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J Immunol 2014; 192:5802-5812; Prepublished online 14 May 2014;
doi: 10.4049/jimmunol.1301898
http://www.jimmunol.org/content/192/12/5802

Supplementary Material http://www.jimmunol.org/content/suppl/2014/05/14/jimmunol.1301898.DCSupplemental

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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 (bNAb) 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 env) 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-binding 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 NAbs that recognize a wide variety of HIV-1 strains.

Successfully licensed antiviral vaccines, such as the yellow fever and measles vaccines, elicit NAbs as correlates of protection (10, 11), and passive transfer of broad neutralizing Abs (bNAb) 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 Ab reactivity against Env was required for the observed vaccine-induced protection of macaques (20), this strongly suggests that the induction of bNAb is a pivotal property of a protective HIV-1 vaccine (8). The recent identiﬁcation of epitopes targeted by new sets of bNAb 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...
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 predominant 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 Envs 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 bNAbs (34). However, we previously noticed the development of NAbs to several SIVs in an attenuated SIVmac239 infection 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.

The conclusion of this study is that the immunological principle put forward in this study is based on repetitive DNA vaccination (clade B envs gp140) followed by boosting with the heterologous recombinant gp140 Env protein (30–32). The heterologous recombinant gp140 Env protein (30–32) was used to repeatedly immunize rabbits with a trivalent DNA vaccination (clade B envs gp140) followed by boosting with the heterologous recombinant gp140 Env protein (30–32).

### 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-1X4T, YU2, ITM1, BIBMC9-9, and HIV-2 (accession no. UC37; AY949474; 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 Hyperslacks (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 polyethylenimine 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 C for 4 h to remove cell debris, and passed through a 0.22 μm filter. After adjustment to pH 8 using 1 M glycine (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 sCD4 starting at 20 nM was used under similar conditions. Buffers, Tween, the sensor surface was regenerated by two 30-s injections of 10 mM glycine, pH 2.0. Data were analyzed in the BIA evaluation software (V 4.0.1). Double referencing was performed using a blank control flow cell, as well as a buffer injection, and kinetic data were fitted to a 1:1 Langmuir binding model.

#### DNA vaccine plasmids

The construction of BX08 gp140 (GenBank JX473289) plasmid used codons from highly expressed human genes as described earlier (46–48) and shares two primary envs from chimpanzees (chim1 [JX473290] and chim2 [JX472391]) (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, 8, and 12 with a mix of the three clade B env-DNA plasmids encoding SIV1 env gp140 intradermally i.d. (i.d.) with the Derma Vax EP device (CytoPulse Sciences/Collectes, 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 time point. At week 16, all DNA primed rabbits received a heterologous protein trimer SIVmac239 (50 μg) 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 priming at weeks 4, 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). These two groups subsequently received a heterologous protein trimer SIVmac239 (50 μg) i.d. injection (no adjuvant), and sera were 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 2 wk later (wk 22). Three animals from Gr. 1 and three from Gr. 2 received a second gp140 boost from HIV-1X4T: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 boost at week 20. A third group (Gr. 3; n = 6) received empty vector plasmids and aluminum hydroxide as protein boost. The fourth group (Gr. 4; n = 6) received only protein trimer SIVmac239 (50 μg) 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-1TM1,4 in cationic adjuvant formulation 01 (57) by i.d. injection...
ELISA
End-point binding titers were determined as described previously (58). In brief, serially diluted serum samples were added to microtiter plates coated with 0.5 μg/ml gp140 (HIV-1C32 or SIVmac239) or HIV-1_CNSA C1 (74-CVPADPNPQEMVLEN, HXB2 numbering), HIV-1C54 V2 (175-LFYRL-DIVPLTKKNY), HIV-1CNSA V3 (300-NTRKSRIPGQFTF), HIV-1CNSA V5 (461-EPNDTETRPPGGDM), SIVmac239 V2 (169-KFTMTGLKRDKTKE-YNETYW), or SIVmac239 V3 (319-VLPVTMSGLVFHSQPINDR) 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), developed with 1-STEP Ultra TMB-ELISA substrate (Thermo Fisher Scientific), 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.

Neutralization assays
IgGs were purified from serum using Protein G HP SpinTrap columns (GE Healthcare) according to the manufacturer’s instructions. Eluted IgGs were quantified 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, conducted in triplicate as described previously (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 Ochsener 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 cells 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), and another set received cells only (background control). Infected Abs were detected with a biotinylated anti-HIV-1 Abs (HGP68, F105, and HR10) and labeled with streptavidin–HRP. OD values were read at 690 nm.

Statistical analyses
Figures and statistical analyses were prepared using GraphPad Prism statistical 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 SIVmac239 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 SIVmac239 gp140 trimer migrated as a high m.w. band under nonreducing SDS PAGE, the HIV-1 gp140 trimers demonstrated at least partial dissociation into monomeric forms in the presence of reducing SDS PAGE (Fig. 1B).

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 concentration of 16 μg/ml before addition of the pseudovirus and a final DMSO concentration of 0.3%. The HIV-1 MN V3 peptide (CPRPNYNKRKRRHRIGGPQAYYTNTIQRQAC, EVA7019), the GPG HIV-1 SP2 clade B V3 peptide (TRKSIYIPGRGAFHTT, ARP7707), the GPG HIV-1 consensus clade A peptide (KSVHERIQGGFAST, ARP78012.1), and the scrambled control (ARP7009) were obtained from the European Vaccine Against Centre for AIDS Reagents, National Institute for Biological Standards and Control (NIBSC; South Mimms, Potters Bar, U.K.).

SIVmac239 gp140 Env CAN BOOST HIV-1 NEUTRALIZATION
FIGURE 1. Trimerization of SIVmac239 gp140 and binding to human CD4. (A) Gel-filtration chromatography analysis of the soluble gp140 trimers from SIVmac239 and HIV-1_YU2. Both complexes elute as a single symmetric peak at the expected elution volume for the trimERIC species demonstrating that the gp140 trimers produced were pure and homogenous. The chromatography trace for HIV-1_YU2 is representative for the different HIV-1 gp140 used in this study. (B) Nonreducing (N/R) and reducing (Red) SDS PAGE analysis of SIVmac239 and HIV-1_YU2 gp140. The SIVmac239 gp140 protein runs as a high m.w. band under nonreducing conditions, indicating that the three polypeptides of the trimer are covalently linked by disulfide bonds between the three gp140 chains. HIV-1_YU2 gp140 runs as a smear under nonreducing conditions, suggesting that the three polypeptides of the trimer are not covalently linked. (C) Surface plasmon resonance analysis of SIVmac239 gp140 and HIV-1_C037 gp140 binding to human CD4 and anti–HIV-1 Nabs. SIVmac239 (right panel) or HIV-1_C037 (left panel) were immobilized and their binding to tetrameric soluble human CD4 (CD4-IgG3), as well as mAb F105, HGP68, and HR10, was measured.
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 Env**

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 emcs (from the clinical isolates HIV-1Bx08, 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 (pFlc) 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-1NBS040.9 (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 AITM1.4 trimer 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 (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 Ab 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 SIVmac239 or HIV-1 UG37 gp140-binding Abs (Fig. 2C, Gr. 3). Importantly, repeated administration of SIVmac239 trimer without HIV-1 DNA priming did not elicit high-titer binding Abs to HIV-1 UG37 gp140 even though responses to SIVmac239 were induced (Fig. 2C, Gr. 4). Conversely, binding IgG responses toward SIVmac239 were induced by the HIV-1 DNA prime before the SIVmac239 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 UG37 and SIVmac239 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 env DNA prime was able to induce Abs capable of binding to both clade A HIV-1 UG37 and SIVmac239.

Trimeric SIV Env protein boost significantly increases NAbS induced by HIV-1 env DNA priming

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$_{50}$ 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 SF162.LS and HIV-1 MN.$n$ viruses after the DNA priming phase (wk 16, DNA) with strongly increased neutralization titers after the first SIVmac239 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 SIVmac239 gp140. To further assess the breadth of the responses after boosting with SIVmac239 gp140, we measured neutralization titers against tier 1 and 2 viruses using the A3R5.7 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 A3R5.7 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, QH0692.42 (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 (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. 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.
FIGURE 5. Epitope mapping reveals preferential binding to C1, C2, V2, V3, and V5. (A) Selected sera (n = 2 per group from the first study) from time points after HIV-1 DNA-prime (wk 16, DNA) and after the first SIVmac239 protein boost (wk 20, DNA/gp140) were subjected to epitope mapping by pepscan peptide arrays using heterologous HIV-1_CN54 peptides. (B) The change in response intensity after SIVmac239 protein boosting is shown for peptides that were undetectable after DNA immunizations (white) or showed positive responses after DNA priming (gray). (C) Percentage of positive peptides detected before (wk 16, DNA) and after the boost (wk 20, DNA/gp140) in the peptide array. (D) ELISA binding titers to indicated peptides from HIV-1 or SIVmac239 were determined after the second protein 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 < 0.05, **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).

in which the animals (n = 4) received clade B env DNA priming followed by a boost with HIV-1_TMT-1_4 gp140 (clade A; Fig. 4A). HIV-1_TMT-1_4 gp140 was selected based on comparative analyses of a large set of HIV-1 gp140s and was found to be one of the most potent for induction of neutralizing activity when given in an adjuvant without prior priming (45). However, in contrast with SIVmac239 gp140, HIV-1_TMT-1_4 gp140 was not able to boost the response against the clade B viruses HIV-1_LPI62_LS and HIV-1_LMN.2. When tested against clade C (MW965) and CRF01 AE (TH023.6), only two of four animals had detectably increased neutralization titers against the CRF01 AE virus after boosting with HIV-1_TMT-1_4 gp140 (Fig. 4B). Visualization of the magnitude-breadth curves of data pooled from both TZM-bl and A3R5.7 assays for the viruses that were tested on all animals revealed a significant increase of neutralizing activity after the SIVmac239 gp140 boost, but not after the HIV-1_TMT-1_4 gp140 boost (Fig. 4C). We then asked the question whether HIV-1 sınav40.9 (clade A) and/or HIV-2 gp140 trimer would be able to further boost neutralization titers induced by the DNA (clade B en1x) prime and SIVmac239 gp140 boost (Fig. 4D–G). As shown in the primary study, boosting with the SIVmac239 gp140 trimer increased neutralization titers induced by the DNA. Interestingly, there was a clear trend for increased neutralization titers against HIV-1 induced by the HIV-1 gp140 trimer that was not observed with HIV-2 gp140.

Taken together, these data demonstrate that the highly heterologous, stable SIVmac239 gp140 trimer was a potent and important component in the induction of high titers of NAb activity against heterologous clade B viruses with evidence of cross-clade NAb activity. Furthermore, the DNA prime–protein boost protocol induced durable Ab responses that lasted for at least 3 mo after final immunization with SIVmac239.

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
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It should be noted that the mechanistic basis for this induction of heterologous neutralization remains unresolved. The HIV-1 antigenicity profile of SIV<sub>mac239</sub> 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 SIV<sub>mac239</sub> 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 SIV<sub>mac239</sub> was recently shown to confer selective protection against repetitive intrarectal challenge with SIV<sub>mac239</sub> (71). A recent study suggested that SIV<sub>mac239</sub> displays increased gp160 trimer stability in vivo and specifically highlighted a thyrusts cysteine 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 SIV<sub>mac239</sub> gp140 used in this study also appeared to be uniformly bound by covalent disulfide bonds between the protomers. This is likely to conferring 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 trimers in immunogens comes from the observation that trimeric HIV-1 gp140 induced more potent NAb 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 SIV<sub>mac239</sub> trimer result in the induction or boosting of cross-reactive Th cell responses, and thus strengthening the primed B cell response.

Even though the mechanism underlying how the SIV<sub>mac239</sub> 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 SIV<sub>mac239</sub> gp140 protein boost to increase HIV-1 NAb titers. 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 elicited 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-1<sub>NIBSC40.9</sub> 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 Env to reach the goal to induce high-tier bNAbs. Sequential immunization using Envs isolated during the course of a natural infection that resulted in high-tier bNAbs is one principle put forward (79). In this study, we adopted repeated immunizations but used highly divergent gp140 Env of SIV<sub>mac239</sub> as one component in a recurrent immunization schedule. Our data indicate that the SIV<sub>mac239</sub> 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 proteomics. This study reports the induction of HIV-1 neutralization through repeated HIV-1 DNA env mixture priming followed by a highly heterologous boost based on a SIV<sub>mac239</sub> gp140 trimer.

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 HIV-1 DNA immunization schedule was based on a previously published optimization (49), and its effectiveness in this study is highlighted by the fact that strong binding titers and significant neutralization were already observed after the DNA immunization phase (Figs. 2C and 3). It is conceivable that the use of attenuated vectors, or more effective DNA delivery systems/adjuvants, may be required for the induction of potent Ab priming in primates (69). The pivotal role of effective priming, for induction of the neutralizing Abs, is indicated by the observation that the top responders after DNA priming were the animals with a tendency of responding with the highest neutralizing activity against HIV-1 after the SIV<sub>mac239</sub> boost (Fig. 3). These results also suggest a genetic impact on the ability of the animals to generate high-titer NAbs.

Discussion

This study reports the induction of HIV-1 neutralization through repeated HIV-1 DNA env mixture priming followed by a highly heterologous boost based on a SIV<sub>mac239</sub> gp140 trimer.

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 HIV-1 DNA immunization schedule was based on a previously published optimization (49), and its effectiveness in this study is highlighted by the fact that strong binding titers and significant neutralization were already observed after the DNA immunization phase (Figs. 2C and 3). It is conceivable that the use of attenuated vectors, or more effective DNA delivery systems/adjuvants, may be required for the induction of potent Ab priming in primates (69). The pivotal role of effective priming, for induction of the neutralizing Abs, is indicated by the observation that the top responders after DNA priming were the animals with a tendency of responding with the highest neutralizing activity against HIV-1 after the SIV<sub>mac239</sub> boost (Fig. 3). These results also suggest a genetic impact on the ability of the animals to generate high-titer NAbs.
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-LMN4 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-LMN4 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 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 gp140 proteins.

In conclusion, this study provides a framework for the induction of HIV-1 Env-specific NAbS against clade B viruses, as well as reactivity against sensitive CRF01 AE and clade C viruses by repeated DNA env mixture priming followed by boosting with distally related and relatively stable trimeric SIVmac239.

Acknowledgments
We thank the European Vaccine Against AIDS Centre for AIDS Reagents, NIBSC for kindly providing reagents and peptides. We thank Prof. Quentin Sattentau, University of Oxford for critically reading the manuscript, and Prof. Sarah Rowland-Jones, University of Oxford, for the HIV-2 expression construct.

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
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