Induction of HIV-1 subtype B and AE-specific neutralizing antibodies in mice and macaques with DNA prime and recombinant gp140 protein boost regimens

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ARTICLE INFO

ABSTRACT

We developed highly expressing clade B and AE DNA and envelope protein (Env) vaccines for evaluation in mice and macaques as DNA prime/protein boost regimens. High levels of Env-specific antibodies were induced in mice, albeit with limited neutralizing activity *in vitro*. A combined clade B and AE regimen induced high titer Env-specific antibody in two pigtail macaques that neutralized several strains of HIV-1. However, upon mucosal challenge with SHIV$_{SF162P3}$ no protection from infection was observed. Although the vaccines tested provide a platform for inducing robust humoral immunity, further refinements to broaden coverage against divergent strains and induce mucosal immunity are needed.

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1. Introduction

Despite being the focus of much research effort, an effective vaccine to prevent HIV infection or ameliorate disease outcome after infection remains elusive. Env is the major viral target for neutralization by antibody. However Env has high sequence variability, extensive glycosylation and many of the more conserved Env structures are recessed, protecting the virus from neutralization. The first phase III clinical trial of an HIV vaccine used monomeric gp120 protein with the aim of generating neutralizing antibodies (nAb) to prevent infection. This vaccine was not protective [1], and it is now accepted that gp120 monomers induce only narrow nAb responses, primarily against CXCR4-tropic laboratory strains. Recently, efforts have focused on the potential of cytotoxic T lymphocyte (CTL) responses to control viremia and lower viral set point during chronic infection, thus preventing or delaying disease progression. Disappointingly, while a number of studies demonstrated the ability of vaccine-generated CTL responses to prevent or delay disease progression in non-human primate models, an adenoviral vector-based phase III clinical trial (STEP trial) recently failed to achieve these objectives [2]. Similarly, a previous approach from our group using a DNA prime and recombinant fowlpox virus boost failed to elicit significant adaptive cellular immune responses in human subjects [3]. In that study, a major truncation of Env (at amino acid 275) abrogated its ability to elicit

anti-Env antibodies in macaques [4]. In the studies reported here we have tested immunogens with VLP-associated full-length Env and soluble Env analogues including the entire ectodomain (amino acids 1–683). VLP immunogens are recognized and taken up by antigen presenting cells, facilitating the generation of CD4 help and B-cell priming, whereas soluble immunogens can efficiently boost immune responses. VLP-producing plasmid DNA immunogens or purified VLP particles administered to macaques in the absence of subsequent heterologous boosting were able to mediate viral load reduction after homologous intravenous challenge, but not prevent infection [5,6].

We have focused on generating broader humoral immune responses to Env. Env derived from both clade B and circulating recombinant form 01 AE (AE) strains were assessed in an attempt to increase the breadth of the immune responses elicited and tailor the vaccine for Thailand and other South East Asian countries where clade B and AE viruses are predominant. We tested our approach firstly in a murine model and then in a pilot study using pig-tailed macaques. Env was presented in DNA prime/trimeric protein boost regimens, as this has proven useful in eliciting strong and durable responses against Env and other immunogens, as reviewed by Lu [7]. DNA priming was designed to present Env in two different configurations: on the surface of virus like particles (VLP) and as a soluble Env analogue lacking the transmembrane and cytoplasmic domains (gp140). Our strategy elicited robust serum antibody responses that in the case of pig-tailed macaques showed some breadth of neutralization. However, immunization did not prevent infection after intrarectal challenge with a simian/human immunodeficiency virus expressing a het-

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erologous HIV-1 Env (SHIV$_{SF162P3}$), suggesting that eliciting broader immune responses in the mucosal compartment may be critical in future vaccine strategies.

2. Materials and methods

2.1. DNA immunogens

The VLP-encoding plasmid pVLP was constructed by replacing the *gag* and *pol* genes of the plasmid pHIS-HIV-B [4] with those of pHIS-HIV-AE [8] using the restriction sites NotI and EcoRI, and restoring the truncated Env coding frame using an Nde1/AvrII fragment from the wild-type AD8 *env* gene [9] (Fig. 1A). To make plasmid vaccine vectors for the expression of soluble Env analogues, a BssHII/BlpI fragment of pDRenv2 [10] spanning the NL4.3 *env* gene was cloned into the same sites of pHIS-HIV-B, removing the *gag* and *pol* genes, to produce pNL-160. Soluble analogues (gp140) of the AD8 and the clade A/E strain 93TH966 (966) [11] were created by introducing a stop codon immediately prior to the transmembrane domain (amino acid K683-stop, HXB2 numbering) and mutating the cleavage motif between the gp120 and gp41 domains (amino acids 508–511, REKR changed to RETG) to ablate cleavage (Fig. 2B). These gp140 genes were used to replace the *env* gene of pNL-160 using the KpnI site and an NheI site engineered immediately after the stop codon to produce pAD8-140 and p966-140. The Env expression cassettes from these plasmids were cloned into the pEGFP-N1 plasmid (Clontech) using the AseI/SmaI restriction sites, removing the *egfp* gene and creating pN1-AD8-140 and pN1-966-140. These vectors have a neomycin-resistance cassette for the selection of stable Env-expressing mammalian cells. The negative control vaccine vector pEGFP was produced by removal of a BssHII/BlpI fragment of pAD8-160, which spans the *env* gene, and insertion of the *egfp* gene into the PstI/NotI sites.

2.2. Protein immunogens

To produce gp140 protein for boosting immunizations, pN1-AD8-140 and pN1-966-140 were transfected into HeLa cells using Lipofectamine 2000 and stable Env-expressing cell clones were isolated and identified by G418 selection, limiting dilution cloning and indirect ELISA (see Section 2.5) using tissue culture supernatants as the source of Env antigen. Stable Env-expressing cells were overlaid with serum-free Opti-MEM supplemented with 2 mM sodium butyrate and incubated for 4–6 days at 37 °C. Env was purified from the supernatant by lentil-lectin affinity chromatography as previously described [12] for use as immunogen for the murine studies. Lentil lectin eluates were concentrated and subject to size exclusion chromatography using a 16/60 Superdex 200 column and an AKTAprime liquid chromatography system (GE Healthcare) at a flow rate of 1 ml/min with PBS as the buffer. Aliquots of pools of two individual fractions were analysed by SDS/PAGE (8% gels), immunoblotted and probed with the sheep polyclonal anti-gp120 antibody D7324 (Aalto Bio Reagents, Ireland). Signal was quantified using Kodak Molecular Imaging software. In the case of macaque immunizations size exclusion chromatography fractions 47–55 that contained only Env oligomers and that were enriched for Env trimers [13] were pooled and concentrated for use in immunizations.

2.3. Transmission electron microscopy

HeLa cells were transfected with the plasmids pVLP or pEGFP using Lipofectamine 2000. After 48 h, cells were fixed in Karnovsky's fixative (2.5% (v/v) glutaraldehyde, 2.4% (v/v) formaldehyde, 0.1 M MOPS, pH 7.4), washed in 0.1 × MPS buffer and
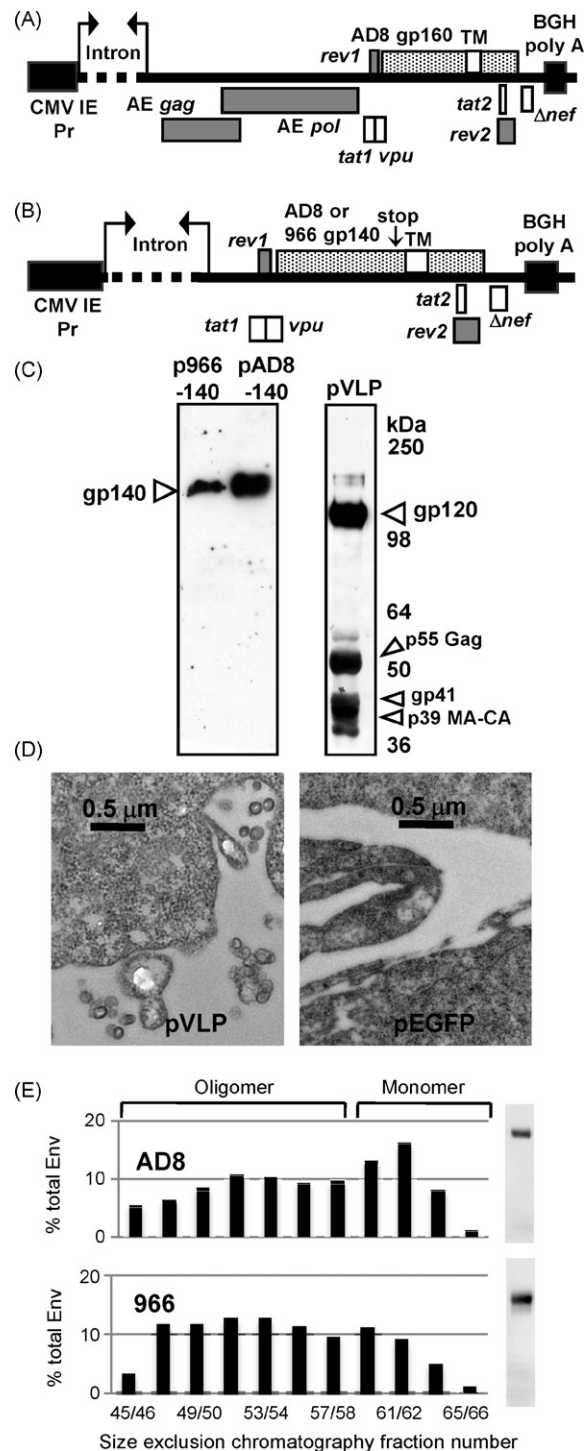

Fig. 1. Env expression from DNA vaccine vectors. (A) Schematic representation of the expression cassette of pVLP. CMV IE Pr = cytomegalovirus immediate early promoter; TM = transmembrane domain; BGH poly A = bovine growth hormone polyadenylation signal. (B) Schematic representation of the expression cassette shared by pAD8-140, p966-140, pN1-AD8-140 and pN1-966-140. (C) Vector-mediated Env expression was assessed by transfecting the indicated plasmids into a human cell line (HeLa) using Lipofectamine 2000. Cell lysates (gp140) or VLP purified on 20% sucrose cushions were analysed by SDS/PAGE (8% gel), immunoblotting and probing with pooled HIV positive sera. Signal was detected by enhanced chemiluminescence. (D) HeLa cells transfected with either pVLP or pEGFP were assessed for the presence of VLP by transmission electron microscopy. (E) Aliquots of size exclusion chromatography fractions (pools of two fractions) of AD8 and 966 gp140 were analysed by SDS/PAGE and immunoblotting with D7324. Signal was plotted as a fraction of total. Fractions containing predominantly oligomeric or monomeric gp140 are indicated [12,13]. Insets show pooled fractions 47–55 that were used for macaque immunizations analysed by SDS/PAGE (8% gels) and immunoblotting with D7324.
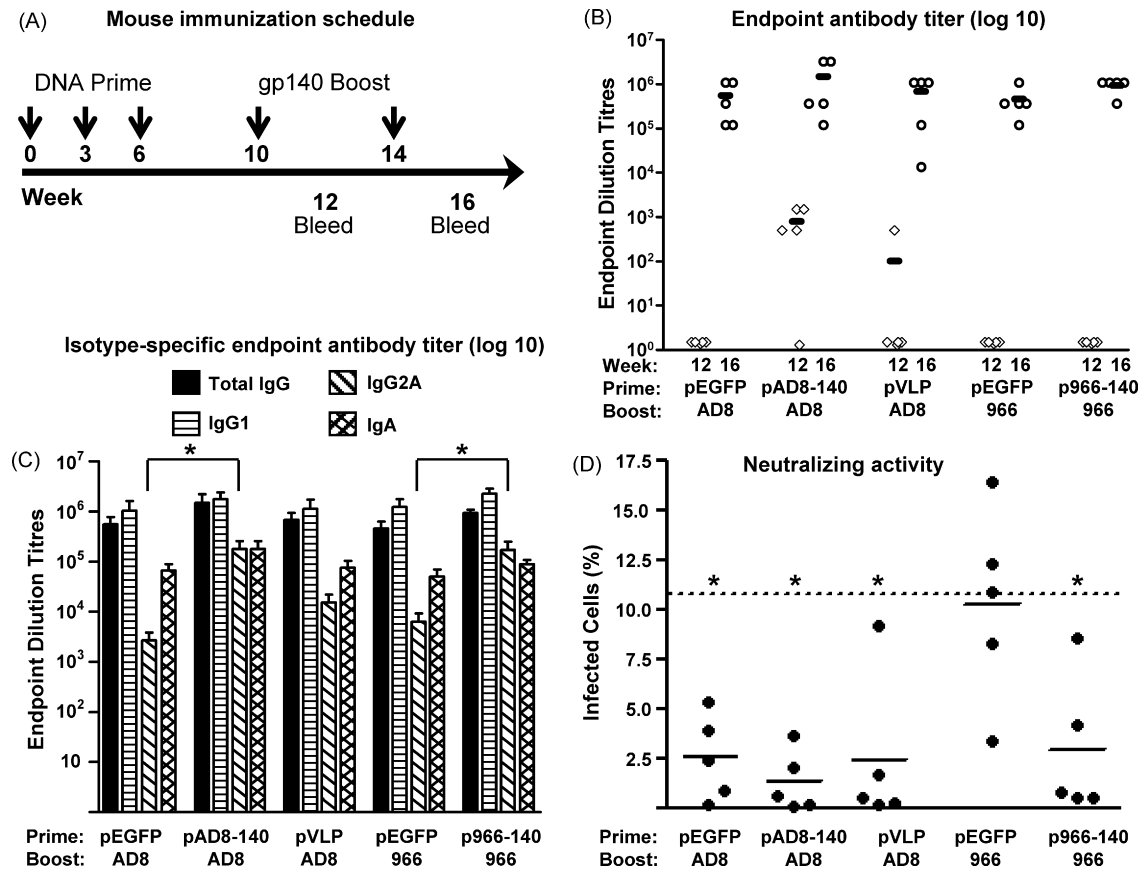
Fig. 2. Immunization of mice with a DNA prime/gp140 protein boost regimen. (A) Immunization schedule. Mice were immunized three times with DNA vectors and twice with gp140 protein at the indicated time points. Blood was collected after each of the protein boost immunizations (weeks 12 and 16). (B) Anti-Env endpoint antibody titers (total IgG) of sera of individual animals tested at week 12, after one protein boost (diamonds) or at week 16, after the second protein boost (circles). Endpoint titer was determined to be the highest serum dilution that gave an OD value of at least three times above background (the OD observed with age-matched sham-vaccinated mouse sera at the same dilution). (C) Anti-Env endpoint antibody titers (total IgG or specific isotypes IgG1, IgG2a or IgA, as indicated) of animal groups tested after the second protein boost (week 16). Results are presented as the mean ± S.E.M. of individual animals ($n = 5$). Endpoint titers were determined as outlined in (B). For (C) and (B), either AD8 or 966 gp140 was used as antigen to match the immunogen used. (D) Neutralizing activity of sera of individual animals tested after the second protein boost (week 16). Pseudoviruses pseudotyped with MN Env were incubated with 1:10 dilutions of sera for 1 h prior to adding to PM1 target cells (6×10^4 well^{-1}) in 96-well plates. The dashed line represents the mean number of infected cells where pseudoviruses were incubated with sera from age-matched, sham-vaccinated mice. For (B), (C) and (D), groups are indicated on the x-axis. Asterisk (*) indicates a p value of <0.05.

dehydrated by serial resuspension in ethanol (30–100%). Samples were then sequentially resuspended in increasing concentrations of phenolic resin (Buehler, IL, USA) until a final incubation in 100% resin. The embedded cells were baked overnight at 60 °C and trimmed using a Leica block trimmer, sectioned using a Leica Ultracut R microtome and embedded on copper grids. Sections were stained with uranyl acetate in 50% ethanol for 5 min and rinsed with ddH2O and images were acquired using a Phillips CM120 BioTWIN transmission electron microscope.

2.4. Immunization

All studies involving the use of animals were approved by the relevant institutional animal ethics committees. Groups of 5 female 6–8-week-old BALB/c × C57BL/6 F1 hybrid mice were immunized as shown in Fig. 2A. Each animal was primed with a total dose of 100 μg of plasmid DNA via intramuscular injection into both hind gastrocnemius muscles. Protein boosting was performed via the intraperitoneal and subcutaneous routes (5 μg each site in 50% (v/v) Freund's incomplete adjuvant). Blood samples were collected from the tail vein. Two healthy pigtail macaques (*Macaca nemestrina*) were obtained from the Australian National Macaque Facility. Animal 6161 was female and animal 6870 was male. The immunization schedule for macaques is shown in Fig. 3A.

DNA priming was via intramuscular injection of 1 mg at one site. Protein boosting was via the intramuscular and subcutaneous routes (0.5 mg protein in 50% (v/v) Montanide ISA 51 VG (Seppic) at each site). Blood samples were collected from the femoral vein.

2.5. ELISA

The presence of Env-specific antibodies in murine or macaque sera was determined by indirect ELISA. The polyclonal sheep anti-gp120 antibody D7324 was coated onto 96-well plates at 200 ng/well overnight at 4 °C. All subsequent steps were performed at room temperature. Wells were washed sequentially in PBS/0.1% Tween 20 and PBS and then blocked with block buffer (PBS/0.1% Tween 20/5% skimmed milk powder) for 1 h. Tissue culture supernatants from stable Env-expressing cell lines were added for 2 h to allow D7324 capture of Env antigens. Wells were washed as described above, followed by the addition of serial dilutions of sera in block buffer. After a 2-h incubation, wells were washed and horseradish-peroxidase-conjugated anti-IgG antibody specific for murine IgG (total), IgG1, IgG2a or IgA or macaque IgG (total) was added and incubated for 1 h. Color reactions were developed using 3,3′-5,5′-Tetramethylbenzidine and absorbance was measured at 405 nm against a reference of 492 nm.
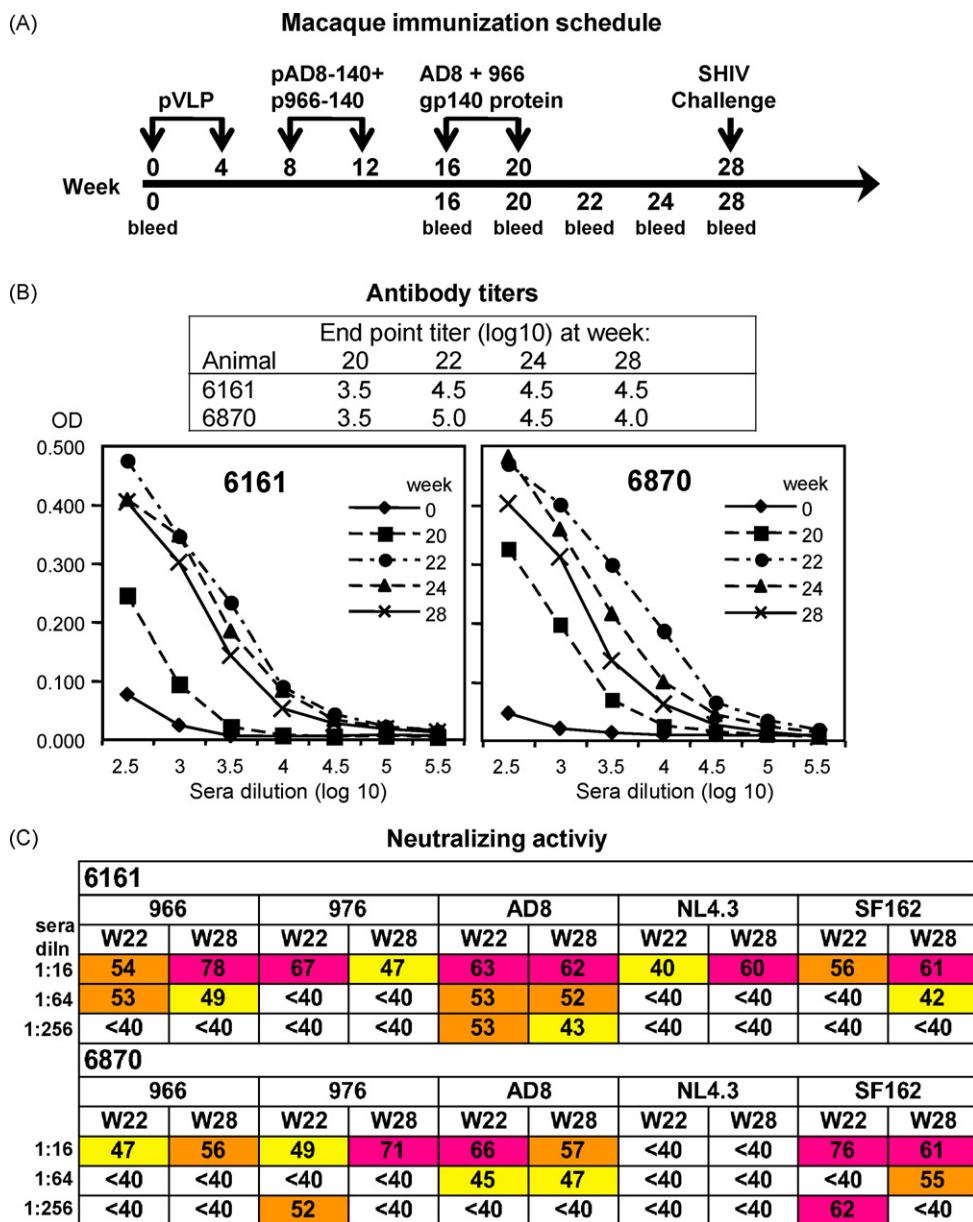
Fig. 3. Immunization of macaques with a DNA prime/gp140 protein boost regimen. (A) Immunization schedule. Animals were immunized twice with pVLP, twice with an equimolar mix of pAD8-140 and p966-140, and twice with an equimolar mix of AD8 and 966 gp140 proteins at the indicated time points. Blood was also collected at the indicated time points. (B) Anti-Env antibody titer using half log 10 serial dilutions of sera of animal 6161 and 6870 tested at the indicated time points. A 1:1 mix of AD8 and 966 gp140 was used as antigen. The upper panel shows the endpoint titer (highest serum dilution that gave an OD value at least threefold higher than that obtained with sera taken prior to vaccination at the same dilution). (C) Neutralizing activity of sera of animals 6161 and 6870 taken at week 22 and week 28 (day of challenge). Pseudoviruses pseudotyped with the indicated Env strain were incubated for 1 h at 37 °C with sera at 1:16, 1:64 and 1:256 dilutions prior to adding to Cf2th-CD4/CCR5/CXCR4 target cells (2×10^4 well^{-1}) in 96-well plates. Percentage neutralization denoted by red ≥60%, orange 50–59% and yellow 40–49%.

2.6. Neutralization assay

The neutralization assay is more fully described elsewhere (Campbell et al., in preparation). Briefly, pseudotyped HIV-1 particles were produced by cotransfecting HeLa cells with an Env-negative HIV-1 EGFP reporter proviral DNA (pNL-4.3ΔenvNef-EGFP) and a vector expressing Env of the test strain. Pseudoviruses were incubated with varying concentrations of sera in duplicate for 1 h at 37 °C prior to the addition of target cells followed by spinoculation at $1200 \times g$ for 2 h at room temperature. Residual pseudovirus and antibody were then removed and 200 µl of fresh media added to the cells, which were cultured for 2 days. The percentage of target cells that were infected (positive for EGFP expression) was determined by flow cytometry (FACSort, Becton-Dickinson). The

% of neutralisation was calculated as follows: $(1 - [\text{virus} + \text{immune sera}/\text{virus} + \text{non-immune sera}]) \times 100$.

2.7. T cell assays, SHIV_{SF162P3} challenge and viral load determination

T cell immunity was evaluated in the macaque study on fresh PBMCs by intracellular cytokine staining, following stimulation with peptide pools, as previously described [14]. Peptides used were overlapping SIV$_{mac239}$ Gag or HIV-1$_{MN}$ clade B Env 15mer peptide pools or HIV-1$_{93TH253}$ clade AE Env peptides spanning the first and last third of this Env protein [8]. Macaques were challenged intrarectally with SHIV$_{SF162P3}$ by atraumatic instillation of 3×10^4 TCID$_{50}$ on 2 consecutive days. SHIV$_{SF162P3}$ viral load in

plasma and peripheral CD4 T cell depletion was monitored as previously described [14]. The viral loads and CD4 T cell counts observed here were compared to the eight previously reported unvaccinated pigtail macaques infected with the same dose of SHIV$_{SF162P3}$ [15].

2.8. Statistical analyses

Comparison of murine isotype-specific endpoint titers was performed using the Mann–Whitney test. The neutralization data with murine sera were analysed using a one-way ANOVA test with significance determined by Dunnett's multiple comparison test. All statistical analyses were performed using GraphPad Prism version 4.0 software. Differences were deemed significant if $p < 0.05$.

3. Results

3.1. In vitro expression from DNA vaccine vectors

A series of DNA and protein vaccines were designed to present native Env trimers and elicit nAb. The expression cassette of pVLP (Fig. 1A) retains approximately two-thirds of the proviral sequence. Multiple mutations or deletions of the *gag*, *pol* and *nef* coding regions and complete removal of the *vpr* and *vif* genes and both long terminal repeat sequences (all derived from the pHIS-HIV-B and pHIS-HIV-AE parental plasmids) ensured maximal safety. The expression cassettes of pVLP (Fig. 1A), pAD8-140 and p966-140 (Fig. 1B) share common features including transgene expression under the control of the strong immediate early CMV promoter, an artificial chimeric CMV/IgG intron and the presence of both *rev* exons in their natural configuration. This permits efficient expression of gp140 from the pAD8-140 and p966-140 vectors and of VLP from the pVLP vector when transiently transfected into HeLa cells (Fig. 1C). Additionally, these vectors have 15 primate-optimized CpG immunostimulatory motifs derived from pHIS-HIV-B. Transmission electron microscopy demonstrated that pVLP-transfected HeLa cells formed VLP, in contrast to pEGFP-transfected negative controls (Fig. 1D). Correctly processed gp120/41 complexes were efficiently incorporated on VLP purified on a sucrose cushion (Fig. 1C). Expression of a full-length 966 A/E Env in the same context resulted in the incorporation mainly of uncleaved gp160 (data not shown). Size exclusion chromatography profiles using a 16/60 Superdex 200 column indicated that 61% and 74% of the total Env protein from lentil lectin-purified AD8 and 966 gp140 preparations respectively were contained in fractions 45–58 (Fig. 1E) that were validated as oligomer-containing in previous biophysical studies using scanning transmission electron microscopy and sedimentation equilibrium analysis [13]. Aliquots of the pooled fractions 47–55 that were used for macaque immunizations were analysed by immunoblotting with D7324 (insets Fig. 1E) and confirm the integrity of the Env protein.

3.2. Murine immunization trial

The ability of our DNA and Env protein vaccines to stimulate Env-specific humoral immune responses was initially examined in mice. Sera were assessed by ELISA at weeks 8, 12 and 16 (Fig. 2A). No groups showed evidence of anti-Env antibody responses at the highest concentration of sera tested (1:100) at week 8 after DNA immunization alone (data not shown). After the first AD8 gp140 protein boost (week 12), four out of five animals that were primed with pAD8-140 had positive antibody titers compared to none of those primed with the pEGFP negative control plasmid and boosted with AD8 protein (Fig. 2B, diamonds). In contrast, mice primed with

p966-140 and boosted once with 966 gp140 protein did not have detectable anti-Env titers (Fig. 2B, diamonds). Similarly, only one mouse that was primed with pVLP had anti-Env antibodies after a single protein boost with AD8 gp140. After the second protein boost (week 16) all mice, including those primed with the pEGFP negative control plasmid, had equivalent and high anti-Env titers (Fig. 2B, circles). DNA priming may affect the immune response qualitatively. In particular, class switching from IgG1 to IgG2a has been described for antigens when presented in a plasmid-based format [16]. We therefore determined isotype-specific anti-Env antibody titers after the second protein boost at week 16 (Fig. 2C). Both pAD8-140 and p966-140 primed mice had IgG2a anti-Env titers after protein boosting that were significantly higher than mice primed with the pEGFP negative control vector. IgG2a anti-Env titers in pVLP-primed mice were not significantly different from the pEGFP-primed mice. Titers for IgG1 and IgA were not significantly different between any of the groups.

Mice sera collected after the second protein boost (week 16) were tested for the ability to neutralize virus pseudotyped with Env derived from different strains of HIV. The dashed line indicates the mean number of infected cells where pseudoviruses were incubated with sera from age-matched, sham-vaccinated mice. Sera from all groups receiving two AD8 gp140 protein boosts gave significant neutralizing responses at a 1:10 dilution against MN pseudovirus (a neutralization sensitive strain) compared with sham vaccinated controls, including sera from mice primed with the pEGFP negative control plasmid (Fig. 2D). Sera from mice primed with p966-140 and boosted with 966 gp140 protein neutralized MN pseudovirus to a significant extent. Sera from pEGFP primed/966 gp140 protein boosted mice reduced infection with MN pseudovirus, however this difference failed to reach statistical significance. Mice sera did not neutralize virus pseudotyped with AD8, 966, YU-2 or NL4-3 Env (data not shown).

3.3. Primate immunization trial

Pig-tailed macaques were immunized twice with pVLP and twice with equal amounts of pAD8-gp140 and p966-gp140, followed by two boosting immunizations with equal amounts of AD8 and 966 gp140 protein (Fig. 3A). No anti-Env antibody titers were detected after DNA immunization alone (data not shown). Four weeks after the first protein immunization (week 20), the endpoint titers for animals 6161 and 6870 were $10^{3.5}$, increasing to $10^{4.5}$ and 10^5 respectively 2 weeks after the second protein boost (week 22) (Fig. 3B). Endpoint titers were subsequently maintained in animal 6161 until challenge but fell in animal 6870 to $10^{4.5}$ and 10^4 at week 24 and at challenge (week 28), respectively. Mucosal samples (rectal and vaginal washes and saliva) collected at week 24 were assessed and found not to contain significant levels of anti-Env-specific IgG or IgA (data not shown).

Sera taken at week 22 (peak binding titer) and week 28 (day of challenge) were tested for neutralizing activity against AD8 (vaccine clade B strain), 966 (vaccine clade AE strain), NL4.3 (clade B), 93TH976 (976, clade AE) and SF162 (clade B). The SF162 strain was the parental Env strain used to generate the SHIV$_{SF162P3}$ challenge virus. Neutralization of all five strains tested was observed (Fig. 3C). NL4.3 pseudotyped virus was the worst neutralized virus, with neutralization seen only at the highest sera concentration tested (1:16) in animal 6161. In contrast, AD8 pseudotyped virus was neutralized to at least the 40% level at a sera concentration of 1:256 in animal 6161 and 1:64 for animal 6870 at both week 22 and week 28. Typically, <40–80% neutralization is seen using patient-derived sera used at a similar dilution range in this assay (up to 90% for sensitive strains such as MN) (data not shown).
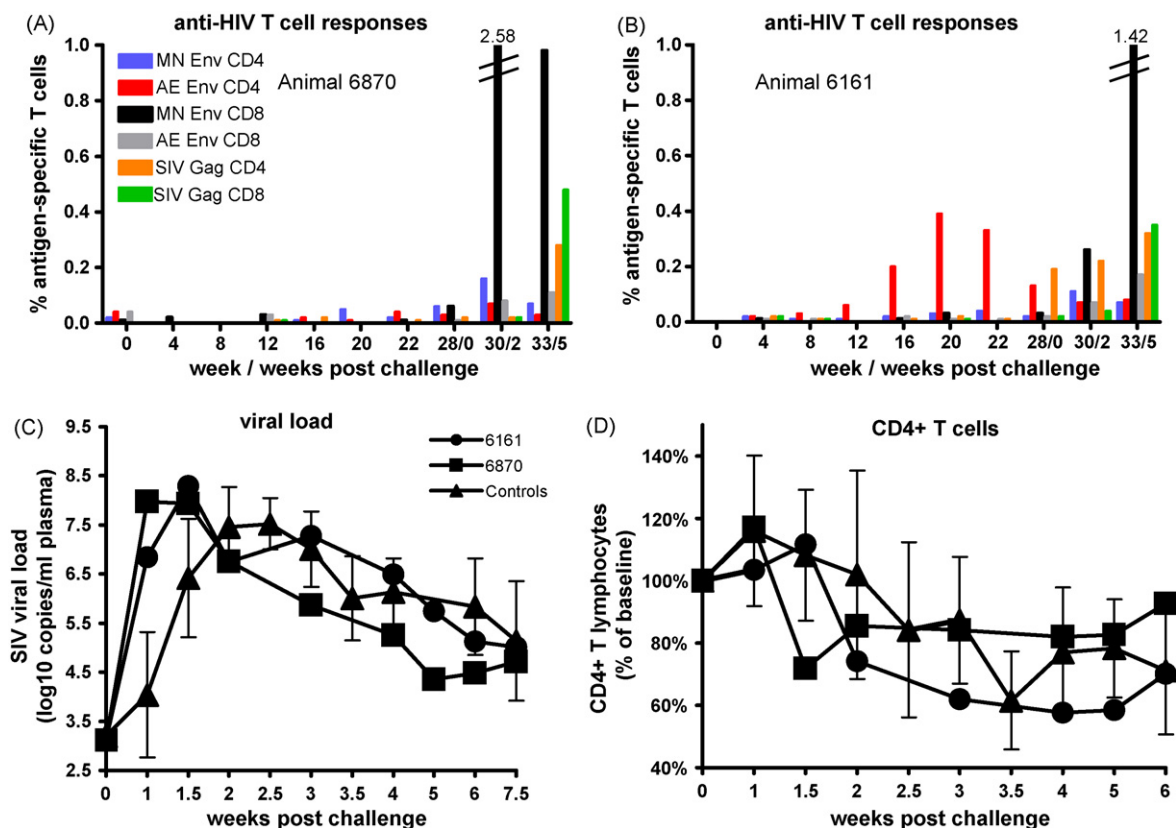
Fig. 4. Cell-mediated immunity and outcome of challenge for vaccinated macaques. IFNγ intracellular cytokine staining was performed on fresh PBMC following stimulation with peptide pools at the indicated time points. The proportion of gated CD4+ or CD8+ T lymphocytes expressing IFNγ was analysed by flow cytometry. (A) Cell-mediated immunity for animal 6870. (B) Cell-mediated immunity for animal 6161. (C) SIV viral load after challenge with SHIV$_{SF162P3}$ compared to unvaccinated controls. (D) CD4 positive T cells expressed as a percentage of lymphocytes after challenge with SHIV$_{SF162P3}$ compared to unvaccinated controls. In (C) and (D), controls were calculated as the mean ± S.E.M. of eight animals.

3.4. T cell responses to vaccination and outcome of SHIV$_{SF162P3}$ challenge

Since the DNA prime/protein boost vaccination regimen has the potential to prime T cell immunity, we also evaluated CD4 and CD8 T cell responses to both clade B (MN strain) and AE Env peptides as well as SIV Gag peptides. Animal 6870 did not show detectable anti-HIV cellular responses prior to SHIV challenge (Fig. 4A). Animal 6161 developed a consistent CD4 T cell response to the AE clade Env peptides after the DNA vaccinations (0.2–0.3% of all CD4+ T cells, Fig. 4B). This Env-specific T cell response did not cross-react with the B clade peptides. No responses to the HIV-1 Gag expressed by the pVLP DNA vaccine were detected (data not shown).

To assess the effectiveness of the vaccination we challenged both animals intrarectally with a heterologous subtype B SHIV$_{SF162P3}$ challenge stock we have previously studied in a large number of pigtail macaques [17]. Both animals developed high viral loads within 1 week of exposure and had persistently elevated viral loads through to chronic infection (Fig. 4C). There was a modest fall in total peripheral CD4 T cells during acute infection that stabilised thereafter (Fig. 4D). Viral loads and CD4 T cell levels in 6161 and 6870 were very similar to those of eight unvaccinated pigtail macaques previously challenged with the same SHIV$_{SF162P3}$ viral stock [15,17], demonstrating that there was no protective immunity.

DNA vaccination may prime T cell immunity that is readily detectable only after boosting with another immunogen or after virus challenge [14]. To evaluate whether priming of T cell immunity occurred in the macaque study, we evaluated T cell immunity before and after SHIV challenge. A strong Env CD8 T cell response

was detected using the MN peptides (clade B) within 2 weeks after challenge in animal 6870 and within 5 weeks in both animals (Fig. 4A and B). The SF162 parental clade B strain used to produce the SHIV challenge has 84% amino acid identity with MN. This response did not cross-react with the subtype AE Env peptides. In contrast, responses to SIV Gag (expressed only by the challenge virus) emerged more slowly and at lower levels after virus challenge. Our extensive previous experience with unvaccinated pigtail macaques, or pigtail macaques vaccinated with both Gag and Env immunogens is that after infection with either SIV or SHIV, Env-specific T cell immunity emerges concurrently or later than Gag-specific T cell immunity [14,17–19]. This strongly suggests that the brisk induction of high-level Env-specific CD8 T cell responses compared to Gag-specific responses observed here is evidence of priming of Env-specific CD8 T cell responses. Not surprisingly, the AE clade Env-specific CD4 T cell response induced in animal 6161 (that did not cross-react with B clade Env peptides) was not boosted by the subtype B SHIV$_{SF162P3}$ challenge. The clade B Env-specific CD4 responses increase transiently 2 weeks post-challenge, and then decline. This decline is likely to be due to infection and death of activated CD4 positive cells.

4. Discussion

In this study, we tested several modes of Env-based vaccine delivery in murine and non-human primate models. All plasmid vectors were tested *in vitro* by transfection of a human cell line, and high-level Env expression was confirmed. The VLP-encoding construct was very efficient at mediating secretion of VLP with

high levels of incorporation of cleaved gp160, similar to that of virion-associated Env [20] indicating the potential utility of this vaccine platform. The murine system allowed us to assess DNA prime/protein boost regimens looking separately at clade B and AE Env in groups large enough to support statistical analysis. After priming with three DNA immunizations and boosting twice with protein, all mice had strong antibody responses. In the case of immunization with the clade B AD8 gp140-encoding plasmid, the antibody response developed more rapidly than in mice primed with a negative control vector, although this was not the case for the clade AE 966 gp140- or the VLP-encoding plasmids. Furthermore, both clade B AD8 and AE 966 gp140 DNA primed mice had higher levels of anti-Env IgG2a than mice primed with the negative control vector, indicating that enhanced class switching was mediated by the DNA prime. This is consistent with previous murine studies using the model influenza hemagglutinin antigen where intramuscular immunization with DNA in saline induced antibody class switching [16]. The *de novo* expression of antigens from DNA vaccines may mimic active viral replication that coupled with the immunostimulatory properties of plasmid DNA could induce the production and secretion of Type-1 cytokines by CD4+ cells and antibody class switching [16,21]. In contrast, protein immunogens generally elicit mixed Type-1/2 or Type-2 biased responses [21 and references therein]. Enhanced class switching may be a useful property for an HIV vaccine, as it has been found that high serum titers of gp41-specific IgG2 were associated with retarded disease progression in HIV infected patients [22]. Additionally, the two patient-derived monoclonal nAbs with the broadest activity (4E10 and 2F5) [23] were initially isolated as IgG3 [24] rather than the more prevalent subtype IgG1. These findings suggest that structural and functional differences between antibody isotypes may influence *in vivo* neutralizing activity and that antibody isotypes other than IgG1 may be important for anti-HIV immunity.

Many of the known patient-derived broadly acting nAbs have long and protruding CDR3 regions that allow them to access recessed epitopes such as the CD4-binding site [25]. Murine antibodies have on average shorter CDR3 regions than human antibodies [26] and tend to neutralize HIV poorly. In the current study murine sera from all DNA primed and protein-boosted animals were able to neutralize the sensitive clade B strain MN, but failed to neutralize the other strains tested. These results prompted us to test this strategy in a pilot study using a non-human primate model. Evidence suggests that human and macaque vaccinees may recognize some of the same conserved neutralizing epitopes [27], and additionally the use of macaques permits the assessment of protection from SHIV challenge. Two pig-tailed macaques were primed twice with a VLP-encoding plasmid and twice with gp140-encoding plasmids prior to protein boosting. The multi-Env approach (clade B and AE) induced some breadth of neutralizing activity in the sera, including activity against one normally resistant clade B strain (vaccine strain AD8) and the two clade AE strains tested (vaccine strain 966 and 93TH976, which has 90.6% amino acid identity to 966). While immune responses to clade B Env have been relatively well characterized, little is known about responses to clade AE Env. It has been suggested that the third variable loop of gp120 is important for the neutralization of AE strains by patient sera [28,29]. The heterologous AE Env that was neutralized in our study (93TH976) had a V3 loop with 88% amino acid identity with the vaccine AE strain (966). This may be reflective of the reported lower amino acid variability in this circulating recombinant form [30], and may have facilitated cross-neutralization of this strain by anti-V3 loop antibodies raised against 966 gp140. In contrast, while clade B patient sera appear to more frequently neutralize via the CD4-binding site [31], AE strains were found to be more resistant to neutralization at this site by soluble CD4 when compared directly to clade B strains [30]. Interestingly the strain that was less neutralized in our study,

NL4.3, is T-cell line adapted and generally more sensitive to neutralization. Unlike the other test or the vaccine strains, NL4.3 is CXCR4- rather than CCR5-tropic, raising the possibility that at least some of the neutralizing antibodies raised targeted the coreceptor-binding site.

When the immunized macaques were challenged with SHIV$_{SF162P3}$ intrarectally (heterologous challenge), both animals became infected and showed a disease course in terms of CD4 counts and viral load that was consistent with non-immunized animals in our previous studies. Some success with multiple-Env cocktail vaccines has been reported for non-human primates in previous reports. Vaccination with multiple-Env strains using a DNA prime, protein boost regimen can give protection from infection by rectal challenge with a SHIV bearing an HIV Env homologous to one of the vaccination strains in a majority of vaccinated macaques [32]. Protection from heterologous challenge has been more difficult to achieve, and a lack of breadth measured by *in vitro* neutralization assay is predictive of lack of protection against heterologous challenge [33 and references therein]. A multiple-Env approach using successive immunization with plasmid DNA, recombinant vaccinia virus and recombinant protein modalities produced broad anti-Env binding and neutralizing antibody responses and improved disease control following heterologous challenge [34]. In some cases however, multiple-Env approaches have achieved coverage against the broader range of vaccine strains rather than heterologous non-vaccine strains [35] or neutralization sensitive rather than more resistant heterologous strains [36,37] and protection against heterologous challenge has not been substantially improved.

The lack of protection seen of our study was despite the ability of sera antibodies to neutralize several strains of HIV, including SF162, the parental strain used to produce the SHIV$_{SF162P3}$ challenge virus. Given that most HIV transmission is via mucosal surfaces, it is likely that mucosal antibodies will be required both for protection against experimental mucosal challenge and for an effective human vaccine. Our regimens may need to include a mucosal delivery mode, such as intranasal immunization, although this has been difficult to achieve with standard DNA vaccine approaches [17]. In terms of the experimental design, protection from infection studies may need to use repeated low dose mucosal challenge rather than two high dose challenges, as used here, to more accurately mimic natural transmission in humans [38]. Additionally, it appears that the timing of vaccination may be critical, as heterologous Env protein boosting of macaques that were previously resistant to homologous challenge failed to elicit protective responses to SHIV bearing the Env homologous with the protein boost [39], possibly due to original antigenic sin. Our regimen may have provided broader anti-Env recognition if both clade B and AE Env were present in the first immunization rather than presenting only clade B Env at that time point.

Despite the lack of protection in the macaque trial, some promising features of these vaccines suggest this platform is worthy of further refinement. We are encouraged that cross-neutralization of R5-tropic HIV strains was generated in vaccinee sera. Since our base platform induces potent levels of Env antibodies in both mice and primates, we can now rationally improve immunogen design to broaden the nAb coverage. Further, the ability of our gp140-encoding DNA vectors to induce IgG2a responses in mice and some T cell responses in primates suggests the potential to generate a combination of humoral and cellular immune responses, which are likely to be needed to provide robust protective immunity in improved versions.

Acknowledgements

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