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Inclusion of a CRF01_AE HIV envelope protein boost with a DNA/MVA prime-boost vaccine; impact on humoral and cellular immunogenicity and viral load reduction after SHIV-E challenge

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Abstract

The current study assessed the immunogenicity and protective efficacy of various prime-boost vaccine regimens in rhesus macaques using combinations of recombinant DNA (rDNA), recombinant MVA (rMVA), and subunit gp140 protein. The rDNA and rMVA vectors were constructed to express Env from HIV-1 subtype CRF01_AE and Gag-Pol from CRF01_AE or SIVmac 239. One of the rMVAs, MVA/CMDR, has been recently tested in humans. Immunizations were administered at months 0 and 1 (prime) and months 3 and 6 (boost). After priming, HIV env-specific serum IgG was detected in monkeys receiving gp140 alone or rMVA but not in those receiving rDNA. Titers were enhanced in these groups after boosting either with gp140 alone or with rMVA plus gp140. The groups that received the rDNA prime developed env-specific IgG after boosting with rMVA with or without gp140. HIV Env-specific serum IgG binding antibodies were elicited more frequently and of higher titer, and breadth of neutralizing antibodies was increased with the inclusion of the subunit Env boost. T cell responses were measured by tetramer binding to Gag p11c in Mamu-A*01 macaques, and by IFN-gamma ELISPOT assay to SIV-Gag. T cell responses were induced after vaccination with the highest responses seen in macaques immunized with rDNA and rMVA. Macaques were challenged intravenously with a novel SHIV-E virus (SIVmac239 Gag-Pol with an HIV-1 subtype E-Env

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CAR402). Post challenge with SHIV-E, antibody titers were boosted in all groups and peaked at 4 weeks. Robust T cell responses were seen in all groups post challenge and in macaques immunized with rDNA and rMVA a clear boosting of responses was seen. A greater than 2 log drop in RNA copies/ml at peak viremia and earlier set point was achieved in macaques primed with rDNA, and boosted with rMVA/SHIV-AE plus gp140. Post challenge viremia in macaques immunized with other regimens was not significantly different to that of controls. These results demonstrate that a gp140 subunit and inclusion of SIV Gag-Pol may be critical for control of SHIV post challenge.

1. Introduction

A considerable effort has been expended over the last twenty years in the quest to develop a safe and effective HIV-1 vaccine; five HIV vaccine efficacy trials have been conducted to date. Two trials tested combinations of recombinant Env proteins in adjuvant (AIDSVAX 003 and AIDSVAX 004 sponsored by VaxGen Inc) and two trials tested Adenovirus 5 (Ad5) based vectors encoding Gag, Pol and Nef (STEP and Phambili trials) through the HIV Vaccine Trials Network and Merck & Co [1,2,3,4]. All of these trials failed to demonstrate efficacy; however, a recent trial conducted in Thailand (RV144 trial) demonstrated a 31.2% level of efficacy with a 'prime-boost' combination consisting of a Canaripox expressing Gag, Pol and Env together with a VaxGen Env protein (AIDSVAX B/E) in adjuvant [5]. This partial success has underscored the importance of prime-boost vaccine regimens for prevention or containment of HIV infection and resulted in renewed interest in poxvirus vectored vaccines boosted by Env proteins in adjuvant [6,7].

Prime-boost vaccine regimens use different vaccine vectors and/or subunit proteins for sequential immunization in order to enhance T cell responses or to enhance both T cell and antibody responses [8,9,10,11,12]. DNA plasmids as a prime followed by Modified Vaccinia Ankara (MVA) as a boost is the most extensively studied prime-boost vaccine approach to date. Both vectors have demonstrated strong safety profiles in humans administered alone or together. Both DNA and MVA vaccines allow for the construction of multi-component vectors potentially suitable for all regions and strains of the virus. In addition, both have already been tested in humans with no reported safety events even at relatively high doses (up to 8 mg of DNA) [13,14,15,16,17,18]. Robust HIV-specific immune responses are induced in humans vaccinated with rMVAs expressing *env* and *gag-pol* [19,20,21,22]. The combination of DNA and MVA or other poxviruses in humans induces a spectrum of immune responses, including polyfunctional T cells and strong vaccine induced antibody responses with a trend indicating that DNA priming provides broader T cell responses whereas multiple MVA vaccinations alone tend to enhance the magnitude of antibody induced responses [15,17,23,24].

Multiple animal pre-clinical immunogenicity and efficacy studies conducted using MVA vaccination strategies in non-human primates have demonstrated consistent and sustained specific CD8 T-cell responses and partial control of pathogenic SIV/SHIV challenges [25,26,27,28,29]. Several studies have demonstrated that the level of control afforded by vaccination is related to and dependent on the nature of the challenge virus [25,26,30,31,32,33]. DNA vaccines encoding HIV/SIV antigens in combination with recombinant poxvirus boosting or combined with cytokines generate robust SIV/SHIV-specific CD8 T-cell responses in non-human primates, however correlates of protection and or control have not been clearly defined [28,34,35,36].

There is general agreement that an HIV vaccine should induce cellular as well as neutralizing antibody (NAbs) responses, but inducing the latter at sufficiently high titers and

breadth to neutralize typical primary HIV isolates has proven to be a difficult task. Substantial enhancement of antibody binding and neutralizing titers in small animals and primates has been achieved after DNA priming by boosting with soluble HIV-1 subunit proteins [37,38]. However, current subunit vaccines have not been effective at inducing antibodies capable of neutralizing primary isolates (tier 2 and 3 viruses) in humans [5,39,40,41,42,43,44,45]. More recent studies using potent adjuvants and other modalities to enhance antibody responses have only provided modest improvements in magnitude of NABs to tier one isolates [15,46,47,48,49]. These data expose the difficulty of constructing a subunit vaccine with proper conformational structure to induce potent neutralizing and other protective antibody responses [50,51,52].

One approach that has shown some promise is the use of modified Env including trimeric and other oligomeric forms in the hope of more closely mimicking the antigenic characteristics of Env protein on the surface of virions and infected cells. Trimeric HIV-1CN54 clade C gp140 Env administered by an intra-vaginal route in macaques elicited antibodies with tier 1 neutralizing activity and recently trimeric SF162 Env has been tested in humans in a DNA prime protein boost study with demonstration of tier 1 neutralization [47,53]. In this study we tested a combination approach to HIV-1 vaccination where heterologous circulating recombinant form CRF01_AE-Env (Env-E) proteins were used in the rDNA (93TH966.8), rMVA-(CM235) and protein subunit (CM235) boost vaccine regimen followed by a SHIV-E (CAR402) challenge. A combined approach of different inter-subtype E immunogens (as described here) or inter-subtype M as described by others may be the best approach to cover variability of Env for elicitation of both cellular and humoral response [23,47,54,55,56,57]. We combined oligomeric gp140 and rMVA and rDNA vectors that express an HIV-1 E env and SIV₂₃₉ gag-pol. The vaccination regimen described in the present report induced high levels of antibodies as well as cellular responses, which varied depending on the vaccine regimen, dose and dosing schedule. Adjuvanted subunit boosts can be added to DNA/MVA prime-boost vaccination regimens to improve antibody responses without compromising CD8 T cell specific responses.

2. Materials and Methods

2.1. Animals

A total of 31 mature Indian-origin rhesus macaques were used for this study; there were 23 females and 8 males with an average weight of 5.7 kg. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations related to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (NRC Publication, 1996 edition). All animal experiments were approved by the WRAIR/NMRC Institutional Animal Care and Use Committee with research conducted in a facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

2.2. Vaccine Antigens

A rMVA encoding SIVmac239 gag-pol and HIV-1 E Env (MVA/SHIV-A_E) was constructed by co-infection of CEF cells with 5 infectious units each of MVA/CM235env [58] and MVA/SIV239-Gag-Pol [30]. Infection with MVA/SIV239-Gag-Pol results in foci with a unique rounded-up appearance. Thus, selection of the double recombinant was based on rounded-up morphology and Env immunostaining. After clonal purification and amplification, expression of both antigens was verified by Western blotting and immunostaining. Construction of the MVA-CMDR containing HIV-1 env, gag and pol genes has been described in full previously [58]. Monkeys were immunized with 1×10^8 pfu by intramuscular (i.m.) injection in the rear hind legs using a dose volume of 1.0 ml. The

plasmid DNA construct containing the HIV-E envelope (subtype CRF01_AE strain Thailand, 93TH966.8) and the plasmid DNA construct containing the SIV Gag-Pol mac239 were formulated in 0.25% bupivacaine. A 2 mg dose of rDNA was administered i.m. in the rear hind legs. Subunit gp140 (from R5-using primary subtype E strain CM235) was produced from recombinant vaccinia virus vWS1 (Sugiura, Earl, Moss, unpublished) as previously described [59]. A dose of 300 µg of protein was formulated in Ras3C (Ribi adjuvant system—three components adjuvant) and administered i.m. in the rear hind leg. Ras3C consists of 2.0% (vol/vol) squalene oil-in-water emulsion containing 250 µg each of MPL, *Mycobacterium phlei* cell wall skeleton and synthetic dicorynomycolate (S-TDCM) per ml (Sigma, St. Louis, MO) [60]. The vaccine components are shown in Table 1.

2.3 Study Schedules

Male and female Indian-origin rhesus macaques were assessed 3 times prior to vaccination to establish baseline safety and immunology parameters. Each group received a total of four vaccinations. Within each group, the first two vaccinations (called prime) were identical as were the second two vaccinations (called boost) (Table 2). Group 1 was primed with gp140 and boosted with gp140 formulated in Ras3C. Group 2 was primed with MVA/SHIV-AE and boosted with MVA/SHIV-AE plus gp140 formulated in Ras3C. Group 3 was primed with DNA/env and DNA/gag-pol and boosted with MVA/SHIV-AE. Group 4 was primed with DNA/env and DNA/gag-pol and boosted with MVA/SHIV-AE plus gp140 formulated in Ras3C. Group 5 was primed with MVA/CMDR and boosted with MVA/CMDR plus gp140 formulated in Ras3C. Group 6 was primed with control DNA control and boosted with control MVA. Group 7 was primed and boosted with control MVA. The study groups are shown in Table 2.

2.4 Humoral Immune responses in Macaques

Serum from macaques from the various timepoints to include 3 pre-immunization timepoints was screened individually for antigen-specific antibody responses. To measure binding to the immunogen, an enzyme immunoassay (EIA) was used as described previously [61,62]. Briefly, a sheep antibody to the carboxy terminus of gp120 (D7324) in PBS (pH 7.4; with 0.01% thimerosal) was coated overnight at 4°C onto Immulon 2 microtiter plates and used to capture gp140. Plates were washed twice with wash buffer (PBS with 0.1% Tween 20 [pH 7.4]) prior to incubation with gp140 (1 µg/ml (diluted in wash buffer with 5% skim milk [pH 7.4]) for 1 h at 37°C followed by two-fold dilutions of monkey sera (diluted in wash buffer with 5% skim milk [pH 7.4]) for 1 h at 37°C. Plates were washed three times with wash buffer and incubated with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (diluted 1:4,000 in wash buffer with 5% skim milk, pH 7.4; Southern Biotechnologies, Birmingham, Ala.). After a 1-h incubation at 37°C, plates were washed three times, after which the substrate ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid); Kirkegaard & Perry, Gaithersburg, MD.] was added. The reaction was stopped with 0.5% sodium dodecyl sulfate after 30 min at 37°C. Serum endpoint titers were determined as the highest serum dilution with enzyme-linked immunosorbent assay (ELISA) optical density (OD) signals greater than twice the mean plus two times the standard deviations of the individual preimmune monkey sera (typically >0.10 OD).

Pre and post-immunization serum samples from each macaque were assayed in several neutralization assay formats, to include PBMC and T cell-line adapted assays, as described below. Serum aliquots were heat inactivated, centrifuged for 10 min at 14,000 rpm, diluted 1:5 in cRPMI, and filtered through a 0.2 µm filter, prior to use in the neutralization assays. For the T cell line adapted (TCLA) assays, viral stocks were propagated in tissue culture medium in the appropriate cell line, as described elsewhere [63,64,65,66]. The CRF01_AE strain NP03 (an X4, syncytium-inducing virus) was propagated in H9 cells, whereas HIV-1

CM235 (CRF01_AE) was grown both in PHA-stimulated PBMC and in the CCR5+ A3/R5-6 T cell line (R McLinden, manuscript in preparation). For the PBMC and A3/R5-6 cell neutralization data, experiments were performed as described elsewhere using p24 protein production as an endpoint [64,67,68,69]. The sera from 2 weeks post the 4th immunization were tested at a final dilution of 1:10, with the percent reduction of p24 production calculated on the basis of a comparison between pre and post vaccine neutralization. A neutralization value of $\geq 50\%$ was defined as positive; each positive serum sample was titrated against that virus, and the 50% end points were estimated by quadratic projection.

2.5. Macaque CD8 T cell Immunogenicity

Mamu-A*01 typing was kindly performed by Dr. David Watkins at the Wisconsin Primate Center, WI. Of the 31 macaques in the study, 18 were Mamu-A01; these were distributed evenly throughout the groups. PBMC were separated by ficoll-hypaque gradient centrifugation and were used immediately for T cell assays, remaining PBMC were cryopreserved in 10% DMSO and stored in liquid nitrogen. SIV Gag-specific CD8 T cell responses were measured by tetramer staining of fresh PBMC using the Mamu-A*01/p11c tetramer provided by the AIDS Research and Reference Reagent Program (AIDSRRRP). The Interferon-gamma (IFN- γ) ELISPOT assay was performed using previously identified Mamu-A*01 specific peptides. The peptides were made using standard Fmoc solid phase chemistry. The gag peptides were as follows (SIV location in parenthesis); LSPRTLNAW (149-157), CTPYDINQM (181-189 = P11C), QNPIPVGNI (254-262), VNPTLEEMLT (340-349) and LAPVPIPF (372-379). The 5 gag peptides were pooled together for stimulation of PBMC in the ELISPOT assay. SIV-Gag-specific T cell responses in all monkeys were measured using SIVmac239 Gag peptides obtained from the AIDSRRRP. A total of approximately 50 twenty-mer peptides spanning the SIV Gag with an 11-mer amino acid overlap were made up into 5 pools.

Antigen-specific CD8 T cell responses were measured at three time points prior to immunization and at 1 and 2 weeks after each immunization. For the tetramer assay, fresh PBMC were washed in DPBS and stained with anti-CD8 clone SK1 and either anti-CD3 clone SP34, or the p-11c tetramer for 30 min in DPBS at 4°C. After washing with cold DPBS, the cells were fixed and analyzed by flow cytometry. The % of CD8/p11C tetramer positive T cells was determined for each macaque. A positive tetramer response was defined as any staining greater than or equal to the mean plus 3 standard deviations of the % CD8/p11C tetramer positive T cells of all Mamu-A01 macaque prior to vaccination. Of 34 measurements over 2 baseline blood draws, this value was 0.034%. All flow cytometry was performed using a FACSCalibur (BD Biosciences, San Jose, CA). Analysis was performed with CellQuest (BD Biosciences, San Jose, CA).

For each ELISPOT assay, 96-well mixed cellulose plates were coated overnight with goat-anti-human-IFN- γ Mab1-DIK (Mabtech, Cincinnati, OH) at 4°C. The plates were washed twice and blocked with 150 μ l RPMI media containing 10% NHS. Different dilutions of fresh macaque PBMC were added in triplicate to the coated wells as follows: 1) 2 μ g/ml SIV Gag or Pol Mamu-A*01 peptides 2) 2 μ g/ml of pooled SIV peptides 3) PMA/Ionomycin; and 4) No antigen. The plates were incubated at 37°C, 5% CO₂ for 24 h and the production of IFN- γ by T cells were detected by addition of 1:800 dilution of biotinylated anti human IFN- γ (Mabtech, Cincinnati, OH). After washing the wells 5 to 6 times with PBS/0.05% tween 20, peroxidase-labeled streptavidin was added. The spots were developed with AEC substrate. The brown spots were visualized after 10 to 15 min under a stereomicroscope and counted using a Zeiss Axiophot Microscope (Zeiss, Thornwood, NJ). The results were expressed as the number of IFN- γ secreting cells/10⁶ or spot forming cells (SFC) per 10⁶ PBMC. For all the ELISPOT data, the background was subtracted so that values are presented as SIV specific IFN- γ SFC/10⁶ PBMC. The quantitative ELISPOT data is

presented as the sum of the SFC for the set of peptide pools. Thus for the 5 overlapping pools of SIV Gag, the geometric mean of the SIV-Gag specific SFC/10⁶ PBMC for each group of macaques was calculated as the SFC sum of SIV Gag pools 1+2+3+4+5. A positive response was defined as SIV specific responses that were greater than 3 times background and >20 SFC/10⁶ PBMC. For the assay to be valid, the PBMC from each macaque had to respond to PMA.

2.6. Tritiated thymidine (³H) antigen-specific lymphocyte proliferation assay (LPA)

The proliferative responses of fresh macaque PBMC were measured at several pre and post vaccine and post challenge times by incubating 1 x 10⁵ cells per well in 96-well U-bottom polystyrene plates with serial dilutions (3 and 1 µg/ml) of Env and Gag proteins. To measure the lymphoproliferative response to the MVA vector 1x10⁶ and 1x10⁷ PFU of empty vector were added to the wells. Tetanus Toxoid (TT) (Statens Serum Institute, Copenhagen, Denmark) was used at 1 µg/ml as an antigen control, and in a separate plate PBMC were cultured with control mitogens: 2.5 µg/ml of pokeweed mitogen and 10 µg/ml of concanavalin A (Sigma-Aldrich, St Louis, MO). After 3 days of incubation with mitogens and 6 days with the antigens, cells were pulsed with 1 µCi/well of [³H]thymidine for 6 hr then harvested, counted and assessed for [³H]thymidine incorporation. The data are expressed as a lymphocyte stimulation index (LSI = (PBMC cpm with antigen/mitogen) / (PBMC cpm with medium)), to define antigen specificity. Individuals were designated as responders to a given antigen if the LSI in response to that antigen ≥ 5. The LSI for each macaque was averaged over multiple time points; the mean of three baseline measurements (−3, −2 and −1 weeks), the mean of week 13 and 14 and the mean of weeks 27, 28 and 30. Week 34 was taken as the only post challenge time point.

2.7. SHIV challenge

Sedated monkeys were inoculated by intravenous saphenous route using a catheter and a 3-way stopcock. Syringes loaded with virus challenge stock or sterile saline for injection were kept on wet ice until used. The challenge was 80 TCID₅₀ given 4 weeks after the final immunization. The challenge stock was in-vivo titrated SHIV-E-P4.1 stock; SIV mac239 Gag-Pol with subtype E HIV-1 envelope (CAR402, CXCR4) [70]. The Env in the rDNA vector (93TH966.8) is 18% divergent to the challenge virus (E_CAR402), the Env protein boost and the Env in the rMVA boost (CM235) are 18.3% divergent compared to the challenge virus. The SHIV-E-P4.1 stock was kindly provided by Drs. P. Luciw and S. Himathongkham (Department of Pathology and Laboratory Medicine, UC Davis, Davis, CA).

2.8. Virus Isolation

Isolation of SHIV from potentially uninfected animals was performed using uninfected human PBMC as substrate cells. After ficoll-separation or separation from tissues, macaque PBMC were counted and 4 x 10⁶ cells were placed into 10 ml culture tubes with 3 x 10⁶ PHA-stimulated uninfected human PBMC in RPMI 1640 medium supplemented with 10% IL-2, 2 mM L-glutamine, gentamicin, Pen-Strep and 10% FBS. On day 3, the cells were fed with complete medium containing human IL-2 at 10% and human PBMC blasts were added at a concentration of 1 x 10⁶/mL. Flasks were fed with a 100% media change every 3 to 4 days and the cell concentration was adjusted to 1 x 10⁶/mL. Virus replication was detected biweekly for Gag antigen using the Coulter SIV ELISA (Hialeah, FL). Flasks were held until after two positive tests were detected or for a maximum of 28 days.

2.9. Flow cytometry for circulating cell populations

100 μ l of whole blood was added to 12 x 75 falcon tubes and incubated with the desired antibodies for 20 min at 4°C in the dark and then washed twice with wash buffer (PBS w/o Ca^{++} or Mg^{++} with 1 percent BSA). The samples were lysed (Coulter Immunolyse, Fullerton, CA), washed 3 times with wash buffer and fixed in 1% formaldehyde for at least 2 hours or overnight. Samples were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA). Analysis was performed with FlowJo software version 4.0 (TreeStar Inc, Ashland OR) and the percent CD4 and CD8 T cells was enumerated.

2.10. Quantification of SIV Viral RNA

SIV was pelleted from 0.4 ml of plasma by centrifugation at 23,800 g for 1 hr at 4°C. Viral RNA was purified by lysis of the virus with guanidine thiocyanate followed by isopropanol precipitation. The purified SIV RNA was resuspended in 80 μ l of diluent (nuclease-free water, 1 μ g/ml yeast t RNA) and stored at -80°C. A negative extraction control was included in each processing run as a monitor for contamination. The quantity of SIV RNA was measured using Real-Time TaqMan RT-PCR (qRT-PCR) methodology. A 159 base-pair region of the SIV *gag* gene was amplified using reagents purchased from Applied Biosystems (TaqMan PCR Core Reagent with Gold kit, Multiscribe Reverse Transcriptase (RT), and RNase Inhibitor: Foster City, CA) following universal amplification parameters for one-step qRT-PCR: 30 min at 48°C, 10 min at 95°C, and 40 cycles at 95°C for 15 sec and 60°C for 1 min with fluorescence measurement at 60°C. Primers and probes for amplification of the *gag* region of SIV239 were selected using Primer Express (Applied Biosystems, Foster City, CA) and Primer Design (Scientific & Educational Software, Durham, NC) programs. The sequences of the primers and probe used in this study are: 1) TGG-GCA-GCA-AAT-GAA-TTA-GAT-AGA (1158-1181 base position, forward), 2) AGA-TGA-CGC-AGA-CAG-TAT-TAT-AAA-GGC (1308-1282 base position, reverse) and 3) FAM-TAC-TTT-CGG-TCT-TAG-CTC-CAT-TAG-TGC-CAA-CAG-BHQ (1231-1263 base position, probe). The concentration of the primers, probe and MgCl_2 were 600 nM, 180 nM and 2.5 mM respectively. A synthetic SIV *gag* RNA stock of known concentration was serially diluted ten-fold as standards and were amplified in duplicate in each qRT-PCR test run. Sample RNAs were amplified in either duplicate or triplicate. Amplification and procedural controls performed were 1) no template, 2) negative specimen RNA and 3) an SIV positive specimen. Real-time fluorescence measurements were performed in a 7700 ABI PRISM Sequence Detector (Applied Biosystems, Foster City, CA). SIV RNA copies in each qRT-PCR reaction were extrapolated from the SIV standard curve. The concentration of the SIV viral RNA (copies/ml) was calculated by dividing the RNA copy value by the calculated input plasma volume and mean values were reported.

2.11. Statistics

For comparisons of IFN- γ ELISPOT and tetramer responses a two tailed Spearman Correlation analysis was applied, graphs and statistics were performed using GraphPad Prism Software V5.0 (La Jolla, CA). Statistical analysis of the viral load, ELISA and CD4/CD8 percentages was done using a Mann-Whitney test using StatView Software (SAS Institute Inc., San Francisco, CA). P values that were <0.05 were considered a significant difference. Positivity criteria and response rates for tetramer, ELISPOT and neutralization assays were independently analyzed by the Data Coordination and Analysis Center at the Henry M. Jackson Foundation using SAS programming (SAS Institute Inc., Cary, NC).

3. Results

3.1. Humoral Immunogenicity

Serum antibody binding to CM235 gp140 for each of the vaccine groups is illustrated in Figure 1. The data shows that monkeys immunized twice (prime) with either gp140, MVA/SHIV-E or MVA-CMDR (groups 1, 2 and 5) developed Env-specific serum IgG. The addition of a gp140 subunit boost to the rDNA/rMVA immunized macaques (groups 3 and 4) substantially increased Env titers after the 3rd and 4th immunizations (boost). Following viral challenge all groups demonstrated equivalent envelope binding antibodies except the rDNA/rMVA control group (Figure 1). While the highest HIV Env-specific antibody binding titers after the final immunization were obtained in monkeys receiving rDNA/rMVA plus gp140 (group 4), the lowest antibody binding titers were observed in the group which received the same rDNA/rMVA constructs but no Env boost (group 3). Pre-challenge there was a significant difference in the titers between groups that received the rDNA/rMVA and gp140 (groups 2, 4 and 5) and the groups that received rDNA/rMVA (group 3) only ($p < 0.05$, Mann-Whitney test). HIV Env-specific antibodies were seen in the control groups only after the SHIV challenge.

Neutralizing antibody responses against TCLA and primary subtype E HIV-1 were assessed (Table 3). HIV-1 NPO3 is an X4 TCLA subtype CRF01_AE HIV-1 isolate. Only those groups that received a gp140 boost developed neutralizing antibodies against the TCLA strains. The majority of the monkeys receiving the gp140 boost developed neutralizing antibody against both the homologous (CM235) and heterologous (NP03) subtype CRF01_AE TCLA isolates. Overall, neutralizing responses were seen most consistently using the two T cell line based assays and especially with the R5-A301T-cell line and testing with the CM235 matched to the protein boost but heterologous to the vaccine challenge. In macaques receiving the protein boost (groups 1, 2, 4 and 5), 12/20 had responses to HIV isolate NP03 and 17/20 to HIV isolate CM235 in the H9 and R5-A301T-cell line based assays respectively. No neutralizing antibodies were detected in the rDNA/rMVA group that did not receive gp140 boosting. No neutralizing antibodies were detected against the primary CM235 isolate using PBMC as target cells except for one control animal in group 7 (Table 3).

Neutralizing activity was also measured using two Thai Env pseudotyped viruses (TH023.6 and NP03.13) in a TZM-bl assay (Duke University Medical School, NC). Due to sample volume limitations and toxicity observed in stored samples, sera were only available for 12 weeks post 3rd vaccine in groups 2, 5 and the control groups whereas sera were available at peak immunogenicity (2-3 weeks post the 4th immunization) from macaques in groups 1, 3 and 4. In this limited set of samples, contrary to what was seen in the TCLA assays, neutralization of env pseudoviruses was also seen in macaques that were immunized with rDNA and rMVA (group 3), particularly against the tier 1 CRF01_AE TH023 virus (data not shown). There was, however, a high level of background activity observed in some of the control groups (groups 6 and 7). ADCC activity was measured at baseline and at 12 weeks post 3rd immunization (groups 2, 5, 6 and 7) or 2-3 weeks post 4th immunization (groups 1, 3 and 4). None of the macaques had any ADCC activity (data not shown).

3.2. CD8 T cell specific Responses

The Mamu-A*01 p11c tetramer was used to detect SIV Gag specific PBMC from immunized rhesus macaques. At two weeks after the 3rd immunization, all (8/8) Mamu-A*01 positive monkeys that were immunized with either MVA-SHIV alone or rDNA-SHIV-E plus rMVA-SHIV-E with or without the subunit boost (groups 2, 3 and 4) were tetramer positive. Likewise after the fourth immunization with the exception of one macaque

in group 2 all Mamu-A*01 positive macaques had tetramer responses (Table 4). Tetramer binding profiles for one macaque in group 2 is shown in Supplementary Figure 1A. None of the 8 Mamu-A*01 positive monkeys immunized with gp140, MVA-HIV or the MVA and DNA controls (groups 1, 5 and 6) were p11c tetramer positive after the 3rd and 4th immunization. The Gag-specific CD8 T cell responses in group 4, DNA prime/MVA+gp140 boost group were subsequently boosted after the 4th immunization while the DNA immunized macaque tetramer responses remained about the same (data not shown). After the 4th shot and at the time of the challenge the highest magnitude of Gag-specific CD8 responses were detected in the rDNA/rMVA + gp140 group (group 4). All macaques had p11c tetramer positive responses after the SHIV-E challenge and Gag-specific CD8 T cell responses were boosted in all macaques (Table 4 and data not shown). In particular, the strongest Gag-specific CD8 T cell responses were detected in the groups that had a vaccine induced responses prior to challenge. The Mamu-A*01 positive monkeys with no vaccine-induced Gag-specific tetramer response developed positive tetramer responses after challenge (8 out of 8). None of the Mamu-A*01 negative monkeys developed positive tetramer responses (data not shown).

The IFN- γ ELISPOT responses were assessed using a pool of 5 defined Mamu-A*01 Gag epitopes (Figure 2A). At the time of challenge the strongest IFN- γ ELISPOT responses were in the monkeys immunized with rDNA/rMVA plus gp140 (group 4) followed by the rMVA plus subunit (group 2) and rDNA/rMVA groups. No positive IFN- γ responses to the 5 defined Mamu-A*01 Gag epitopes were detected at the time of challenge in the non-Mamu-A*01 monkeys (data not shown). The IFN- γ ELISPOT and tetramer responses correlated with each other in Mamu-A*01 monkeys for both magnitude and positivity criteria. We assessed the correlation between the magnitude of the Gag-p11c tetramer specific CD8 T cell responses and IFN- γ ELISPOT responses measured by Gag-10mers SFC/10⁶ at 2 weeks post last vaccine and 2 weeks after the challenge in all Mamu-A*01 macaques. At these 2 time points the Spearman's correlation coefficient was 0.8616 ($p < 0.0001$) and 0.7179 ($p < 0.0026$) respectively (Supplementary Figure 1B). In addition, at 2 weeks post last vaccine, of the 7 Mamu-A*01 monkeys that were positive by tetramer staining, 6 were also positive by IFN- γ ELISPOT. Non-Mamu-A*01 positive monkeys also developed IFN- γ ELISPOT responses when the entire Gag-peptide pools were used as shown in Figure 2B. The magnitude of the response using the entire Gag pool across the groups mirrored that seen with the p11c tetramer and the Mamu-A01 Gag peptides. One week after the last boost, the geometric mean Gag specific SFC/10⁶ spleen cells in groups 2, 3 and 4 were the highest (126, 179 and 182 respectively) compared to groups 1, 5 and the controls (8, 23 and 46 respectively). After challenge, the highest Gag responses were seen in groups 2, 3, 4 and 5. In summary, the magnitude of SIV-Gag specific T cell responses as measured by IFN- γ

ELISPOT assays and tetramer was modest after completion of the vaccine immunizations regardless of regimen, however after the SHIV challenge all responses were increased, in particular groups 2 and 4 where macaques were immunized with 3 rMVAs and protein and 2 rDNAs, rMVA and protein respectively. Responses were durable up to 2 years after challenge in all groups (data not shown). These data indicate SIV Gag specific T cell responses are induced by the rDNA/rMVA prime-boost vaccination strategy.

3.3. Antigen-specific lymphocyte proliferation

Numerous HIV and SIV antigens were used to assess the ability of PBMC from macaques to proliferate. In most cases there were no antigen specific responses or there was notable non-specific proliferation prior to vaccination. The most consistent responses were to Env CM244, prior to vaccination the average LSI to Env CM235 was 1 across all groups. Post vaccination, Env CM244-specific proliferation was observed consistently in group 2 (3 doses of rMVA + gp140) after the prime, boost and challenge (average LSI of 15, 9 and 16

respectively), Env CM244-specific responses were seen sporadically in other groups and all macaques had responses to ConA (Supplementary Table 1 and Supplementary Figure 2). All macaques had responses to PWM and as expected lacked responses to tetanus (data not shown).

3.4 SHIV-E Challenge Results

Monkeys were challenged 4 weeks after the final immunization with 80 TCID₅₀ of subtype E SHIV. SHIV plasma RNA results are presented in Figure 3 for individual macaques and by group in Figure 4. The control monkeys had detectable SHIV-E plasma RNA copies at 1 week after intravenous SHIV-E challenge. These copy numbers peaked at 1×10^7 RNA copies/ml at two weeks post challenge and dropped to undetectable levels by 8 weeks post challenge. The greater than 2 log drop in RNA copies at peak viremia were significantly lower ($p < 0.02$, Mann-Whitney test) in the monkeys immunized with rDNA/rMVA + subunit (group 4) than the control group (Figure 4). Viral loads at week 1, 3 and 4 were also significantly lower in the monkeys immunized with rDNA/rMVA + subunit than the control group with p values of 0.0043, 0.0317 and 0.0043 respectively. The rDNA/rMVA plus subunit resulted in a reduction in peak SHIV RNA levels and a more rapid drop to below detectable RNA levels. In group 4, the number of animals with viral load ≤ 10 copies/ml were 3/5, 5/5 and 5/5 at 4, 6 and 8 weeks after challenge whereas in the control groups the number of animals with viral load ≤ 10 copies/ml were 0/6, 3/6 and 1/6 at 4, 6 and 8 weeks after challenge. PBMC CD4 levels prior to and after SHIV challenge are summarized as Figure 5A. The control monkeys challenged with SHIV-E experienced reductions in both their CD4 percentage and CD4/CD8 ratios. The CD4 percentages and CD4/CD8 ratios for the monkeys immunized with rDNA/rMVA plus subunit showed less decline compared to the controls, consistent with and supportive of the viral load measurements (Figure 5B). At multiple time points post SHIV challenge (weeks 3, 4, 5 and 6) the vaccinated monkeys had statistically significantly higher CD4/CD8 ratios than controls ($p < 0.05$, Mann-Whitney test).

4. Discussion

The choice of which is the best non-human primate model for HIV vaccines has been the source of much debate over the years and as new data comes out and different vaccine regimens are pioneered and tested in humans, the decisions continue to be actively discussed [71,72,73,74]. The route, frequency, viral stock and titer of the challenge are all potentially critical parts of the choice of a non-human primate model for HIV vaccines. SHIV challenges went out of favor and the gold standard was considered to be SIVmac239 delivered intra-rectally as a low dose challenge. There have not been many new SHIV challenge stocks available until recently [75,76,77]. However, there is renewed interest in SHIV challenges after the results of the RV144 study were announced since it appears likely that antibody responses correlated with the observed protection [78,79]. The prevalent and incident HIV-1 strain in Thailand is CRF01_AE; Phase I, II and III vaccine trials in Thailand have used vaccine immunogens from subtype E most notably in the successful RV144 trial [5]. In this study we thus used a heterologous challenge using a subtype E SHIV.

DNA/MVA prime-boost vaccination strategies have been successful in eliciting SHIV specific CD8 T cell responses and providing modest viral load reduction in rhesus macaques from disease after pathogenic SHIV challenge [29,30,77]. Studies using the SIVmac239 challenge model have been less successful perhaps attributable to the relative resistance of SIVmac239 to antibody-mediated neutralization and greater stringency of the challenge [80,81]. However, using more physiological challenges such as oral exposure via breast milk, low dose intra-rectal or intra-vaginal challenges, pox-vectored vaccines have been shown to

prevent infection or control of viremia [28,36,82]. In addition new DNA prime-boost vaccine regimens offer protection from infection and or delay to infection and control of SIV after challenge [83,84]. To support development of the MVA-E/A HIV (MVA-CMDR) vaccine for use in humans, we tested its safety and immunogenicity in Indian-origin rhesus macaques. In parallel, we constructed an MVA expressing the same subtype E Env together with SIV Gag-Pol (MVA-SHIV-E). We obtained DNA expressing subtype E HIV Env and a separate plasmid expressing SIV Gag-Pol. We sought to determine whether this non-subtype B rDNA/rMVA prime-boost vaccination induced SHIV-specific CD8 T cell responses and viral load reduction after a non-subtype B (SHIV-E) heterologous intra-venous challenge. At the time this study was performed intra-rectal or intra-vaginal challenges were not feasible. Additionally, to some of the groups we added a subunit gp140 boost to determine whether the subunit could boost humoral immunity while not diminishing the CD8 T cell specific responses. The rDNA/rMVA $\pm$ subunit vaccination induced SIV Gag-specific CD8 T cell responses as measured both by tetramer staining as well as IFN- γ ELISPOT. Since both 20-mer and 8-mer peptides were used in the IFN- γ ELISPOT assays, it seems likely that both CD4 and CD8 responses were detected in response to the vaccine regimens though this was not able to be confirmed by flow cytometry. In pre-clinical and clinical trials both CD4 and CD8 T cell responses are detected when similar prime-boost regimens are used [23, 24]. HIV Env-specific binding and neutralizing antibody were elicited earlier and of higher titer with the inclusion of the subunit boost.

Correlates of protection from infection and viral load control are numerous and complex and likely to involve CD4 and CD8 responses as well B cell memory and antibody mediated effects [85,86,87]. Complete protection against intrarectal challenge with SHIV (SF162P4) was observed in a study where macaques received intramuscular virus like particles encoding SIV GagPol and HIV-1 SF162 gp140 (V2 deleted) prime plus an HIV-1 SF162 oligomeric o-gp140 V2 deleted adjuvanted Env protein boost. In this study, a statistically significant association was observed between the titer of virus neutralizing and binding antibodies as well as the avidity of anti-Env antibodies measured pre-challenge and protection from infection [88]. Correlates of viral control are more often associated with T cells, however several new studies point to a role of antibodies, a strong inverse correlation was found between the avidity of anti-Env Abs and peak post-challenge viremia in macaques immunized with a rDNA + rMVA [89]. In another study induction of strong T cell mediated and humoral immunity in macaques immunized with DNA delivered by electroporation along with RANTES was associated with control of SIV infection [77]. A non-replicative virosome vector expressing the MPER region of gp41 and whole gp41 delivered i.m. and intranasal in a prime boost regimen completely protected macaques from a repeated vaginal SHIV-SF162P3 exposure. In macaques receiving just the i.m. virosomes delayed time to infection was observed and reduction in viral load. The correlates of protection were identified as gp41-specific vaginal IgA with the ability to block HIV-1 transcytosis, and vaginal IgG with neutralizing and/or antibody dependent cellular cytotoxicity activities against HIV-1[90]. Finally in another prime boost regimen in macaques, adenovirus type 5 host range recombinants expressing SIVGag-Pol and HIVEnv followed by boosting with a gp140 envelope protein enhanced acute-phase protection against intravenous simian/human immunodeficiency virus (SHIV)89.6P. The enhanced control was associated with ADCC [91]. Thus all these macaque studies together with the new data from the correlates of protection for RV144 point to an important role of antibodies not only in protection from infection but also in post infection control of virus. Although we were not able to assess correlates of viral load reduction, we believe that the group 4 regimen of rDNA plus rMVA plus protein was critical. Rapid induction of high-titer antibody responses was seen in groups 1, 2 and 5 where gp140 alone was given 4 times or rMVA x 2 and gp140 x 2, but it is likely that the rDNA prime was critical for induction of effective T cell and antibody responses seen in the macaques in group 4 with the greatest

control of viral load and stabilized CD4 T cell numbers. In summary, we believe DNA + vector + protein boost regimens will be important in future vaccine strategies.

There are numerous HIV neutralization assays available and the relative merits of each format are under much debate, recently there has been a concerted effort to re-evaluate neutralization assays and both the PBMC assay and the R5-A301T-cell line which has modest levels of surface expressed CCR5 may represent more relevant neutralization targets [92,93]. Inclusion of the subunit boost increased both the Env-specific binding and neutralizing antibody levels. The neutralizing antibody responses were limited to TCLA HIV-1 isolates tested in T cell line based assays. Neutralizing antibodies were not detected in the vaccinated macaques when a more stringent PBMC assay and the CM235 isolate matched to the immunogen was used. Monkeys immunized with the rDNA/rMVA plus subunit Env vaccine where 5/5 macaques had responses to the CM235 HIV isolate had significantly lower peak plasma viral load (Figures 3 and 4) and stabilized PBMC CD4 T cell numbers (Figure 5) after SHIV challenge.

These results demonstrate both that cGMP manufactured MVA-CMDR is safe and immunogenic in non-human primates and that adjuvanted subunit boosts can be added to rDNA/rMVA prime-boost vaccination regimens without compromising CD8 T cell specific responses. Vaccination with rMVA or rDNA/rMVA induced SHIV-specific CD8 T cell responses and inclusion of gp140 boost to rDNA/rMVA preserved CD8 antigen-specific responses and enhanced antibody binding and neutralization capacity. rDNA/rMVA +subunit vaccination strategy significantly decreased viral RNA and increased CD4 counts after heterologous subtype E SHIV intravenous challenge. Further modifications are required in the subunit boost for development of more functional neutralizing and non-neutralizing antibodies [50,51,52]. Nonhuman primate models offer the best system for preclinical evaluation of these approaches [71,72,73,74]. New studies in nonhuman primates are providing a better understanding of the protection seen in RV144 and are being used to try to identify the immune responses that are responsible for protection and viral load control and accelerate research efforts towards a more effective vaccine [77,83,84,85,88,89,90,91].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

ADCC	Antibody dependent cellular cytotoxicity
CEF	chick embryo fibroblasts

cGMP	current good manufacturing processes
CRF	circulating recombinant form
CTL	cytotoxic T lymphocytes
DR	double recombinant
IFN-γ	interferon gamma
IP	immunoprecipitation
IVS	<i>in-vitro</i> stimulation, Lymphoproliferation assay
LPA	LSI: lymphocyte stimulation index
mAb	monoclonal antibody
rMVA	recombinant Modified Vaccinia Ankara
Nab	neutralizing antibody
RLU	relative light units
pfu	plaque forming units
PMA	phorbol 12-myristate 13-acetate
TCLA	tissue culture laboratory adapted

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highlights

- Rhesus macaques immunized with rDNA and rMVA expressing SIV Gag-Pol and CRF01_AE Envelope followed by a subunit gp140 Env-E protein in adjuvant developed robust binding antibodies, T cell responses and HIV neutralizing antibodies
- After intravenous challenge with a SHIV-E virus, these macaques had a 2-log decrease in viral load compared to controls
- Use of DNA + poxvirus + protein boosts is a promising approach to developing an HIV vaccine

Figure 1. Serum antibody binding to subtype E gp140 was assessed prior to immunization and at multiple time points after the priming and boosting immunizations. The data is presented as the mean titer of the 5 monkeys in each of the groups. Immunizations are described in Table 1 and the materials and methods. Arrows indicate immunizations.

Figure 2.

IFN- γ ELISPOT. PBMC from all macaques were collected at baseline, 2 weeks post the second, third and forth immunizations (weeks 0, 6, 14, and 26), on the day of challenge, 2 weeks pre and post challenge (weeks 28, 30 and 32) and 2 years after challenge. PBMC were stimulated with either pools of 5 SIV Gag 10-mer Mamu-A*01 binding peptides (Figure 2A) or pools of SIV Gag 20-mer peptides representing the whole of Gag (Figure 2B). The bars represent the mean SFC/10⁶ PBMC $\pm$ the standard error of the mean for 2-3 Mamu-A*01 macaques in figure 2A and 5-6 macaques in group 2B. For each group, the bars from left to right are for each of the indicated time points with the clear and hashed bars representing pre and post challenge responses respectively. The vaccines are indicated in the boxes on the x-axis and are detailed in Table 1, the macaques in the control groups 6 and 7 were combined.

Figure 3. Plasma Viral RNA load in individual macaques. Immunizations are described in Table 1 and materials and methods. Monkeys were challenged intravenously 4 weeks after the final immunization with 80 TCID-50 SHIV-E. Each line represents the viral loads of individual monkeys.

Figure 4.

Plasma Viral RNA load averages by immunization group. Immunizations are described in Table 1 and materials and methods. Monkeys were challenged intravenously 4 weeks after the final immunization with 80 TCID-50 SHIV-E. * $p < 0.02$ at weeks 1, 2, 3 and 4 between rDNA/rMVA gp140 and controls

Figure 5. Mean CD4 cell counts after challenge. Monkeys were challenged intravenously 4 weeks after the final immunization with 80 TCID-50 SHIV-E, levels of CD4 and CD8 were measured using flow based assays. The % CD4 are shown in figure 5A and CD/CD8 ratios in figure 5B. Significant stabilization of CD4 T cells and CD4/CD8 ratios were obtained at weeks 3, 4, 6 and 8 weeks post-challenge ($p < 0.05$ shown by *).

Table 1

Vaccine and challenge virus descriptions

Immunogen and challenge type	Immunogen name	Insert(s)	HIV/SIV isolate
MVA	MVA/CMDR	HIV env	HIV CM235
		HIV gagpol	HIV CM240
	MVA/SHIV-A_E	HIV env	HIV CM235
		SIV gagpol	SIV mac239
DNA	DNA/env	HIV env	93TH966.8
	DNA/gagpol	SIV gagpol	SIV mac239
subunit protein	gp140-A_E	Not Applicable	HIV CM235
Challenge name	SHIV-E	HIV env	CAR402
		SIV gagpol	SIV mac239

Table 2

Study groups.

Study Group	Prime month 0 & 1	Boost Month 3 & 6
1	gp140	gp140
2	¹ MVA/SHIV-A_E	¹ MVA/SHIV-A_E gp140
3	DNA/env ¹ DNA/gagpol	¹ MVA/SHIV-A_E
4	DNA/env ¹ DNA/gagpol	¹ MVA/SHIV-A_E gp140
5	² MVA/CMDR	² MVA/CMDR gp140
6	control DNA	control MVA
7	control MVA	control MVA

¹The 'backbone' DNA or MVA construct is gagpol SIVmac239

²The 'backbone' MVA construct is gag/pol HIV CM240.

Macaques were challenged at week 31 with SHIV-E-P4.1 (SIVmac239 gagpol, HIV env CAR402).

Table 3

HIV-1 Neutralizing antibodies from immunized macaques

¹ Group #	NP03	CM235	CM235	Any Response
	² H9	² R5-A301	² PBMC	(%)
1	3/5	4/5	0/5	7/15 (47%)
2	3/5	5/5	0/5	8/15 (53%)
3	0/5	0/5	0/5	0/15 (0%)
4	3/5	4/5	0/5	7/15 (47%)
5	3/5	4/5	0/5	7/15 (47%)
6	0/3	0/3	0/3	0/9 (%)
7	0/3	0/3	1/3	1/9 (11%)

¹Details of the macaque immunizations are given in Table 2 and the materials and methods.

²Neutralization at 2 weeks post 4th immunization was defined as a 50% reduction in p24 antigen concentration in HIV culture supernatants as compared to matched pre-immunization serum. Final serum dilution was 1:10 in the presence of virus. The cell type and virus isolate is indicated above each column.

Table 4

Frequency of Gag-specific CD8 T cells as detected by SIV gag p-11C tetramer staining in rhesus macaques

¹ Group #	² MAMU-A*01 tetramer positive (#/total)			
	Post 2nd	Post 3rd	Post 4th	Post challenge
1	0/3	0/3	0/3	3/3
2	3/3	3/3	2/3	3/3
3	0/2	2/2	2/2	2/2
4	0/3	3/3	3/3	3/3
5	0/2	0/2	0/2	2/2
6	0/3	0/3	0/3	3/3

¹ Details of the macaque immunizations are given in Table 2 and the materials and methods.

² A positive tetramer response was defined as any staining greater than or equal to the mean plus 3 standard deviations of the % CD8/p11C tetramer positive T cells of all Mamu-A01 macaque prior to vaccination ($\geq 0.034\%$). Tetramer staining was conducted at 1 and 2 weeks after each immunization or challenge. A positive response was scored if the macaques had a response at either time point.