membrane localization of CD107a. (D) Env-specific CD3+, CD4+ IL-2 expression (E) Env-specific CD3+, CD8+IL-2 expression. (F) Env-specific CD3+ CD8+ T cell membrane localization of CD107a. Dotted line represents 97% positivity threshold for the indicated T cell subset and cytokine.

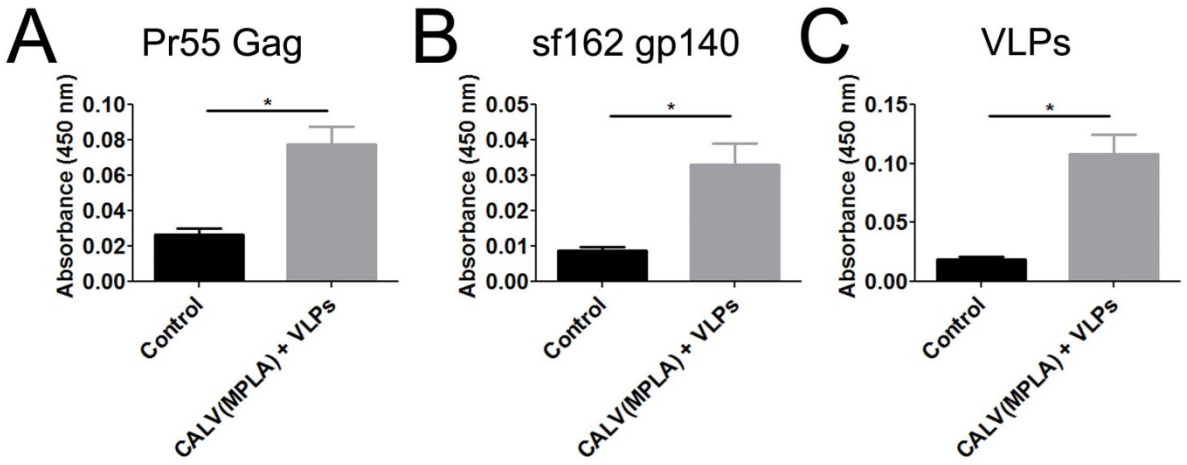

Figure 7: Pre-challenge immunogen-specific ELISA antibody titers. Pre-challenge IgG ELISA of plasma diluted 1:100 from control and CALV(MPLA) +VLPs immunized macaques specific (A) Pr55 Gag, (B) sf162 gp140, and (C) VLPs. * indicates $p < 0.05$ by Welch's t-test. Assay repeated in triplicate, total n=9 per animal.

SHIV VLPs vaccination induces partial protection from SHIV viral challenge

The rhesus macaques were intrarectally challenged on week 52 with a single dose SHIVsf162.P3 viral challenge of 1,000 TCID50. SIV copy number and CD4 count were monitored daily and then weekly with peek viremia occurring 2 weeks after challenge (Figure 8A). There was no difference between groups in peak viremia; however, but no detectable levels of SIV were detected at any time point after challenge in 10-195 (Figure 8B). Similar to SIV copy number, there was no difference in CD4 copy number between groups at the time points measured (Figure 8C and 8D). The macaques were monitored for 51 weeks after challenge, sacrificed, and cytokine staining was performed to analyze CD8 cellular function (Figure 9). Gag specific IFN-γ and CD107a CD8+ T cells were observed in 6-189 after peptide stimulation. Env specific IL-2 and CD107a CD8+ T cells were also observed in 6-189 after peptide stimulation. Gag specific CD107a CD8+ cells were also detected in the 8-127, of the control group. Of note, rhesus H083 was sacrificed before the expected endpoint due to neurological issues; it is unclear if this

was due to neuro-AIDS or her advanced age (17 years old) compared to the other macaques.

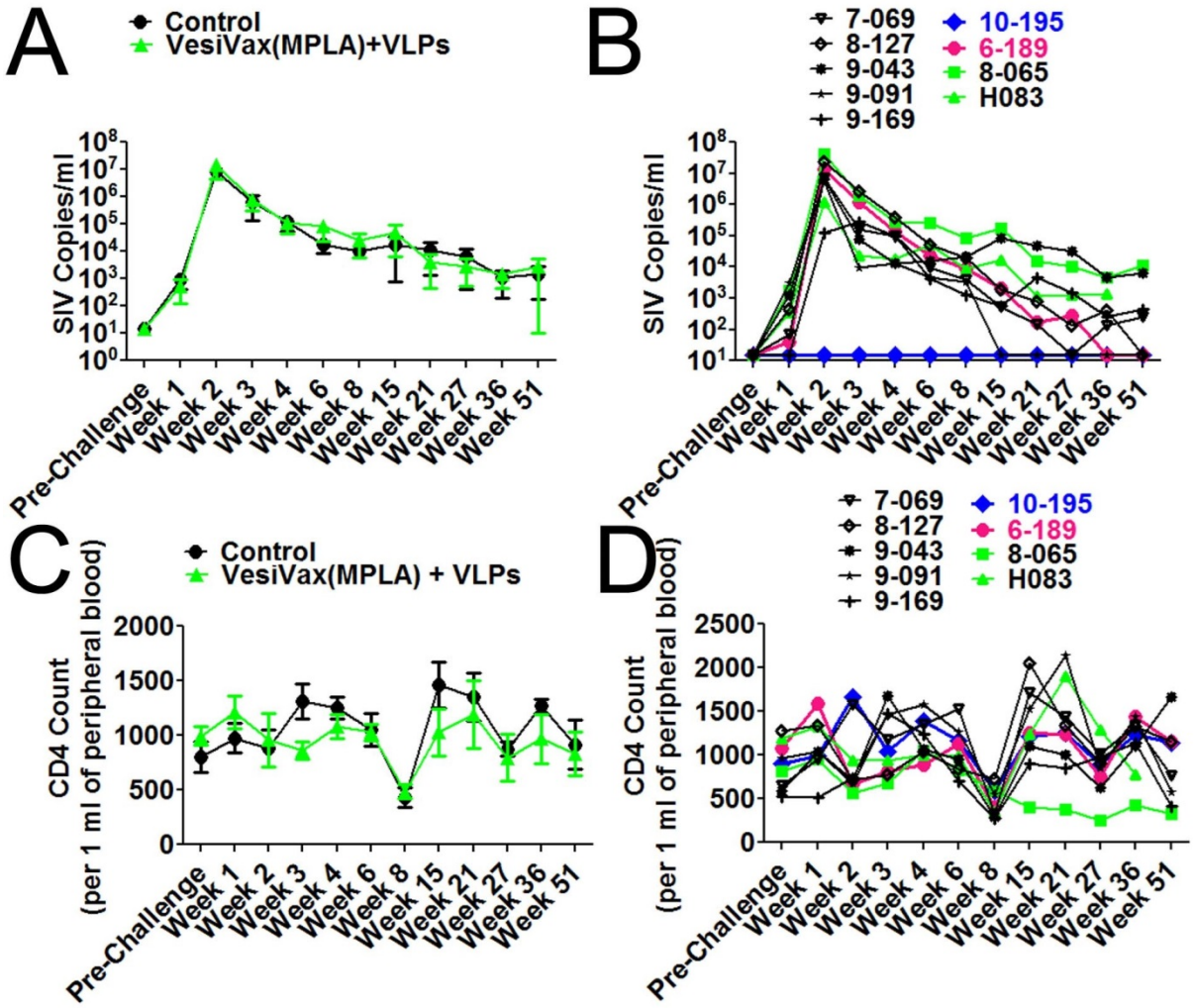

Figure 8: Post Challenge SIV Viral Load and CD4 Count. (A) Grouped plasma SIV viral load of control and CALV(MPLA)+VLPs immunized macaques. (B) Individual plasma SIV viral load of control and CALV(MPLA)+VLPs immunized macaques. (C) Number of CD4+ T cells per 1 ml of whole blood in control and CALV(MPLA)+VLPs immunized macaques. (D) Individual CD4+ T cells per 1 ml of whole blood in control and CALV(MPLA)+VLPs immunized macaques.

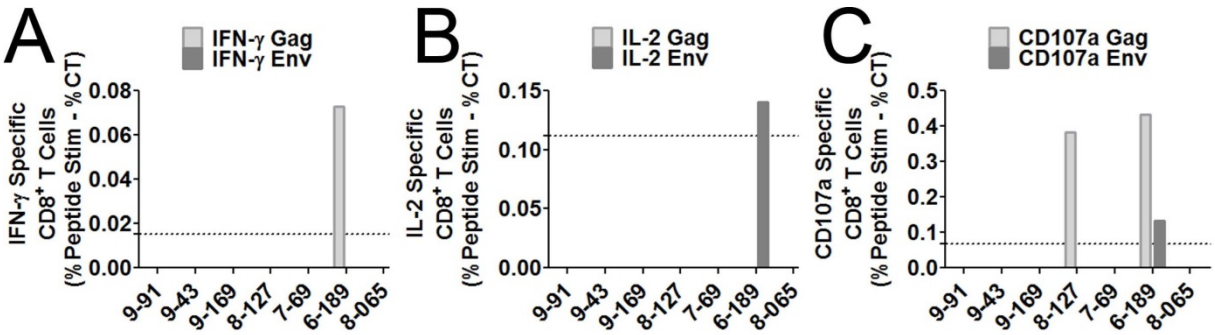

Figure 9: Cytokine Staining at Experiment Termination. (A) IFN-γ expression in CD8+ PBMCs after stimulation with pooled SIV Gag or sf162 Env peptides (2 μg/ml). (B) IL-2 expression in CD8+ PBMCs after stimulation with pooled SIV Gag or sf162 Env peptides (2 μg/ml). (C) CD107a membrane localization in CD8+ PBMCs after stimulation with pooled SIV Gag or sf162 Env peptides (2 μg/ml). Dotted line represents 97% positivity threshold for the indicated T cell subset and cytokine.

DISCUSSION AND CONCLUSION

In this study we have demonstrated that vaccination with SHIV/HIV VLPs offers partial protection and efficient viral load control in a non-human primate SHIV challenge model. Four macaques were immunized with our novel route of administration of intranasal prime followed by three sub-cheek

boosts with baculovirus expression system produced SIV Gag VLPs presenting sf162 gp120/gp41 and human CD40L on their surface, and a final boost of mammalian expression system produced HIV Gag VLPs presenting BaL gp120/gp41 on their surface also administered *via* the sub-cheek route. Of the four macaques, 10-195, which exhibited both cellular and humoral immune responses, was not infected after high-dose SHIVsf162.P3 virus challenge, and 6-189, in which we observed robust effector T cell function, had undetectable SIV copy number by the completion of the study. Although only 25% of the vaccine group was protected from virus challenge, an additional 25% of the vaccine group could effectively control viral load within the experimental time frame, a detailed dissection of each immune cell population's phenotype and functional analysis in each of the individuals could offer valuable information for a future large cohort study.

Our VLPs induced a minimal cellular response; with no difference in central or effector memory CD8+ T cell percentages compared to controls; however, we did observe central memory CD8+ T cell Gag specific CD107a membrane localization upon peptide stimulation in one of the four macaques (10-195), the one who finally protected from viral challenge. Previous studies in rhesus macaques immunized with SIV or SHIV VLPs have reported specific Gag, or Gag and Env CTL responses induced by VLP immunization [25-27]. Although these studies showed that VLP immunization induced an SHIV cytotoxic response, the observations were made with target: effector assays and were not broken down into central or effector memory CD8+ T cells. The importance of Gag specific effector CD8+ T cells has been repeatedly demonstrated to either control or prevent SHIV infection [9,12,28-30]. Although central memory CD8+ T cells have not been shown to prevent SHIV infection, Gag specific central memory CD8+ population of elite human controllers have maintained viral control even with the effector memory CD8+ T cells depleted [31,32]. The induction of functional central memory CD8+ T cells instead of effector CD8+ T cells by our vaccine could be due to a number of factors, in particular the presence of CD40L on the VLP surface or the strength of the antigen. CD40L has been shown to promote the formation of central memory CD8+ T cells, while in mice central memory CD8+ T cells have been shown to develop over effector memory CD8+ T cells based on the potency of the antigen, with less potent antigens leading to differentiation into central memory, and more potent antigens favoring effector memory differentiation [33,34]. Therefore, our VLPs elicited a weak CD8 response directed at Gag; however, 10-195 presented CD107a specific central memory cells and 6-189, in which SIV copy number was undetectable 7 months after infection, had Gag specific central memory IL-2 before challenge and had developed Gag specific CD8 effector function by the termination of the study.

VLPs are thought to be better inducers of humoral immunity compared to cellular immunity, supported by previous studies of VLPs in macaques showing the induction of neutralizing antibodies and strong titers against envelope protein [26,27,35,36]. Similarly, our VLPs induced Gag and VLP specific antibodies in all four immunized macaques, while three of the four immunized macaques showed increased titers towards sf162 gp140 envelope during the immunization period. Prior to virus challenge, three of the immunized macaques had 2 to 6-fold higher sf162 gp140 titers compared to control macaques (H083's titers were no different than controls). 10-195, which had the highest titer, was protected from virus challenge. Because our sample size was four macaques and we saw protection in only one, it is difficult to find correlations between our immune parameters and protection. However, Barouch et al thoroughly examined correlations between protection and humoral mechanisms and found the strongest correlate of protection to be Env-specific antibody binding, followed by neutralizing and non-neutralizing mechanisms [8].

HIV vaccines have uncovered a collection of anti-envelope antibody derived protective mechanisms against HIV/SHIV infection, including neutralizing antibodies, antibody dependent immune systems (e.g. ADVI), and concerted actions between antibodies and other immune cells (e.g. ADCC) [19,37]. HIV neutralizing antibodies have been shown repeatedly to provide protection from virus challenge; in addition, both plasma neutralizing antibodies and localized mucosal antibodies have been shown to correlate to protection from virus challenge in macaques [8,10,38]. While no plasma neutralizing antibodies were detected in any of the four macaques, localized neutralizing antibodies were detected in two of the immunized macaques prior to challenge. However, they were not protected and their peak viremia was similar to that of controls. In humans, the RV144 trial indicated the importance of non-neutralizing anti-envelope antibodies directed at the V1V2 region, as well as ADCC [39,40]. No V1V2 binding or ADCC was detected in the pre-challenge plasma of the four macaques (data not shown).

Of the four macaques in our immunized group, one (10-195) was protected from virus challenge and a second (6-189) macaque had improved viral control compared to the average of the control group. While control of virus was likely due to 6-189's development of cellular immunity, 10-195 protection is more likely due to her anti-envelope humoral immunity. Our studies' small sample size limits the scope of our mechanistic understanding, but it does demonstrate that VLPs can offer both protection and improved viral control against SHIV. Additional studies with a larger sample size and VLPs capable of inducing and maintaining a more robust anti-envelope response would allow for better characterization of virus protection induced by VLPs.

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REFERENCES

1. Zhang S, Cubas R, Li M, Chen C, Yao Q. Virus-like particle vaccine activates conventional B2 cells and promotes B cell differentiation to IgG2a producing plasma cells. Mol Immunol. 2009;46:1988–2001.
2. Buonaguro L, Tornesello ML, Tagliamonte M, Gallo RC, Wang LX, Kamin-Lewis R, et al. Baculovirus-derived human immunodeficiency virus type 1 virus-like particles activate dendritic cells and induce *ex vivo* T-cell responses. J Virol. 2006;80: 9134–43.
3. Sailaja G, Skountzou I, Quan FS, Compans RW, Kang SM. Human immunodeficiency virus-like particles activate multiple types of immune cells. Virology. 2007;362: 331–41.
4. Zhang R, Zhang S, Li M, Chen C, Yao Q. Incorporation of CD40 ligand into SHIV virus-like particles (VLP) enhances SHIV-VLP-induced dendritic cell activation and boosts immune responses against HIV. Vaccine. 2010;28: 5114–5127.
5. Harouse JM, Gettie A, Eshetu T, Tan RC, Bohm R, Blanchard J, et al. Mucosal transmission and induction of simian AIDS by CCR5-specific simian/human immunodeficiency virus SHIV(SF162P3). J Virol. 2001;75: 1990–1995.
6. Nehete PN, Nehete BP, Hill L, Manuri PR, Baladandayuthapani V, Feng L, et al. Selective induction of cell-mediated immunity and protection of rhesus macaques from chronic SHIV(KU2) infection by prophylactic vaccination with a conserved HIV-1 envelope peptide-cocktail. Virology. 2008;370: 130–141.
7. Reimann KA, Li JT, Veazey R, Halloran M, Park IW, Karlsson GB, et al. A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after *in vivo* passage in rhesus monkeys. J Virol. 1996;70: 6922–6928.
8. Barouch DH, Stephenson KE, Borducchi EN, Smith K, Stanley K, McNally AG, et al. Protective efficacy of a global HIV-1 mosaic vaccine against heterologous SHIV challenges in rhesus monkeys. Cell. Elsevier Inc.; 2013;155: 531–539.
9. Hansen SG, Vieville C, Whizin N, Coyne-Johnson L, Siess DC, Drummond DD, et al. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. Nat Med. 2009;15: 293–299.
10. Li H, Liu J, Carville A, Mansfield KG, Lynch D, Barouch DH. Durable mucosal simian immunodeficiency virus-specific effector memory T lymphocyte responses elicited by recombinant adenovirus vectors in rhesus monkeys. J Virol. 2011;85: 11007–11015.
11. Barouch DH, Alter G, Broge T, Linde C, Ackerman ME, Brown EP, et al. Protective efficacy of adenovirus/protein vaccines against SIV challenges in rhesus monkeys. Science. 2015;349: 320–324.
12. Genescà M, Rourke T, Li J, Bost K, Chohan B, McChesney MB, et al. Live attenuated lentivirus infection elicits polyfunctional simian immunodeficiency virus Gag-specific CD8+ T cells with reduced apoptotic susceptibility in rhesus macaques that control virus replication after challenge with pathogenic SIVmac239. J Immunol. 2007;179: 4732–4740.
13. Fazilleau N, Mark L, McHeyzer-Williams L, McHeyzer-Williams M. Follicular Helper T Cells: Lineage and Location. Immunity. 2009;30: 324–335.
14. Kuhrt D, Faith S, Hattemer A, Leone A, Sodora D, Picker L, et al. Naïve and memory B cells in the rhesus macaque can be differentiated by surface expression of CD27 and have differential responses to CD40 ligation. J Immunol Methods. 2011;363: 166–176.
15. Neumann B, Klippert A, Raue K, Sopper S, Stahl-Hennig C. Characterization of B and plasma cells in blood, bone marrow, and secondary lymphoid organs of rhesus macaques by multicolor flow cytometry. J Leukoc Biol. 2015;97: 19–30.
16. Shibata R, Igarashi T, Haigwood N, Buckler-White A, Ogert R, Ross W, et al. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat Med. 1999;5: 204–210.
17. Ferrari G, Pollara J, Kozink D, Harms T, Drinker M, Freel S, et al. An HIV-1 gp120 envelope human monoclonal antibody that recognizes a C1 conformational epitope mediates potent antibody-dependent cellular cytotoxicity (ADCC) activity and defines a common ADCC epitope in human HIV-1 serum. J Virol. 2011;85: 7029–7036.
18. Holl V, Peressin M, Decoville T, Schmidt S, Zolla-Pazner S, Aubertin A-M, et al. Nonneutralizing antibodies are able to inhibit human immunodeficiency virus type 1 replication in macrophages and immature dendritic cells. J Virol. 2006;80: 6177–6181.
19. Asmal M, Sun Y, Lane S, Yeh W, Schmidt SD, Mascola JR, et al. Antibody-dependent cell-mediated viral inhibition emerges after simian immunodeficiency virus SIVmac251 infection of rhesus monkeys coincident with gp140-binding antibodies and is effective against neutralization-resistant viruses. J Virol. 2011;85: 5465–5475.
20. Rosenberg ES, Billingsley JM, Caliendo AM, Boswell SL, Sax PE, Kalams SA, et al. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. Science. 1997;278: 1447–1450.
21. Letvin NL, Mascola JR, Sun Y, Gorgone DA, Buzby AP, Xu L, et al. Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. Science. 2006;312: 1530–1533.
22. Poteet E, Lewis P, Li F, Zhang S, Gu J, Chen C, et al. A Novel Prime and Boost Regimen of HIV Virus-Like Particles with TLR4 Adjuvant MPLA Induces Th1 Oriented Immune Responses against HIV. PLoS One. 2015;10: e0136862.
23. Montefiori DC. Measuring HIV neutralization in a luciferase reporter gene assay. Methods Mol Biol. 2009;485: 395–405.
24. Donaldson MM, Kao S-F, Eslamizar L, Gee C, Koopman G, Lifton M, et al. Optimization and qualification of an 8-color intracellular cytokine staining assay for quantifying T cell responses in rhesus macaques for pre-clinical vaccine studies. J Immunol Methods. 2012;386: 10–21.
25. Paliard X, Liu Y, Wagner R, Wolf H, Baenziger J, Walker CM. Priming of strong, broad, and long-lived HIV type 1 p55gag-specific CD8+ cytotoxic T cells after administration of a virus-like particle vaccine in rhesus macaques. AIDS Res Hum Retroviruses. 2000;16: 273–282.

26. Wagner R, Teeuwsen VJ, Deml L, Notka F, Haaksma AG, Jhagjhoorsingh SS, et al. Cytotoxic T cells and neutralizing antibodies induced in rhesus monkeys by virus-like particle HIV vaccines in the absence of protection from SHIV infection. Virology. 1998;245: 65–74.
27. Notka F, Stahl-Hennig C, Dittmer U, Wolf H, Wagner R. Accelerated clearance of SHIV in rhesus monkeys by virus-like particle vaccines is dependent on induction of neutralizing antibodies. Vaccine. 1999;18: 291–301.
28. Liu J, O'Brien KL, Lynch DM, Simmons NL, La Porte A, Riggs AM, et al. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. Nature. 2009;457: 87–91.
29. Acierno PM, Schmitz JE, Gorgone DA, Sun Y, Santra S, Seaman MS, et al. Preservation of functional virus-specific memory CD8+ T lymphocytes in vaccinated, simian human immunodeficiency virus-infected rhesus monkeys. J Immunol. 2006;176: 5338–5345.
30. Genescà M, Skinner PJ, Hong JJ, Li J, Lu D, McChesney MB, et al. With minimal systemic T-cell expansion, CD8+ T Cells mediate protection of rhesus macaques immunized with attenuated simian-human immunodeficiency virus SHIV89.6 from vaginal challenge with simian immunodeficiency virus. J Virol. 2008;82: 11181–1196.
31. Ndhlovu ZM, Proudfoot J, Cesa K, Alvino DM, McMullen A, Vine S, et al. Elite controllers with low to absent effector CD8+ T cell responses maintain highly functional, broadly directed central memory responses. J Virol. 2012;86: 6959–6969.
32. Ndhlovu ZM, Stampouloglou E, Cesa K, Mavrothalassitis O, Alvino DM, Li JZ, et al. The Breadth of Expandable Memory CD8+ T Cells Inversely Correlates with Residual Viral Loads in HIV Elite Controllers. J Virol. 2015;89: 10735–10747.
33. van Faassen H, Saldanha M, Gilbertson D, Dudani R, Krishnan L, Sad S. Reducing the stimulation of CD8+ T cells during infection with intracellular bacteria promotes differentiation primarily into a central (CD62LhighCD44high) subset. J Immunol. 2005;174: 5341–5350.
34. Trella E, Raafat N, Mengus C, Traunecker E, Governa V, Heidtmann S, et al. CD40 ligand-expressing recombinant vaccinia virus promotes the generation of CD8(+) central memory T cells. Eur J Immunol. 2016;46: 420–431.
35. Koup RA, Douek DC. Vaccine design for CD8 T lymphocyte responses. Cold Spring Harb Perspect Med. 2011;1: 1–15.
36. Iyer SS, Gangadhara S, Victor B, Shen X, Chen X, Nabi R, et al. Virus-like particles displaying trimeric SIV envelope gp160 enhance the breadth of DNA/MVA SIV vaccine induced antibody responses in rhesus macaques. J Virol. 2016;90:8842-54.
37. Fouts TR, Bagley K, Prado IJ, Bobb KL, Schwartz JA, Xu R, et al. Balance of cellular and humoral immunity determines the level of protection by HIV vaccines in rhesus macaque models of HIV infection. Proc Natl Acad Sci U S A. 2015;112: E992-E999.
38. Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, et al. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med. 2000;6: 207–210.
39. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N Engl J Med. 2012;366: 1275–1286.
40. Sellhorn G, Caldwell Z, Mineart C, Stamatatos L. Improving the expression of recombinant soluble HIV Envelope glycoproteins using pseudo-stable transient transfection. Vaccine. 2009;28: 430–436.