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Boosting of HIV-1 Neutralizing Antibody Responses by a Distally Related Retroviral Envelope Protein

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Our knowledge of the binding sites for neutralizing Abs (NAb) that recognize a broad range of HIV-1 strains (bNAbs) has substantially increased in recent years. However, gaps remain in our understanding of how to focus B cell responses to vulnerable conserved sites within the HIV-1 envelope glycoprotein (Env). In this article, we report an immunization strategy composed of a trivalent HIV-1 (clade B envs) DNA prime, followed by a SIVmac239 gp140 Env protein boost that aimed to focus the immune response to structurally conserved parts of the HIV-1 and simian immunodeficiency virus (SIV) Envs. Heterologous NAb titers, primarily to tier 1 HIV-1 isolates, elicited during the trivalent HIV-1 env prime, were significantly increased by the SIVmac239 gp140 protein boost in rabbits. Epitope mapping of Ab-reactivity revealed preferential recognition of the C1, C2, V2, V3, and V5 regions. These results provide a proof of concept that a distally related retroviral SIV Env protein boost can increase pre-existing NAb responses against HIV-1. The Journal of Immunology, 2014, 192: 5802–5812.

The high antigenic variability of HIV-1 is one of the major problems in the development of an effective HIV-1 vaccine. However, even HIV-1 has its Achilles heel, because segments of the virus cannot be changed without deleterious consequences for viral replication (1). Accordingly, studies have revealed that 10–30% of HIV-1–infected individuals develop broad serum neutralizing Ab (NAb) activity, and that a small fraction (~1%) develop very potent responses (2–9), clearly demonstrating that the immune system can generate NAb that recognize a wide variety of HIV-1 strains. Successfully licensed antiviral vaccines, such as the yellow fever and measles vaccines, elicit NAb as correlates of protection (10, 11), and passive transfer of broad neutralizing Abs (bNAbs) can protect macaques against virus infection with chimeric simian immunodeficiency viruses (SIVs) encoding the HIV-1 envelope glycoprotein (Env) (12–19). Taken together with the recent finding that the induction of bNAbs is a pivotal property of a protective HIV-1 vaccine (8). The recent identification of epitopes targeted by new sets of bNAbs from HIV-1–infected individuals represented a major achievement for our understanding of the vulnerable sites on the HIV-1 Env (21). Target sites include the membrane-proximal epitope region in gp41, the CD4 binding site (CD4bs), and some of the conserved elements in the variable regions V1/V2 and V3, including the integrin (α4β7) and CCR5 binding sites (2, 22).

Eliciting responses against the conserved regions in Env may be critical for a vaccine to achieve protection against a broad spectrum of viral species, overcome viral immune escape, and circumvent individual host variation. However, despite the increased understanding of the different target sites for effective neutralizing activity, we still lack knowledge on how to design immunogens and immunization regimens with the capacity to elicit Ab responses focused toward such structurally conserved sites that are crucial for viral function. During natural HIV-1 infection, the NAb responses

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The sequences presented in this article have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession number KJ579955.

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Abbreviations used in this article: AAC, allogeneic activated cell; bNAbs, broad neutralizing Abs; CD4bs, CD4 binding site; CRF01, circulating recombinant forms 01; Env, envelope glycoprotein; Gr., group; i.d., intradermal; NAb, neutralizing Ab; NIBSC, National Institute for Biological Standards and Control; SIV, simian immunodeficiency virus.

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generally fail to neutralize concurrent viral isolates (23–26), and multiple rounds of Ab selection and viral escape occur over several years before bNAb responses are induced (4, 21, 25, 27). Therefore, the eventual expansion of B cells with high specificity for subdominant constant regions of viral proteins appears to be required, but lags behind viral evolution, and generally occurs too late to be of substantial benefit for the infected individual (21, 28, 29).

The immunological principle put forward in this study is therefore based on recurrent host exposure to divergent Env and the novel use of the highly divergent gp140 Env from SIVmac239 as protein boost. We repeatedly immunized rabbits with a trivalent DNA vaccination (clade B envs gp140) followed by boosting with the heterologous recombinant gp140 Env protein (30–32). The novelty of our concept was to use a highly divergent gp140 Env from SIVmac239 for the protein boost. SIVmac239 is a highly pathogenic virus in macaques that causes rapid depletion of CD4+ T cells and destruction of the immune system, a similar picture to human AIDS (33). Hence natural infection with SIVmac239 generally does not induce bNAb (34). However, we previously noticed the development of NABs to several SIVs in an attenuated SIV vaccine model when animals were treated with daily tenofovir between 10 d and 4 mo after inoculation (35). The SIVmac239 virus is very resistant to NABs (36), and the macaques displayed potent neutralization to sensitive heterologous SIVs before the appearance of neutralization to homologous SIVmac239 (35). This attenuated SIVmac239 infection study additionally revealed neutralization of HIV-1 in sera from the macaques (35), even though the HIV-1 and SIVmac239 gp140 proteins have only ∼30% sequence identity and divergent antigenicity. We therefore hypothesize that the neutralization-resistant SIVmac239 Env may have immunogenic features suitable for the induction of NABs, of which some appear cross-reactive between HIV-1 and SIVmac239. Accordingly, they both bind human CD4 and display significantly conserved topological architectures (37). In addition, the higher stability of SIVmac239 trimers when compared with those generally produced from HIV-1 Env (38–40) is likely to provide additional advantages during immunization. In conclusion, the vaccination strategy designed in this study made use of repetitive DNA priming using HIV-1 gp140 and a highly heterologous SIVmac239 gp140 boost, and resulted in high-tier heterologous NABs against clade B viruses and activity against circulating recombinant forms 01 (CRF01) AE and clade C viruses, including HIV-1 Env-specific responses to conserved epitopes primarily in the C1, C2, V2, V3, and V5 regions.

Materials and Methods

Animals

New Zealand white (male and female) rabbits (10–12 wk of age at start of experiment, ∼3 kg) were housed at the animal facility of the Swedish Institute for Infectious Control according to directives and guidelines of the Swedish Board of Agriculture and the Swedish Animal Protection Agency. The study was performed under approval of the Stockholm North Ethical Committee on Animal Experiments.

Expression and purification of recombinant gp140

SIVmac239, HIV-1LAV/IIIB, YU2, ITM1_4, NIBSC40-9, and HIV-2 (accession no. UC37; AY494974; YU2: M93258; ITM1_4: FM165626; NIBSC 40-9: K579955; HIV-2 is JN863894) (41–45) gp140 were produced following transient transfection of 293T cells cultured in multilayer Cell Bind Hypertalks (Corning) in high-glucose DMEM (Sigma) supplemented with 10% FCS (Sigma) and penicillin-streptomycin solution (Sigma). Two milligrams plasmid DNA was incubated with 3.6 mg polyethyleneimine in media without FCS for 30 min to allow complex formation. This was added to cells and brought to 500 ml with DMEM containing 2% FCS. Supernatants were collected after 48 h, and fresh media, containing 10% FCS, was added to the cells for a further 48 h, at which point the media were exchanged once again. All supernatant was centrifuged at 7000 × g for 4 h to remove cell debris, and passed through a 0.2-μm filter. After adjustment to pH 8 using 1 M HCl or 1 M NaOH (Sigma), media were passed over a cobalt chloride metal-affinity column made of Talon Superflow resin (Clontech). After washing with 2 column volumes of 0.015 M TBS (Sigma), protein was eluted with 250 mM imidazole. The eluted gp140 was concentrated and separated by gel filtration chromatography using a Superdex200 26/60 size-exclusion column (GE Healthcare). Fractions corresponding to the trimers were identified and further purified using GNA-lectin (Vector Labs) to specifically bind the glycoprotein. A further SEC fractionation allowed separation of pure gp140 (Fig. 1).

Surface plasmon resonance

Surface plasmon resonance experiments were performed on a BIAcore 3000 (BIACore, GE Healthcare) in HBS-EP buffer (BIACore) at 37°C. Seven hundred response units of gp140 were immobilized on a CMS sensor chip using standard amine coupling at pH 4.5. Binding responses were measured by injecting mAbs or CD4-IgG over the surfaces for 5 min at 50 μl/min followed by a 5-min dissociation phase. For kinetic analysis, a 2-fold dilution of xCD4 starting at 20 nM was used under similar conditions. Both antigens were captured and induced to undergo conformational changes to more closely resemble the epitope recognized by bNAb. Changes in the sensorgram were monitored and the association and dissociation rate constants were determined using the BIAevaluation software (V 4.0.1). Surface plasmon resonance was measured using an immobilized control that was not coated with antigen (background level).

DNA vaccine plasmids

The construction of BX08 gp140 (GenBank JX473289) plasmid used codons from highly expressed human genes as described earlier (46–48) and two other primary Env gnels from Danish patients (ctl21 – JX473290) and ethyl (JX473291) (49) were similarly codon optimized and cloned in the same expression plasmid with the key elements CMV IE promoter-enhancer, tissue plasminogen activator secretion signal, and a bovine growth hormone poly A signal. Clones were verified by sequencing.

Immunizations

Groups of rabbits (52 animals in total), 10–12 wk old, were immunized as shown in Fig. 2A. Animals were immunized at weeks 0 (twice first week), 4, and 8 with a mix of the three clade B env-DNA plasmids encoding SIV- env, gp140 intradermally (i.d.) with the Derma Vax EP device (CytoPulse Sciences/Cellectis, Romainville, France; Fig. 2A). A total concentration of 200 μg DNA was injected i.d. at two injection sites, as described by Roos et al. (50). Blood for collection of sera and PBMCs was taken from the ear vein before immunizations (time point 0 [wk 0]) and 4 wk after the last DNA immunization (wk 16). DNA immunizations were administered in 12 animals (group [Gr.] 1) without adjuvant and in 12 animals (Gr. 2) with a cellular adjuvant consisting of allogeneic activated cells (AACs) in the first study. The AACs were prepared as previously described (51–55) using allogeneic rabbit splenocytes activated in vitro with Con A (2.5 μg/ml; Sigma) for 48 h before apoptosis induction. The gamma-irradiated cells were diluted in PBS and mixed with the DNA before administration. In total, each rabbit received 10 × 10⁶ AACs per injection site. At week 16, all DNA primed animals received a heterologous protein trimer SIVgp120 (50 μg) by i.d. injection without adjuvant, and sera were collected 4 wk later (wk 20). A second identical boost was performed 4 wk after the first protein immunization, and blood was collected 2 wk later (wk 22). Three months later, blood samples were analyzed for memory responses (wk 34). A repeat study with 24 animals divided into 4 groups was performed: the first group (Gr. 1, study II, n = 6) received DNA-env DNA-vaccine DNA-priming at weeks 0, 8, and 12 delivered by electroporation; a second group (Gr. 2, study II; n = 6) received the addition of a TLR5 agonist encoding flagellin (pFlIC) as previously described (56). Two of these groups subsequently received a heterologous protein trimer SIVmac239 (50 μg) by i.d. injection (no adjuvant), and sera was collected 4 wk later (wk 20). A crossover experiment with three animals from Gr. 1 and 2 (study II) was then performed 4 wk after the first protein immunization (wk 20), and blood was collected 4 wk later (wk 22). Three animals from Gr. 1 and three from Gr. 2 received a second gp140 boost from HIV-1 NIBSC 40-9 clade A at week 20. The other three animals in Gr. 1 and 2 received gp140 from HIV-2 (primary isolate CA65330.5) as the second protein boost at week 20.

The third group (Gr. 3; n = 6) received empty vector plasmids and alum as protein boost. The fourth group (Gr. 4; n = 6) received only protein trimer SIVmac239 (50 μg) by i.d. injection at weeks 0, 8, and 16. A comparative group (Gr. 5; n = 4) received the DNA-env clade B mix at weeks 0, 4, 8, and 12 delivered by electroporation followed by a gp140 from HIV-1 ITM-1_4 in cationic adjuvant formulation 01 (57) by i.d. injection.
tivity in A3R5.7 cells. The ID50 was calculated as the reciprocal serum
titration levels were determined after 4 d by measuring Renilla luciferase ac-
added to each well. One set of control wells received cells + virus (virus
DIVPLTKKNY), HIV-1CN54 V3 (300-NTRKSIRIGPGQTF), HIV-1CN54V5
(461-EPNDTETFRPGGGDM), SIVmac239 V2 (169-KFTMTGLKRDKTKE-
YNETWY), or SIV mac239 V3 (319-VLPVTIMSGLVFHSQPDNR) peptides
and blocked with 2% BSA in washing buffer (0.05% Tween 20 in PBS).
Bound Abs were detected with anti-rabbit IgG-HRP (Sigma-Aldrich), de-
veloped with 1-STEP Ultra TMB-ELISA substrate (Thermo Fisher Scien-
tific), and OD values were read at 450 nm. Data were analyzed by subtracting
the background followed by fitting of a sigmoidal dose–response curve to
each data set. End-point titers were defined as the concentration at which the
curve reached the threshold (0.01), which was greater than 2 SDs above
background. HIV peptides were obtained from the Eurovac Consortium.

Peptide scanning
Overlapping linear 15-mer and circularized 15-mer peptides based on gp140
of HIV-1CN54 were tested for reactivity against heat-inactivated rabbit sera
by Pepscan Therapeutics. Positive responses were defined as higher than
two times the median of all peptides tested. The effect of boosting was
determined by dividing the ELISA value after boosting by the ELISA value
after DNA prime for each peptide. Peptide binding breadth for each animal
was calculated as the percentage of peptides that showed positive responses.
For graphical representation, average ELISA values of 9-aa windows
were calculated for each position as described (59).

Neutralization assays
IgGs were purified from serum using Protein G HP SpinTrap columns (GE
Healthcare) according to the manufacturer’s instructions. Eluted IgGs were
quantitated spectrophotometrically with yields ranging from 2 to 4 mg/ml.
The purified IgG or sera were assayed in pseudovirus neutralization assays
using TZM-bl cells, expressing CD4 and both CCR5 and CXCR4, con-
ducted in triplicate as described previously (60) (http://www europrise.org/
neutnet_sops.html; SOP10). A panel of pseudoviruses was used and pre-
pared as previously described (60, 61). Infection levels were determined
after 48 h by measuring firefly luciferase activity in TZM-bl. Neutralizing
Ab activity was also assessed using A3R5.7 cells (62). A3R5.7 cells (63)
were obtained from Drs. Jerome Kim and Robert McLinden at the U.S.
Medical HIV Research Program. This is a human CD4+ lymphoblastoid
cell line (CEM/A3.01) (64) that was engineered at the U.S. Medical HIV
Research Program to express CCR5. Infectious molecular clones of HIV-1
carrying the entire ectodomain of the viral env of choice and a Tat-
regulated LucR reporter gene (65) were obtained from Drs. Christina
Ochsenbauer and John Kappes at the University of Alabama, Birmingham.
In brief, a dose of virus that generates ~50,000 relative luminescence units
after 4 d of infection was incubated with serial 3-fold dilutions of test
sample in duplicate in a total volume of 150 μl for 1 h at 37˚C in 96-well,
flat-bottom culture plates. Exponentially dividing A3R5.7 cells (90,000
in 100 μl growth medium containing 25 μg/ml DEAE dextran) were
added to each well. One set of control wells received cells + virus (control
virus), and another set received cells only (background control).
Infection levels were determined after 4 d by measuring Renilla luciferase ac-
tivity in A3R5.7 cells. The ID50 was calculated as the reciprocal serum
dilution causing 50% reduction of relative light units compared with the
virus alone (without test sample). Magnitude-breadth curves were prepared
as previously described (66). A Mantel–Cox log rank test was used to
compare the different curves.

Peptide competition neutralization assays
Peptide inhibition of IgG neutralization was measured using a modified
pseudovirus neutralization assay (43) in which the purified IgGs were
preincubated for 30 min with peptide dissolved in DMSO at a final con-
centration of 16 μg/ml before addition of the pseudovirus and a final
DMSO concentration of 0.3%. The HIV-1 MN V3 peptide (CTRPNY-
NKKRHIHGPRAYTKTINIGTIRQAC, EVA0719), the GPG-1 HIV-1
SF2 clade B V3 peptide (TRKSYIGPGRFHTT, ARP7971), the GPG
HIV-1 consensus clade A peptide (KSVHIGHPQAYET, ARP7012.1), and
the scrambled control (ARP7099) were obtained from the Eurovac
Centre Against AIDS for Reagents, National Institute for Biological
Standards and Control (NIBSC; South Mimms, Potters Bar, U.K.).

Statistical analyses
Figures and statistical analyses were prepared using GraphPad Prism sta-
tistical software version 4.03 (GraphPad Software, La Jolla, CA) according
to the statistical tests described in the figure legends.

Results
Production of stable covalent SIVmac239 gp140 for heterologous boost
All gp140 trimers used in this study were produced in HEK 293T cells
using transient expression plasmid DNA transfection. The SIV mac239
and the HIV-1 gp140 trimers were highly pure and uniformly trimeric
in solution as analyzed by gel filtration chromatography (Fig. 1A).
However, whereas the SIV mac239 gp140 trimer migrated as a tight
high m.w. band under nonreducing SDS PAGE, the HIV-1 gp140
trimers demonstrated at least partial dissociation into monomeric
species (Fig. 1B). The analyzed proteins migrated as single monomeric species under reducing conditions (Fig. 1B). Together, this indicates that both HIV-1 and SIVmac239 yielded uniformly trimeric gp140 and that the SIVmac239 trimer is stabilized by covalent disulfide bonds between the monomers. Surface plasmon resonance binding analysis revealed that HIV-1 and SIVmac239 gp140s bind to soluble recombinant human CD4. However, no binding to SIVmac239 gp140 was detected using a panel of mAbs against the CD4bs as well as the V2 and V3 regions of HIV-1 gp160 including F105, VRC01, HGP68, and HR10 (Fig. 1C, Supplemental Fig. 1A and 1B). Hence these findings suggest poor HIV-1 antigenicity of SIVmac239 gp140, and that SIVmac239 binds with a lower affinity to human CD4 compared with HIV-1UG37.

**Induction of binding Abs to clade A HIV-1UG37 and SIVmac239 EnvS after trivalent clade B Env immunization**

In a previous study, we optimized a DNA prime regimen in which clade B gp140 enV was delivered as a mixture composed of three synthetic codon–optimized enVs (from the clinical isolates HIV-1 Bx08, ctl21, and ctl27) with the aim to increase the proportional concentration of epitopes derived from shared regions (49). In this study, we used the same DNA prime followed by a highly heterologous trimeric SIVmac239 gp140 protein boost, hypothesizing that such a regimen might focus the response to more conserved structures. Sequence alignments comparing enV from Bx08, ctl21, ctl27, and SIVmac239 are shown in Supplemental Fig. 1C. Although the overall sequence identity between HIV-1 enV and SIVmac239 enV is only in the order of 30%, certain parts of Env display shared motifs including the V3 region.

To mimic the long-term antigenic exposure required for the development of bNAbS in HIV-1–infected individuals, we adopted an intense immunization regimen, which consisted of repeated DNA immunizations of rabbits over a 4-mo period before boosting with SIVmac239 or HIV-1 (clade A) gp140 Env. Plasmid DNA was delivered by i.d. injections in the first week (days 0 and 2), as well as at weeks 4, 8, and 12 (Fig. 2A). Each i.d. injection was followed by electroporation, which previously has been demonstrated to substantially increase immunogenicity in both animal and human studies (67). Two SIVmac239 gp140 boosts were delivered i.d. at weeks 16 and 20 (50 μg/animal/time point) without adjuvant in both Gr. 1 and 2. In addition, we also evaluated whether an adjuvant, consisting of activated apoptotic cells that engage TLR4 (52–54), would increase DNA priming efficiency (study I, Gr. 2, n = 12; Fig. 2A). Gr. 1 (study I, n = 12) did not receive any adjuvant. Subsequently, a second independent study with six animals in each group was performed in which Gr. 2 received a TLR5 agonist that was delivered as a DNA plasmid (pFlIc) together with the DNA prime (study II, Gr. 2, n = 6; Fig. 2A) (56), whereas Gr. 1 (study II, n = 6) did not receive any adjuvant. All animals in Gr. 1 and 2 from the second study also received an i.d. SIVmac239 gp140 boost at week 16. In addition, a crossover experiment with three animals from each of these two groups was performed at week 20 to evaluate whether a recombinant gp140 trimer from HIV-1B NIBSC 40-9 clade A (n = 6) or HIV-2 (n = 6) could be used as a second protein boost. Furthermore, the second study included a negative control group receiving empty plasmid and albumin (Gr. 3, n = 6), as well as a group that received only the recombinant SIVmac239 trimer (Gr. 4, n = 6). Finally, a comparative group (Gr. 5) was included to compare the boosting capacity of a gp140 HIV-1 clade Btrimer with the results obtained by the SIVmac239 boost.

We first assessed the prevalence of Env-specific binding IgGs in serum against both SIVmac239 gp140 and heterologous clade A HIV-1UG37 gp140 by ELISA. High titers of binding Abs against both Ags were detected in all animals after the protein boost (wk 22), without any demonstrable effect of the AAC adjuvant (Fig. 2B). These high IgG titers were confirmed in the second study as high ELISA titers were already detected after DNA immunizations followed by one i.d. injection with HIV-1 ITM-1_4 gp140 (white bars) or heterologous HIV-1UG37 gp140 (black bars) as measured by ELISA analyzing sera obtained before immunizations (wk 0) and after immunizations at wk 22 from the first study. Data are depicted as median and range.
SIV<sub>mac239</sub> gp140 Env CAN BOOST HIV-1 NEUTRALIZATION

Sera were analyzed for neutralizing activity against a panel of tier 1 viruses in the TZM-bl assay, as previously defined by Seaman et al. (68), and responses are displayed as ID<sub>50</sub> titers (Fig. 3). Serum neutralization responses in Gr. 1 and 2 of the first study are displayed together because no significant differences in NAb activity were found in the group receiving the AAC adjuvant (Supplemental Table 1). A significant induction of neutralizing activity was measured against heterologous HIV-1<sub>SF162.LS</sub> and HIV-1<sub>MN.3</sub> viruses after the DNA priming phase (wk 16, DNA) with strongly increased neutralization titers after the first SIV<sub>mac239</sub> gp140 boost (wk 20, DNA/gp140; Fig. 3). Measurements with purified serum IgG confirmed the induction of neutralization activity (Fig. 3C and 3D). Furthermore, neutralizing activity was also detected against tier 1 viruses from clade C (MW965.26) and CRF01 AE (TH023.6; Fig. 3E and 3F) after boosting with SIV<sub>mac239</sub> gp140. To further assess the breadth of the responses after boosting with SIV<sub>mac239</sub> gp140, we measured neutralization titers against tier 1 and 2 viruses using the A3RS5.7 assay, as well as against HIV-2 (Fig. 3G, 3H, and Supplemental Table 1). Significant neutralization was induced against RHPA.LucR (tier 1B clade B) and SC22.3C2.LucR (tier 2 clade B) using the A3RS5.7 assay and also against HIV-2 in the TZM-bl assay. The sera did not, however, show neutralization to viruses such as JR-FL, Q23.17, 6535.3, QHO692.42 (data not shown). An arbitrary color code was used in Fig. 3 where the top eight neutralization responders against SF162.LS are depicted in red, the eight animals with the lowest neutralization titers to SF162.LS in blue, and the intermediate responders in yellow (n = 8). The lowest responders to SF162.LS neutralization (blue) seemed to also be among the low responders against the other viruses, whereas the best SF162.LS neutralizers (wk 16) and found to be generally increased by the protein boost (Fig. 2C). Similarly to the results obtained with AAC, no difference in binding Abs titers was detected between the groups that did or did not receive the pFliC adjuvant during the DNA priming phase (Fig. 2C, Gr. 1 and 2). The empty vector and albumin protein control group did not mount any SIV<sub>mac239</sub> or HIV-1<sub>UG37</sub> gp140-binding Abs (Fig. 2C, Gr. 3). Importantly, repeated administration of SIV<sub>mac239</sub> trimer without HIV-1 DNA priming did not elicit high-titer binding Abs to HIV-1<sub>UG37</sub> even though responses to SIV<sub>mac239</sub> were induced (Fig. 2C, Gr. 4). Conversely, binding IgG responses toward SIV<sub>mac239</sub> were induced by the HIV-1 DNA prime before the SIV<sub>mac239</sub> Env boost (Fig. 2C, Gr. 1 and 2). The comparative group receiving a gp140 HIV-1 boost after priming (Gr. 5) mounted similar binding titers to HIV-1<sub>UG37</sub> and SIV<sub>mac239</sub> but tended to be lower when compared with those of Gr. 1 and 2 (Fig. 2C). Conclusively, these findings show that the trivalent clade B<sub>env</sub> DNA prime was able to induce Abs capable of binding to both clade A HIV-1<sub>UG37</sub> and SIV<sub>mac239</sub>-env.

**FIGURE 3.** Induction of high-titer neutralizing Abs against clade B viruses through the highly heterologous DNA-prime protein-boost regimen. Data are shown for individual animals from Gr. 1 and 2 combined (n = 24) (A–H). Lines represent median. Sera were collected before immunization (wk 0), 4 wk after last DNA immunization (DNA), 4 wk after first SIV<sub>mac239</sub> gp140 boost (DNA/gp140), and 2 wk after the second SIV<sub>mac239</sub>gp140 boost (DNA/gp140/gp140). Three months later, sera were analyzed for the longevity of responses (3 mo after last boost). High ID<sub>50</sub> neutralizing titers were detected in a TZM-bl assay against tier 1 isolates from clade B SF162.LS (A) and MN.3 (B). Purified IgGs were tested in a TZM-bl assay against HIV-1 clade B SF162 and BX08 (C and D). Percent neutralization is shown using 130 μg/ml IgG final concentration approximately corresponding to a 1/80 serum dilution, and dotted line indicates ID<sub>50</sub>. Neutralizing titers in sera were detected in a TZM-bl assay against tier 1 isolates from clade C (MW965.26) (E) and CRF01 AE (TH023.6) (F). An arbitrary color code indicates the animals with the highest titers against SF162.LS in red (n = 8), lowest in blue (n = 8), and those in between in yellow (n = 8). These colors are kept throughout the figure to be able to track the animals and their respective response to other viruses. ID<sub>50</sub> titers in sera from Gr. 1 and 2 were tested in the A3RS5.7 assay against clade B viruses RHPA.LucR (G) and SC22.3C2.LucR (H). Neutralization titers obtained in the TZM-bl assay for the control groups that received control DNA and albumin (Gr. 3, displayed in green) or only SIV<sub>mac239</sub> protein without a DNA prime (Gr. 4, displayed in blue) are shown for neutralization of HIV-1 MN.3 (I) and the laboratory-adapted sensitive TCLA-SIV<sub>mac251</sub> isolate (J). Significant values are indicated by *p < 0.05, **p < 0.01, ***p < 0.001 using one-way ANOVA with multiple-comparison test.
neutralizers (red) tended to also be high responders against the other viruses. Interestingly, the second SIVmac239 protein boost delivered 4 wk after the first boost did not always significantly increase the neutralization titers. Nevertheless, when long-term persistence of neutralization was evaluated 3 mo after the final SIVmac239 protein boost (wk 34), responses were still detected against both tier 1A viruses (Fig. 3A and 3B) and the tier 1B clade B virus RHPA.LucR (Fig. 3G). In line with the measured Ab binding titers (Fig. 2C), SIVmac239 gp140 boosting without prior HIV-1 priming (Gr.4) did not induce NAbs against HIV-1 viruses (Fig. 3I), whereas significant responses against a sensitive TCLA-SIVmac251 were observed (Fig. 3J). Although the trivalent clade B env DNA immunization induced binding Abs to SIVmac239, it was not sufficient to induce a neutralization response to TCLA-SIVmac251, whereas boosting with SIVmac239 resulted in a detectable SIVmac251 neutralization (Fig. 3J, Gr. 1 + 2). Hence the SIVmac239 gp140 was able to induce significant neutralization to a neutralization-sensitive clone of TCLA-SIVmac251.

To further analyze and compare the boosting effect of the highly heterologous gp140 SIVmac239, we included a comparative group,
followed by a boost with HIV-1 ITM-1_4 gp140 (clade A; Fig. 4A). HIV-1 ITM-1_4 gp140 was selected based on comparative analyses indicated by *p phase (Gr. 2, n = 6) or with a cellular adjuvant AAC during the priming phase (wk 20, DNA/gp140) were used. After HIV-1 DNA-prime (wk 16, DNA) and after the first boost (wk 22) in animals immunized without (Gr. 1, n = 6) or with a cellular adjuvant AAC during the priming phase (Gr. 2, n = 6; mean ± SEM). Significant values are indicated by **p < 0.01, ****p < 0.0001, or were not significant (ns) using a Mann–Whitney U test (B), a paired t test (C), or a one-way ANOVA of log-transformed data with a Bonferroni multiple-comparison test (D).

Epitope mapping reveals preferential Ab binding to C1, C2, V2, V3, and V5

Epitope mapping using linear and circularized 15-mer HIV-1_CN54 peptides was performed to obtain additional insights into the specificities of the Ab-binding response. Sera obtained after the DNA priming schedule (wk 16, DNA) primarily recognized epitopes in the C1 and V3 regions and, to a lesser extent, in the V2, C2, and V5 regions (Fig. 5A). Notably, almost all of the observed Ab responses increased after the SIVmac239 gp140 boost (wk 20, DNA/gp140; Fig. 5A–C). Indeed, Ab-binding responses that were already positive after priming displayed, on average, a significantly stronger increase after the boost (Fig. 5B). In addition, Ab binding to some peptides that were not recognized after DNA priming was detected after the protein boost, demonstrating an increased breadth after the SIVmac239 boost (Fig. 5B and 5C).

Peptides from regions in C1, V2, V3, and V5 were used in ELISA to assess the impact of the AAC adjuvant on binding titers against individual epitopes. Although the AAC adjuvant did not improve binding Ab titers against the complete gp140 trimer as measured by the ELISA, it significantly increased responses against all the HIV-1 peptides tested (Fig. 5D). Altogether, these results indicate that the SIVmac239 protein strongly boosted primed HIV-1 responses and led to broadening of the recognition, whereas the addition of AAC during the priming phase induced higher Ab-binding titers to C1, V2, V3, and V5.

The neutralization of HIV-1 SF162 was assessed in the presence of a cyclic peptide that comprises the entire V3 region of MN HIV-1 gp120 (residues 266–301) in sera obtained following two protein boosts (wk 22; Fig. 6A and Supplemental Fig. 2). The neutralization inhibition assay demonstrated recognition of the peptide in sera from all of the animals tested and blocking of a major part of
It should be noted that the mechanistic basis for this induction of heterologous neutralization remains unresolved. The HIV-1 antigenicity profile of SIVmac239 gp140 was poor, as defined by lack of binding using a panel of mAbs against HIV-1 gp160 including F105, HGP68, and HR10, which recognize the CD4bs and the V2 and the V3 regions, respectively. Nevertheless, HIV-1 Env has been repeatedly shown to possess different antigenicity compared with immunogenicity, as recently reviewed by Kong and Sattentau (70). Therefore, it appears possible that the SIVmac239 trimer used in this study possessed favorable immunogenic features for the boosting of anti–HIV-1 Ab responses irrespective of the lack of conserved antigenicity with HIV-1. Furthermore, the immunogenic profile of SIVmac239 was recently shown to confer selective protection against repetitive intraepithelial challenge with SIVmne660 (71). A recent study suggested that SIVmac239 displays increased gp160 trimer stability in vivo and specifically highlighted a tryptophan motif located in V2 to be responsible for disulfide bonding between the protomers and the higher stability (38–40). It is therefore interesting to note that the recombinant SIVmac239 gp140 used in this study also appeared to be uniformly bound by covalent disulfide bonds between the protomers. This is likely to confer higher stability to the gp140 trimer, which has been demonstrated to be of pivotal importance for other protein-based recombinant vaccines, such as the human papillomavirus vaccine (72, 73). Further support for the importance of stable trimeric immunogens comes from the observation that trimeric HIV-1 gp140 induced more potent NAbs responses compared with monomeric gp120 (74). Although beyond the scope of this study, it would be highly interesting to evaluate whether the favorable properties of the SIVmac239 trimer result in the induction or boosting of cross-reactive T cell responses, and thus strengthening the primed B cell response.

Even though the mechanism underlying how the SIVmac239 trimer boost provided such robust and broad increases of B cell responses against HIV-1 remains unclear, the results presented in this article highlight the ability of this highly heterologous SIVmac239 gp140 protein boost to increase HIV-1 NAb titters. A recent study using a gp120 HIV-1 DNA vaccine prime followed by an adjuvanted formulation of a heterologous gp120 protein (BG505 Env) elicited tier 1 NAbs in rabbits (75). Our study confirms and extends the observation that heterologous prime-boost regimens not only elicit high-level but may also improve quality of Ag-specific Ab responses, and thus offer a new platform for eliciting bNAbs to HIV-1 (76–78).

Further boosting of the neutralization responses was observed with HIV-1NBSG40.9 clade A gp140, but not with a gp140 trimer from HIV-2 gp140. Hence more work is required to understand how to optimally expose the immune system with repeated exposures to different Envgs to reach the goal to induce high-titer bNAbs. Sequential immunization using Envgs isolated during the course of a natural infection that resulted in high-titer bNAbs is one principle put forward (79). In this study, we adopted repeated immunizations but used highly divergent gp140 Env of SIVmac239 as one component in a recurrent immunization schedule. Our data indicate that the SIVmac239 protein largely boosted primed responses and alone was not able to induce either HIV-1 binding or HIV-1 NAbs. We have attempted to reveal epitopes induced by the vaccination regimen by cross-competition ELISA. Using this assay, we were unable to detect Abs against the CD4bs and a range of other epitopes tested (data not shown). We were, however, able to demonstrate inhibition of neutralization using peptides against V3.

In our opinion, this study provides a proof of principle for an immunization strategy that induces immune responses, including the induction of Ab recognition to conserved elements in HIV-1.
Env, and, in particular, to the C1, V2, V3, and V5. A recent study by Luo et al. (80) used a similar approach in which the use of SIV–HIV-1 cross-immunization induced reactivity against C1, V3, and V5, as well as broad neutralizing activity with low magnitude. However, a fundamental difference from our study is the use by Luo et al. (80) of SIVmac239 for DNA priming and HIV-1 Env CAN gp140 for the boost. The use of reversed order of HIV-1–SIV cross-immunization could be beneficial because the NAb response reported in this article comprised high-magnitude responses (in the order of serum titers of 1/10,000) to tier 1 viruses. It should be noted that we could detect significant neutralization even 3 mo after the final SIVmac239 protein boost. Although the neutralizing activity against HIV-1 Env CAN V3 was largely mapped to the V3 region as expected (22), it is interesting to note that a significant portion of the neutralization activity was not inhibited by the V3 peptide in at least 3 of the 12 animals tested, indicating that other neutralization specificities were present in the sera. In addition, it is conceivable that the neutralizing activity against tier 2 viruses may be governed by other regions such as conserved glycan epitopes that may be present on both HIV-1 and SIVmac239.

In conclusion, this study provides a framework for the induction of HIV-1 Env-specific NAb to clade B viruses, as well as reactivity against sensitive CFF01 AE and clade C viruses by repeated DNA env mixture priming followed by boosting with distally related and relatively stable trimeric SIVmac239.

Acknowledgments

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Disclosures

A.-L.S. and G.S.-J. are authors on a patent application related to this publication. The patent was submitted by and owned by Oxford University. J.W.B. is employed by Pepscan Therapeutics.

References


Supplementary FIGURE 1. Surface plasmon resonance analysis of mAb and soluble CD4 binding to SIV<sub>mac239</sub>.

(A) Interaction of an extended panel of mAb with HIV-1<sub>UG37</sub> or SIV<sub>mac239</sub> is shown as in Fig.1C. (B) Kinetic data of monomeric soluble CD4 interaction with HIV-1 and SIV<sub>mac239</sub> gp140s was recorded and fitted to a 1:1 Langmuir binding model. Traces are plotted in black with curves showing the fit results overlain in red. (C) Sequence alignment of the gp160 sequences from the HIV-1 variants ITM1_4, YU2, UG37 Bx08, ctl21 and ctl127 as well as from SIV<sub>mac239</sub> shaded by sequence conservation. The V1/V2, V3 regions as well as the V4 and V5 loops are highlighted.
Supplementary FIGURE 2. Entire panel of animals tested in the peptide inhibition assay.

Individual results of the peptide inhibition assay as presented in Fig.6A. Percent neutralization is depicted on the y-axis and concentration of purified IgG on the x-axis. (A) Neutralization is displayed for individual animals in the absence (black) or presence of a peptide covering the V3 region from HIV-1 clade B virus MN.3 (dark grey) or a scrambled control peptide (light grey). Peptide controls without IgG did not result in significant enhancement or neutralization of the virus.
### Supplementary Table 1. Potent neutralization ID<sub>50</sub> titres after immunization.

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Serum ID<sub>50</sub> titres obtained from individual animals at week 22 from the first study (Group (Gr) 1 (no adjuvant) and 2 (AAC adjuvant)) are color coded as follows: white boxes, titre<20; green boxes, titre<100; yellow boxes, titre<500; orange boxes, titre<1000; and red boxes, titre >1000. Grey boxes, not determined.

Additional viruses tested in TZM-bi assay and all samples below 100: JR-FL, Q23.17, 6535.3, QH0692.42.