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

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Abstract

Our knowledge of the binding sites for neutralizing antibodies (NAbs) 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). Here we report an immunization strategy composed of a trivalent HIV-1 (clade B *envs*) DNA prime, followed by a SIV_{mac239} gp140 Env protein boost that aimed to focus the immune response to structurally conserved parts of the HIV-1 and SIV Envs. Heterologous NAb titres, primarily to tier 1 HIV-1 isolates, elicited during the trivalent HIV-1 *env* prime, were significantly increased by the SIV_{mac239} gp140 protein boost in rabbits. Epitope mapping of antibody 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.

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Keywords

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Introduction

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, since 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 NAb activity and that a small fraction (approximately 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 bNAbs can protect macaques against virus infection with chimeric SIVs encoding the HIV-1 Env (SHIV) (12–19). Taken together with the recent finding that antibody reactivity against Env was required for the observed vaccine-induced protection of macaques (20) this strongly suggests 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 (MPER), the CD4 binding site (CD4bs) and some of the conserved elements in the variable regions V1/V2 and V3 including the integrin ($\alpha 4\beta 7$) 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 regimes with the capacity to elicit antibody responses focused towards 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 antibody 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 lag behind viral evolution and generally occurs too late in order to be of substantial benefit for the infected individual (21, 28, 29).

The immunological principle put forward here is therefore based on recurrent host exposure to divergent Envs and the novel use of the highly divergent gp140 Env from SIV_{mac239} 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 SIV_{mac239} for the protein boost. SIV_{mac239} 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 SIV_{mac239} generally does not induce bNAbs (34). However, we previously noticed the development of NAbs to several SIVs in an attenuated SIV_{mac239} infection model when animals were treated with daily tenofovir between ten days and four months following inoculation (35). The SIV_{mac239} virus is very resistant to NAbs (36) and the macaques displayed potent neutralization to sensitive heterologous SIVs before the appearance of neutralization to homologous SIV_{mac239} (35). This attenuated SIV_{mac239} infection study, additionally revealed neutralization of HIV-1 in sera from the macaques (35) even though the HIV-1 and SIV_{mac239} gp140 proteins have only about 30% sequence identity and divergent antigenicity. We therefore here hypothesize that the neutralization resistant SIV_{mac239} Env may have immunogenic features suitable for the induction of NAbs of which some appear cross-reactive between HIV-1 and SIV_{mac239}. Accordingly, they both bind human CD4 and display significantly conserved topological architectures (37). Additionally, the higher stability of SIV_{mac239} trimers when compared to 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 SIV_{mac239} gp140 boost and resulted in high titre heterologous NAbs against clade B viruses and activity against 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 weeks of age at start of experiment, approximately 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

SIV_{mac239}, HIV-1_{UG37}, YU2, ITM1_4, NIBSC40-9 and HIV-2 (accession numbers, UG37: AY494974; YU2: M93258; ITM1_4: FM165626; NIBSC 40-9: KJ579955; HIV-2 is JN863894) (41–45) gp140 were produced following transient transfection of 293T cells cultured in multilayer Cell Bind Hyperflasks (Corning) in high glucose DMEM (Sigma) supplemented with 10% FCS (Sigma) and Penicillin-Streptomycin solution (Sigma). Two mg plasmid DNA was incubated with 3.6 mg PEI in media without FCS for 30 minutes to allow complex formation. This was added to cells and brought to 500 ml with DMEM containing 2% FCS. Supernatants were collected after 48 hours and fresh media, containing 10% FCS, was added to the cells for a further 48 hours at which point the media was exchanged once again. All supernatant was centrifuged at 7000 × g for 4 hours to remove cell debris, and passed through a 0.22 µm filter. After adjusting to pH 8 using 1 M Tris HCl (Sigma), media was passed over a cobalt chloride metal-affinity column made of Talon superflow resin (Clontech). After washing with 2 column volumes of 0.015 M Tris Buffered Saline (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 trimer were identified and further purified using GNA-lectin (Vectorlabs) 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 Inc., Sweden) in HBS-EP buffer (Biacore Inc.) at 37°C. 700 response units (RUs) of gp140 were immobilized on a CM5 sensor chip using standard amine coupling at pH 4.5. Binding responses were measured by injecting mAbs or CD4-IgG₂ 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. Between cycles, the sensor surface was regenerated by two 30-second 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 two other primary *envs* from Danish patients (ctl21 (JX473290) and ctl27 (JX473291) (49) were similarly codon optimized and cloned in the same expression plasmid with the key elements CMV IE promoter-enhancer, tPA 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 weeks 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 HIV-1 *env* gp140 i.d. with the Derma Vax EP device (CytoPulse Sciences/Collectis, Romainville, France) (Fig. 2A). A total concentration of 200 µg DNA was injected i.d. at two injection sites, as described in (50). Blood for collection of sera and PBMC was taken from the ear vein before immunizations (time point 0 (wk 0)) and four weeks after the last DNA immunization (wk 16). DNA immunizations were administered in 12 animals (group 1 (Gr.1)) without adjuvant and in 12 animals (group 2) with a cellular adjuvant consisting of allogeneic activated cells (AAC) in the first study. The AAC was prepared as previously described (51–55) using allogeneic rabbit splenocytes activated *in vitro* with Con A (2,5µg/ml) (Sigma) for 48 hours 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 AAC per injection time point. At week 16, all DNA primed animals received a heterologous protein trimer SIV_{mac239} (50 µg) by i.d. injection without adjuvant and sera were collected 4 weeks later (wk 20). A second identical boost was performed 4 weeks after the first protein immunization and blood was collected 2 weeks 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 (group 1 study II; n=6) received the DNA-*env* clade B mix

priming at weeks 0 (twice first week), 4, 8 and 12 delivered by electroporation; a second group (group 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 SIV_{mac239} (50 µg) by i.d. injection (no adjuvant) and sera was collected 4 weeks later (wk 20). A cross-over experiment with three animals from groups 1 and 2 (study II) was then performed 4 weeks after the first protein immunization (wk 20) and blood was collected 2 weeks later (wk 22). Three animals from group 1 and three from group 2 received a second gp140 boost from HIV-1_{NIBSC 40-9} clade A at week 20. The other three animals in groups 1 and 2 received gp140 from HIV-2 (primary isolate CA65330.5) as the second protein boost at week 20.

The third group (group 3; n=6) received empty vector plasmids and albumin as protein boost. The fourth group (group 4; n=6) received only protein trimer SIV_{mac239} (50 µg) by i.d. injection at weeks 0, 8 and 16. A comparative group (group 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 CAF01 adjuvant (57) by i.d. injection at week 14 and sera was collected 2 weeks later (wk 16). Sera were separated and stored at -20°C until IgG purification or direct analysis.

Enzyme-linked immunosorbent assay (ELISA)

Endpoint binding titres were determined as described previously (58). Briefly, serially diluted serum samples were added to microtitre plates coated with 0.5 µg/ml gp140 (HIV-1_{UG37} or SIV_{mac239}) or HIV-1_{CN54} C1 (74-CVPADPNPQEMVLEN, HXB2 numbering), HIV-1_{CN54} V2 (175-LFYRLDIVPLTKKNY), HIV-1_{CN54} V3 (300-NNTRKSIRIGPGQTF), HIV-1_{CN54} V5 (461-EPNDTETFRPGGDM), SIV_{mac239} V2 (169-KFTMTGLKRDKTKKEYNETWY) or SIV_{mac239} V3 (319-VLPVTIMSGLVFHSQPINDR) peptides and blocked with 2% bovine serum albumin in washing buffer (0.05% Tween-20 in PBS). Bound antibodies 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 dataset. Endpoint titres were defined as the concentration at which the curve reached the threshold (0.01), which was greater than two standard deviations above background. HIV peptides were obtained from the EU Eurovac consortium.

Peptide Scanning

Overlapping linear 15-mer and circularized 15-mer peptides based on gp140 of HIV-1_{CN54} were tested for reactivity against heat-inactivated rabbit sera by Pepscan Therapeutics (Netherlands). 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 9aa windows were calculated for each position, as described previously (59).

Neutralization assays

IgG were purified from serum using Protein G HP SpinTrap columns (GE Healthcare) according to the manufacturer's instructions. Eluted IgG 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 as well as both CCR5 and CXCR4, conducted in triplicates as described previously (60) (<http://www.europrise.org/neutnetsops.html>; SOP10). A panel of pseudoviruses was used and prepared as previously described (60, 61). Infection levels were determined after 48 hours by measuring firefly luciferase activity in TZM-bl. Neutralizing antibody activity was also assessed using A3R5.7 cells (62). A3R5.7 cells (63) were obtained from Drs. Jerome Kim and Robert McLinden at the US Medical HIV Research Program (MHRP). This is a human CD4⁺ lymphoblastoid cell line (CEM/A3.01) (64) that was engineered at the US MHRP 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. Briefly, a dose of virus that generates approximately 50,000 relative luminescence units (RLU) after 4 days of infection was incubated with serial 3-fold dilutions of test sample in duplicate in a total volume of 150 μ l for 1 hour at 37°C in 96-well flat-bottom culture plates. Exponentially dividing A3R5.7 cells (90,000 cells in 100 μ l of growth medium containing 25 μ g/ml DEAE dextran) were added to each well. One set of control wells received cells + virus (virus control) and another set received cells only (background control). Infection levels were determined after four days by measuring Renilla luciferase activity in A3R5.7 cells. The ID₅₀ was calculated as the reciprocal serum dilution causing 50% reduction of relative light units compared to 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 pre-incubated for 30 min with peptide dissolved in DMSO at a final concentration of 16 μ g/ml prior to addition of the pseudovirus and a final DMSO concentration of 0.3%. The HIV-1 MN.3 V3 peptide (CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC, EVA7019), the GPGR HIV-1 SF2 clade B V3 peptide (TRKSIYIGPGRAFHTT, ARP797), the GPGQ HIV-1 consensus clade A peptide (KSVHIGPGQAFYAT, ARP7012.1) and the scrambled control (ARP7099) were obtained from the EVA Centre for AIDS Reagents, NIBSC, UK.

Statistical analyses

Figures as well as statistical analyses were prepared using Graph Pad 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 SIV_{mac239} gp140 for heterologous boost

All gp140 trimers used in the present 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 molecular weight band under non-reducing 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 SIV_{mac239} yielded uniformly trimeric gp140 and that the SIV_{mac239} trimer is stabilized by covalent disulfide bonds between the monomers. Surface plasmon resonance binding analysis revealed that HIV-1 and SIV_{mac239} gp140s bind to soluble recombinant human CD4. However, no binding to SIV_{mac239} gp140 was detected using a panel of mAb against HIV-1 gp160 including F105, VRC01, HGP68 and HR10 which recognize the CD4 binding site, the V2 and the V3 regions, respectively (Fig. 1C and Suppl. Fig. 1 A and B). Hence, these findings suggest poor HIV-1 antigenicity of SIV_{mac239} gp140 and that SIV_{mac239} binds with a lower affinity to human CD4 compared with HIV-1_{UG37}.

Induction of binding antibodies to clade A HIV-1_{UG37} and SIV_{mac239} 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 the present study we used the same DNA prime followed by a highly heterologous trimeric SIV_{mac239} gp140 protein boost, hypothesizing that such a regime might focus the response to more conserved structures. Sequence alignments comparing *env* from Bx08, ctl21, ctl27 and SIV_{mac239} are shown in Suppl. Fig. 1 C. Although the overall sequence identity between HIV-1 *env* and SIV_{mac239} *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, an intense immunization regimen was adopted, which consisted of repeated DNA immunizations of rabbits over a four month period prior to boosting with SIV_{mac239} or HIV-1 (clade A) gp140 Env. Plasmid DNA was delivered by intradermal (i.d.) injections in the first week (day 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 SIV_{mac239} gp140 boosts were delivered i.d. at weeks 16 and 20 (50 µg/animal/time-point) without adjuvant in both groups 1 and 2. Additionally, we also evaluated if an adjuvant, consisting of activated apoptotic cells (AAC) that engage TLR4 (52–54), would increase DNA priming efficiency (Study I Gr. 2 n=12) (Fig. 2A). Group 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 group 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), while group 1 (Study II n=6) did not receive any adjuvant. All animals in groups 1 and 2 from the second study also received an i.d. SIV_{mac239} gp140 boost at week 16. Additionally, a cross-over experiment with three animals from each of these two groups was then performed at week 20 to evaluate whether a recombinant gp140 trimer from HIV-1 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 which received only the recombinant SIV_{mac239} trimer (Gr. 4 n=6). Finally a comparative group (Gr.5) was included in order to compare the boosting capacity of a gp140 HIV-1 clade A ITM-1_4 trimer with the results obtained by the SIV_{mac239} boost.

We first assessed the prevalence of Env-specific binding IgGs in serum against both SIV_{mac239} gp140 and heterologous clade A HIV-1_{UG37} gp140 by ELISA. High titres of binding antibodies against both antigens were detected in all animals following the protein boost (wk 22), without any demonstrable effect of the AAC adjuvant (Fig. 2B). These high IgG titres were confirmed in the second study as high ELISA titres 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 antibody titres 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_{mac239} or HIV-1_{UG37} gp140-binding antibodies (Fig. 2C, Gr. 3). Importantly, repeated administration of SIV_{mac239} trimer without HIV-1 DNA priming did not elicit high titre binding antibodies to HIV-1_{UG37} gp140 even though responses to SIV_{mac239} were induced (Fig. 2C, Gr. 4). Conversely, binding IgG responses towards SIV_{mac239} were induced by the HIV-1 DNA prime prior to the SIV_{mac239} Env boost (Fig. 2C, Gr. 1 and 2). The comparative group receiving a gp140 HIV-1 boost after priming (Gr. 5) mounted similar binding titres to HIV-1_{UG37} and SIV_{mac239} but tended to be lower when compared to those of groups 1 and 2 (Fig. 2C). Conclusively, these findings show that the trivalent clade B *env* DNA prime was able to induce antibodies capable of binding to both clade A HIV-1_{UG37} and to SIV_{mac239}.

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 50% inhibitory dose (ID₅₀) titres (Fig. 3). Serum neutralization responses in groups 1 and 2 of the first study are displayed together as no significant differences in NAb activity were found in the group receiving the AAC adjuvant (Suppl. Table 1). A significant induction of neutralizing activity was measured against heterologous HIV-1_{SF162.LS} and _{MN.3} viruses following the DNA priming phase (wk 16, DNA) with strongly increased neutralization titres after the first SIV_{mac239} gp140 boost (wk 20, DNA/gp140, Fig. 3). Measurements with purified serum IgG confirmed the induction of neutralization activity (Fig. 3C–D). Furthermore, neutralizing activity was also detected against tier 1 viruses from clade C (MW965.26) and CRF01 AE (TH023.6) (Fig. 3E–F) after boosting with SIV_{mac239} gp140. To further assess the breadth of the responses after boosting with SIV_{mac239} gp140,

neutralization titres were measured against tier 1 and tier 2 viruses using the A3R5.7 assay as well as against HIV-2 (Fig. 3G–H and Suppl. Table1). 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 and the eight animals with the lowest neutralization titres to SF162.LS in blue and the intermediary 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, while the best SF162.LS neutralizers (red) tended to also be high responders against the other viruses. Interestingly, the second SIV_{mac239} protein boost delivered four weeks after the first boost did not always significantly increase the neutralization titres. Nevertheless, when long-term persistence of neutralization was evaluated three months after the final SIV_{mac239} protein boost (wk 34), responses were still detected against both tier 1A viruses (Fig. 3A and B) and the tier 1B clade B virus RHPA.LucR (Fig. 3G). In line with the measured antibody binding titres (Fig. 2C), SIV_{mac239} 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-SIV_{mac251} were observed (Fig. 3J). Although the trivalent clade B *env* DNA immunization induced binding antibodies to SIV_{mac239}, it was not sufficient to induce a neutralization response to TCLA-SIV_{mac251} while boosting with SIV_{mac239} resulted in a detectable SIV_{mac251} neutralization (Gr.1+2 Fig. 3J). Hence, the SIV_{mac239} gp140 was able to induce significant neutralization to a neutralization sensitive clone of TCLA-SIV_{mac251}.

To further analyze and compare the boosting effect of the highly heterologous gp140 SIV_{mac239}, a comparative group, in which the animals (n=4) received clade B *env* DNA priming followed by a boost with HIV-1_{ITM-1_4} gp140 (clade A) was included (Fig. 4A). HIV-1_{ITM-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 to SIV_{mac239} gp140, HIV-1_{ITM-1_4} gp140 was not able to boost the response against the clade B viruses HIV-1_{SF162.LS} and _{MN.3}. When tested against clade C (MW965) and CRF01 AE (TH023.6) only two out of four animals had detectably increased neutralization titres against the CRF01 AE virus after boosting with HIV-1_{ITM-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 SIV_{mac239} gp140 boost but not after the HIV-1_{ITM-1_4} gp140 boost (Fig. 4C). We then asked the question whether HIV-1_{NIBSC40-9} (clade A) and/or HIV-2 gp140 trimer would be able to further boost neutralization titres induced by the DNA (clade B *env*s) prime and SIV_{mac239} gp140 boost (Fig 4D–G). As seen in the primary study, boosting with the SIV_{mac239} gp140 trimer increased neutralization titres induced by the DNA. Interestingly, there was a clear trend for increased neutralization titres 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 SIV_{mac239} gp140 trimer was a potent and important component in the induction of high titres of NAb activity against heterologous clade B viruses with evidence of cross-clade NAb activity. Furthermore, the DNA prime – protein boost protocol induced durable antibody responses that lasted for at least three months after final immunization with SIV_{mac239}.

Epitope mapping reveals preferential antibody 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 antibody 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 the observed antibody responses increased following the SIV_{mac239} gp140 boost (wk 20, DNA/gp140) (Fig. 5A–C). Indeed, antibody-binding responses that were already positive after priming displayed on average a significantly stronger increase after the boost (Fig. 5B). In addition, antibody binding to some peptides that were not recognized after DNA priming was detected after the protein boost, demonstrating an increased breadth following the SIV_{mac239} boost (Fig. 5B and C).

Peptides from regions in C1, V2, V3 and V5 were used in ELISA in order to assess the impact of the AAC adjuvant on binding titres against individual epitopes. Although the AAC adjuvant did not improve binding antibody titres 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 SIV_{mac239} protein strongly boosted primed HIV-1 responses, and led to broadening of the recognition, while the addition of AAC during the priming phase induced higher antibody binding titres 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, Suppl. 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 the NAb responses in eight out of the twelve animals tested. Further neutralization assays were performed with shorter peptides that were localized centrally on the V3-crown motifs of clade B (TRKSIYIGPGRFHTT) or clade A (KSVHIGPGQAFYAT) (Fig. 6B). This revealed a limited but significant heterologous inhibition of the NAb responses by the clade A peptide in addition to the potent inhibition by the clade B peptide with the GPGR motif (Fig. 6B).

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_{mac239} 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 titres as well as

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 antibody priming in primates (69). The pivotal role of effective priming, for induction of the neutralizing antibodies, 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_{mac239} boost (Fig. 3). These results also suggest a genetic impact on the ability of the animals to generate high titre NAb.

It should be noted that the mechanistic basis for this induction of heterologous neutralization remains unresolved. The HIV-1 antigenicity profile of SIV_{mac239} gp140 was poor as defined by lack of binding using a panel of mAb against HIV-1 gp160 including F105, HGP68 and HR10 which recognize the CD4 binding site, 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 in (70). Therefore, it appears possible that the SIV_{mac239} trimer used in this study possessed favourable immunogenic features for the boosting of anti-HIV-1 antibody responses irrespectively of the lack of conserved antigenicity with HIV-1. Furthermore, the immunogenic profile of SIV_{mac239} was recently shown to confer selective protection against repetitive intra-rectal challenge with SIV_{smE660} (71). A recent study suggested that SIV_{mac239} displays increased gp160 trimer stability *in vivo* and specifically highlighted a twin-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_{mac239} 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 papilloma virus vaccine (72, 73). Further support for the importance of stable trimeric immunogens comes from the observation that trimeric HIV-1 gp140 induced more potent NAb responses compared to monomeric gp120 (74). Beyond the scope of this study it would be highly interesting to evaluate if the favourable properties of the SIV_{mac239} trimer result in the induction or boosting of cross-reactive T helper cell responses and thus strengthening the primed B-cell response.

Even though the mechanism underlying how the SIV_{mac239} trimer boost provided such robust and broad increases of B-cell responses against HIV-1 remains unclear, the results presented here highlight the ability of this highly heterologous SIV_{mac239} gp140 protein boost to increase HIV-1 NAb titres. 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 NAb in rabbits (75). Our study confirms and extends the observation that heterologous prime-boost regimens do not only elicit high level but may also improve quality of antigen-specific antibody responses and thus offer a new platforms for eliciting bNAbs to HIV-1 (76–78).

Further boosting of the neutralization responses was observed with HIV-1_{NIBSC40-9} 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

Envs to reach the goal to induce high titre bNAbs. Sequential immunization using Envs isolated during the course of a natural infection that resulted in high titre bNAbs is one principle put forward (79). Here we adopted repeated immunizations but used highly divergent gp140 Env of SIV_{mac239} as one component in a recurrent immunization schedule. Our data indicate that the SIV_{mac239} 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 regime by cross-competition ELISA. Using this assay, we were unable to detect antibodies 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 antibody 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* 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 (80). However, a fundamental difference from our study is the use by Luo *et al.* (80) of SIV_{mac239} for DNA priming and HIV-1_{CN54} gp140 for the boost. The use of reversed order of HIV-1-SIV cross-immunization could be beneficial as the NAb response reported here comprised high magnitude responses (in the order of serum titres of 1/10,000) to tier 1 viruses. It should be noted that we could detect significant neutralization even three months after the final SIV_{mac239} protein boost. While the neutralizing activity against HIV-1_{SF162} 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 three of the twelve 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 SIV_{mac239} gp140 proteins.

In conclusion, the present 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 SIV_{mac239}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NAbs	neutralizing antibodies
bNAbs	broad NAbs
Env	Envelope glycoprotein
MPER	membrane-proximal epitope region in gp41
CD4bs	CD4 binding site
AAC	allogeneic activated cells
RLU	relative luminescence units
ID₅₀	50% inhibitory dose
RUs	response units
Gr	group

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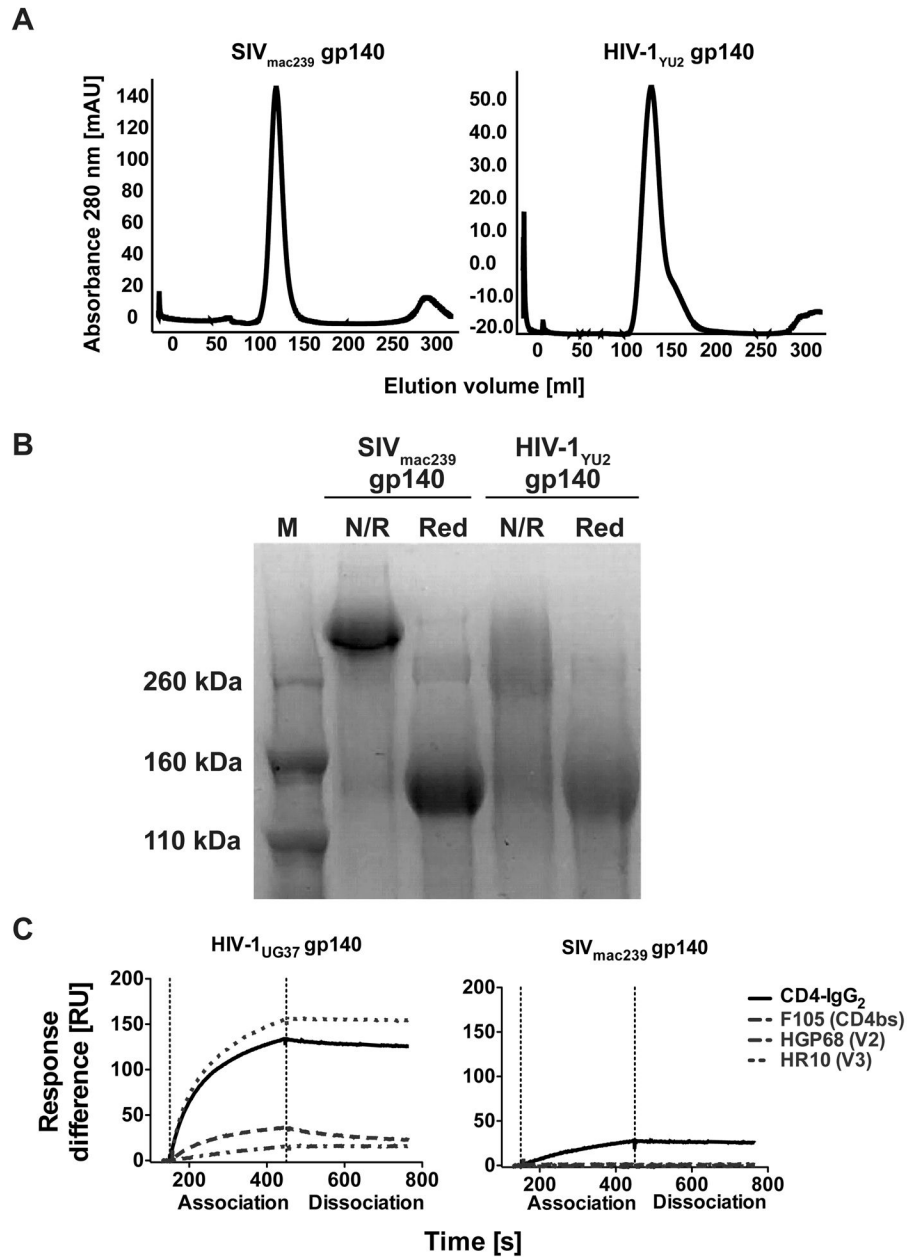


FIGURE 1. Trimerization of SIV_{mac239} gp140 and binding to human CD4

A) Gel filtration chromatography analysis of the soluble gp140 trimers from SIV_{mac239} 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_{YU2} is representative for the different HIV-1 gp140 used in this study. B) Non-reducing (N/R) and reducing (Red) SDS PAGE analysis of SIV_{mac239} and HIV-1_{YU2} gp140. The SIV_{mac239} gp140 protein runs as a high molecular weight band under non-reducing 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 non-reducing conditions suggesting that the three

polypeptides of the trimer are not covalently linked. C) Surface plasmon resonance analysis of *SIV*_{mac239} gp140 and HIV-1_{UG37} gp140 binding to human CD4 and anti-HIV-1 NAbs. *SIV*_{mac239} (right panel) or HIV-1_{UG37} (left panel) were immobilized and their binding to tetrameric soluble human CD4 (CD4-IgG₂) as well as mAb F105, HGP68 and HR10 was measured.

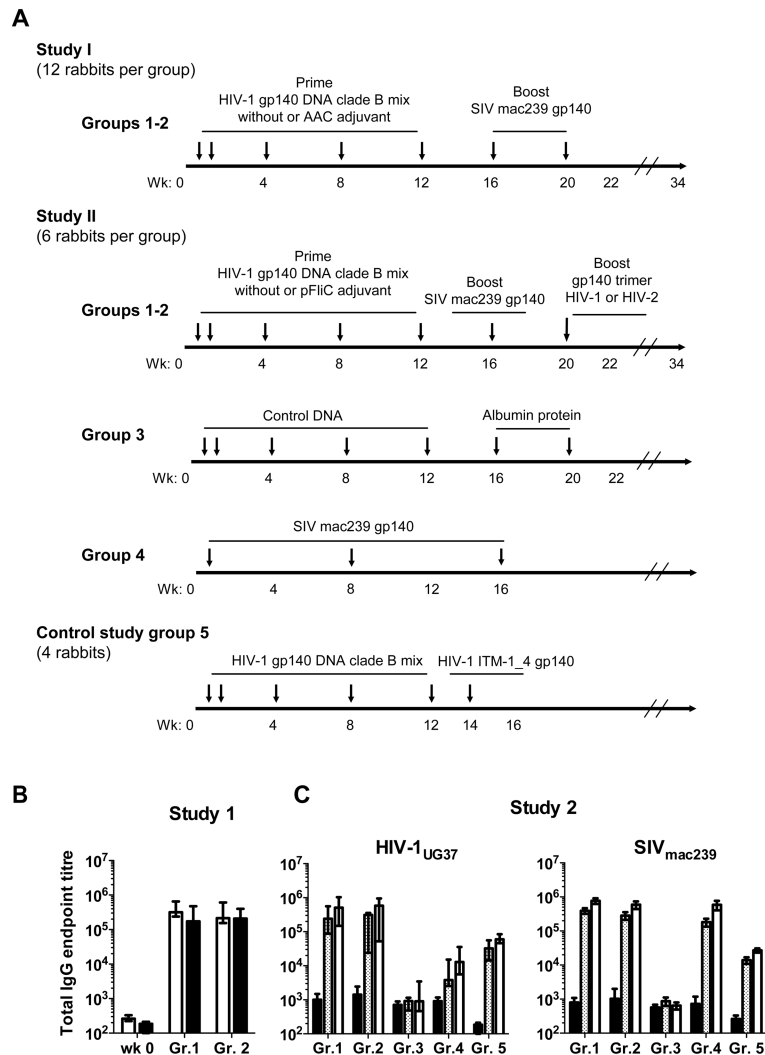


FIGURE 2. Schematic representation of the immunization regimens and binding antibodies elicited

A) Schematic immunization schedules of two independent studies ($n=48$) and a comparative group 5 ($n=4$) with arrows depicting immunizations. Sera were collected before immunization (wk 0). Animals (New Zealand white rabbits) received a mix of three clade B *env*-DNA plasmids at weeks 0 (twice first week), 4, 8 and 12 (200 μ g DNA by i.d. electroporation). Sera were collected 4 weeks after last DNA immunization (wk 16). Animals then received a heterologous protein SIV_{mac239} gp140 by i.d. injection and sera were collected 4 weeks after first protein boost (50 μ g)(wk 20). Animals received a second protein boost with SIV_{mac239} gp140 4 weeks after the first protein immunization and sera were collected 2 weeks after second protein boost (50 μ g) (wk 22). Three months later sera were analyzed for memory responses (wk 34). In the first study, twelve animals were included in Gr. 1 and the second study entailed six animals in this group. In the first study, Gr. 2 ($n=12$) received AAC adjuvant during the DNA priming phase and in the second study Gr. 2 ($n=6$) received a TLR5 agonist pFliC. Gr. 3 ($n=6$) received control DNA and albumin protein and Gr. 4 received three immunizations with SIV_{mac239} gp140 protein by i.d.

injection (n=6). Gr.5 received the mix of three clade B *env* DNA immunizations followed by one i.d. injection with HIV-1_{ITM-1_4} gp140 (n=4). (B) Endpoint titres (log₁₀) of IgG binding to SIV_{mac239} gp140 (white bars) or heterologous HIV-1_{UG37} 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. (C) Endpoint IgG titres from the second study are shown for binding to heterologous HIV-1_{UG37} gp140 and SIV_{mac239} gp140, as marked, in sera obtained at wk 0 (black bars), after the DNA priming (grey bars), and after the first gp140 boost (white bars) from the second study. Statistically significant lower endpoint IgG titres against SIV_{mac239} were found in Gr. 3 and against HIV-1_{UG37} in Groups 3 and 4 when compared with Groups 1 and 2 (One-way ANOVA using Bonferroni's Multiple Comparison Test p<0.05).

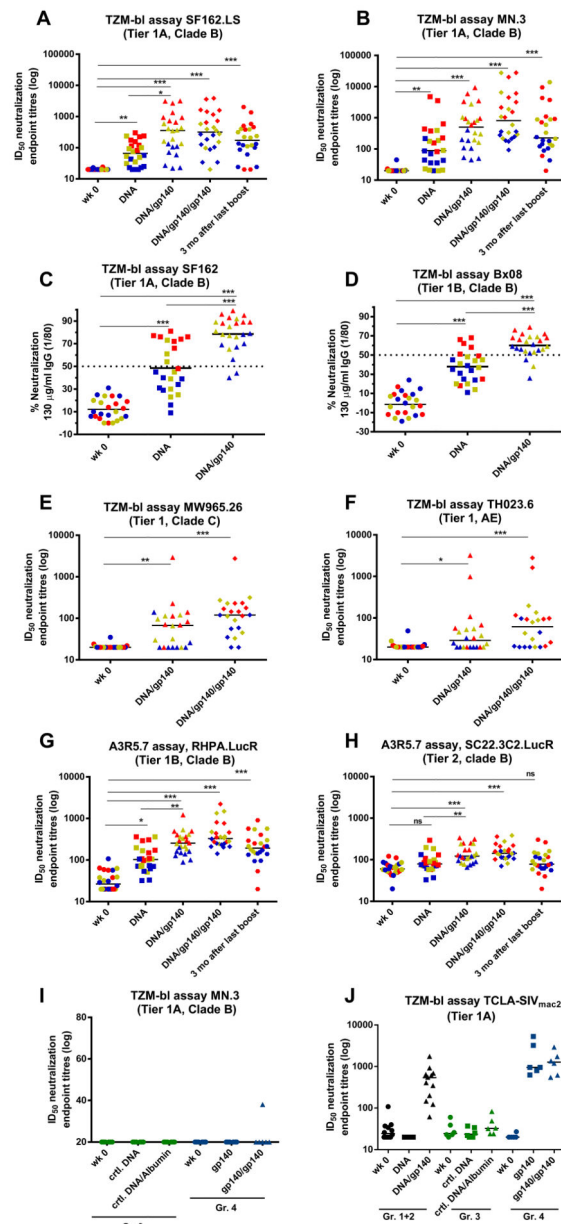


FIGURE 3. Induction of high-titre neutralizing antibodies against clade B viruses through the highly heterologous DNA-prime protein-boost regime

Data are shown for individual animals from groups 1 and 2 combined (n=24) (A–H). Lines represent median. Sera were collected before immunization (wk 0), 4 weeks after last DNA immunization (DNA), 4 weeks after first $SIV_{mac239gp140}$ boost (DNA/gp140) and 2 weeks after the second $SIV_{mac239gp140}$ boost (DNA/gp140/gp140). Three months later sera were analyzed for the longevity of responses (3 mo after last boost). High ID_{50} neutralizing titres 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 $\mu\text{g/ml}$ IgG final concentration approximately corresponding to a 1/80 serum dilution and dotted line indicates ID_{50} .

Neutralizing titres 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 indicate the animals with the highest titres 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. ID₅₀ titres in sera from groups 1 and 2 were tested in the A3R5.7 assay against clade B viruses RHPA.LucR (G) and SC22.3C2.LucR (H). Neutralization titres obtained in the TZM-bl assay for the control groups that received control DNA and albumin (Gr.3, displayed in green) or only SIV_{mac239} protein without a DNA prime (Gr.4, displayed in blue) are shown for neutralization of HIV-1 MN.3 (I) and the lab-adapted sensitive TCLA-SIV_{mac251} isolate (J). Significant values are indicated by *** p<0.001, **p<0.01 and *p<0.05 using One-Way ANOVA with Multiple Comparison Test.

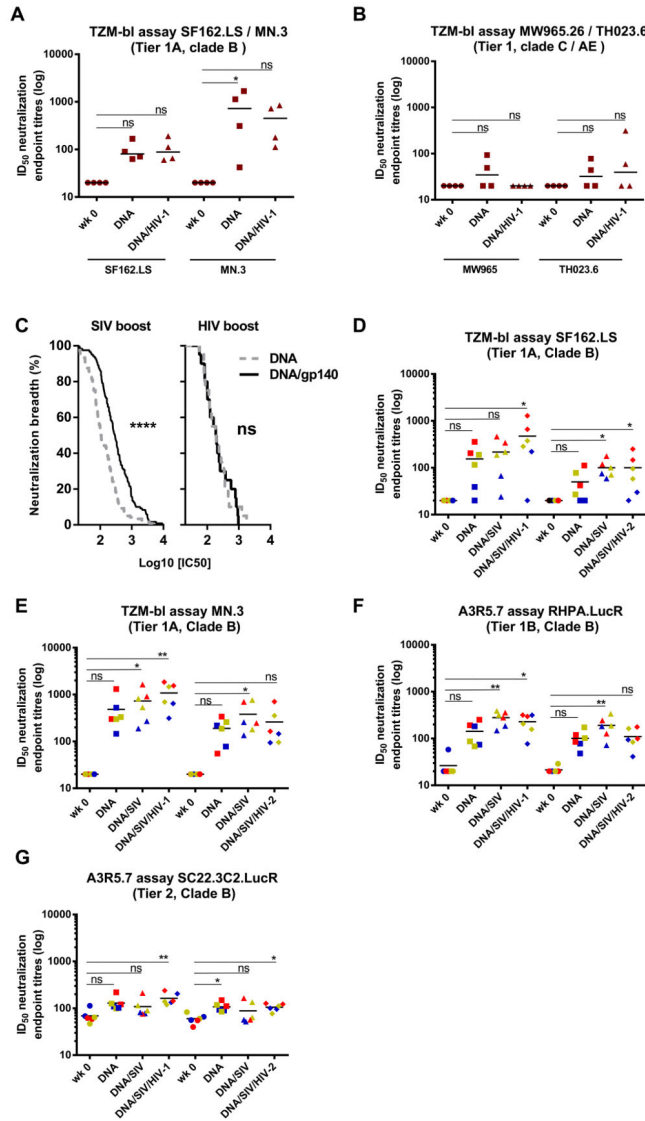


FIGURE 4. SIV_{mac239} can boost HIV-1 neutralization better than HIV-1 clade A ITM-1_4 while HIV-1 NIBSC40.9 clade A can support SIV_{mac239} responses
 (A, B). A comparative arm that received DNA clade B priming followed by the HIV-1 clade A ITM-1_4 gp140 boost (Gr.5), was tested against SF162.LS, MN.3, MW965 and TH023.6 with ID₅₀ titres displayed in brown. (C) Magnitude-Breadth curves show pooled data from both TZM-bi and A3R5.7 assays for the viruses that were tested on all animals (HIV-1 clade B isolates MN.3, SF162.LS, 9020.A13.LucR., RHPA.LucR., SC22.3C2.LucR.). Data obtained after DNA prime and after one protein boost are shown for sera from groups 1 and 2 (left panel) as well as the HIV-1 comparative arm (group5) (right panel). A Mantel-Cox log-rank test was used for statistical comparison. **** p<0.0001; ns= not significant. (D–G) Sera were collected in another comparative study before immunization (wk 0), 4 weeks after last DNA immunization (DNA), 4 weeks after first SIV_{mac239}gp140 boost (DNA/SIV) and 2 weeks after the second gp140 protein boost ((DNA/SIV/HIV-1) or (DNA/SIV/HIV-2)). An arbitrary color code indicate the animals with the highest titres against SF162.LS in red

(n=2), lowest in blue (n=2) and those in between in yellow (n=2). These colors are kept throughout the figure to be able to track the animals and their respective response to other viruses. Significant values are indicated by *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ as well as non-significant (ns) using One-Way ANOVA, Friedman's test with Dunns's Multiple Comparison Test.

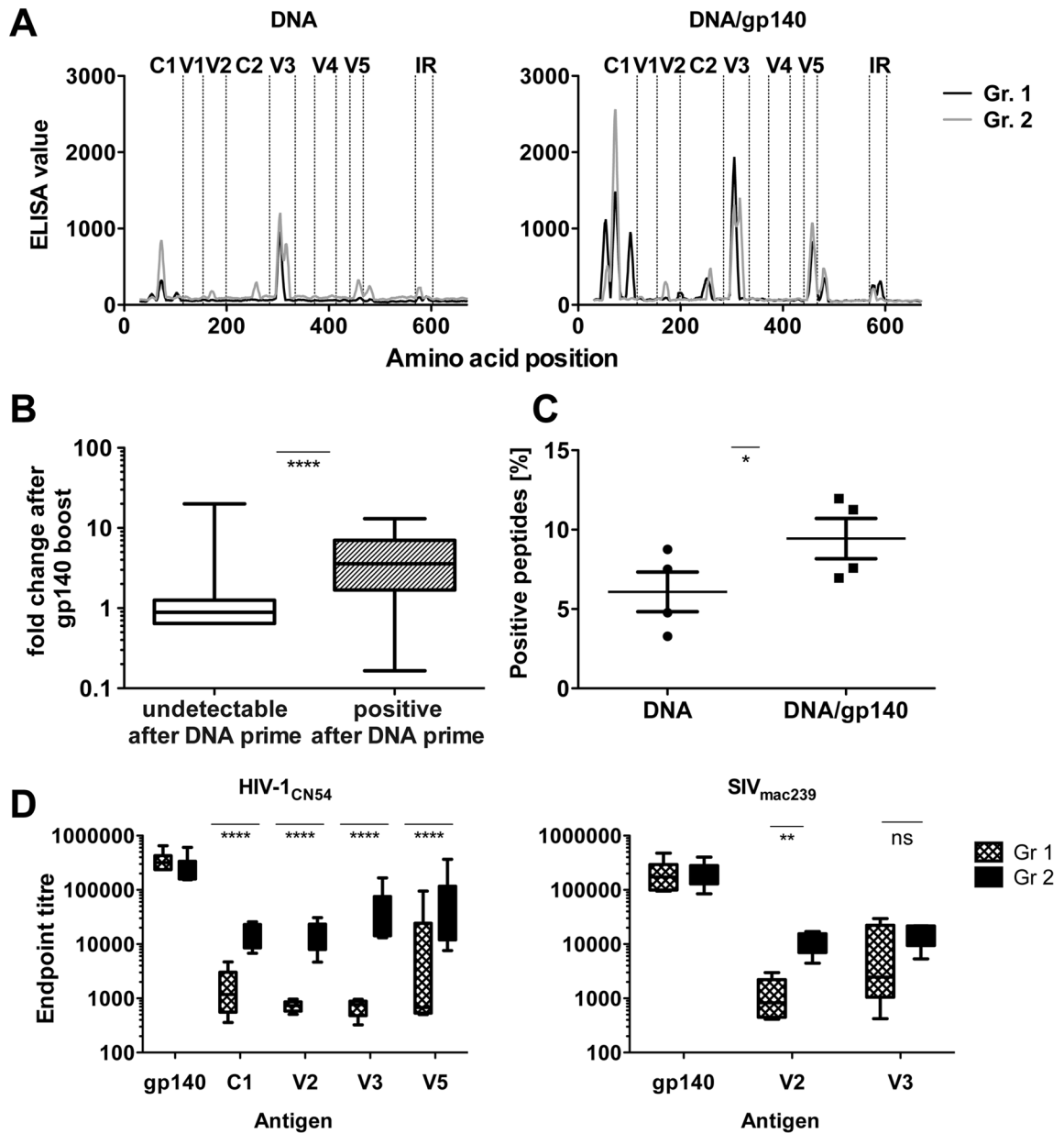


FIGURE 5. Epitope mapping reveals preferential binding to C1, C2, V2, V3 and V5
 (A) Selected sera ($n=2$ per group from the first study) from time points after HIV-1 DNA-prime (wk 16, DNA) and after the first SIV_{mac239} protein boost (wk 20, DNA/gp140) were subjected to epitope mapping by pepsan peptide arrays using heterologous HIV-1_{CN54} peptides. (B) The change in response intensity after SIV_{mac239} protein boosting is shown for peptides that were undetectable after DNA immunizations (white) or showed positive responses after DNA priming (grey) (C) Percentage of positive peptides detected before (wk 16, DNA) and after the boost (wk20, DNA/gp140) in the peptide array. (D) ELISA binding titres to indicated peptides from HIV-1 or SIV_{mac239} 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 * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$ as well as ns=not significant using a Mann-Whitney test (B), a paired t test (C), or a one-way ANOVA of log-transformed data with a Bonferroni's Multiple Comparison Test (D).

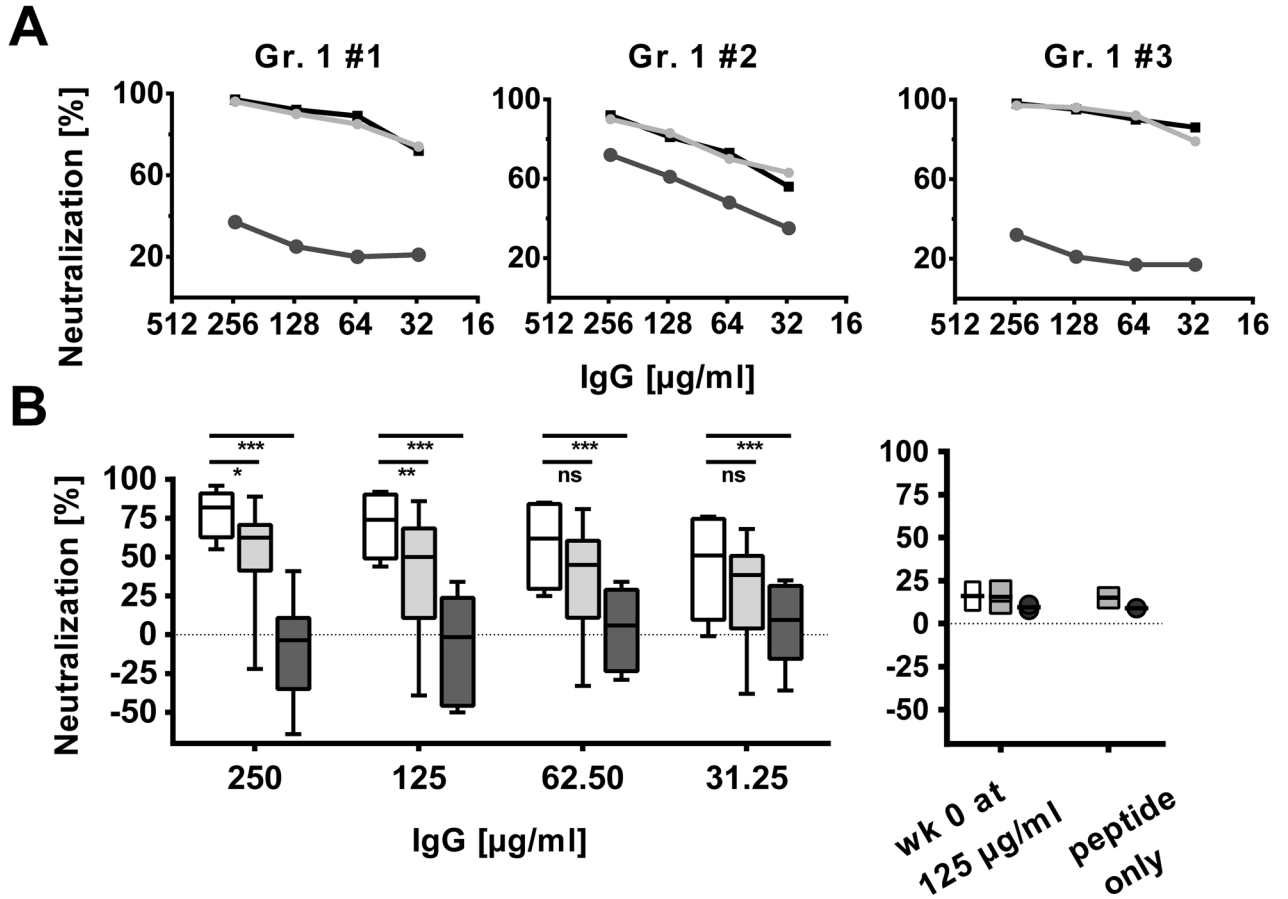


FIGURE 6. Neutralization against the SF162 virus is targeted to the V3 region in majority of animals
 IgG from animals from the first study were assessed for their capacity to neutralize HIV-1_{SF162} in the TZM-bl assay in the presence of blocking peptides and representative individual results are displayed. IgG were purified from sera two weeks after the second SIV_{mac239} gp140 boost. Percent neutralization is depicted on the y-axis and concentration of purified IgG on the x-axis.
 (A) Neutralization is displayed for three representative 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 (Suppl. Fig. 2)
 (B) Pooled analysis of the neutralization (no peptide, white) and inhibition by a short V3 peptide from clade A containing the GPGQ motif (light grey) or clade B containing the GPR motif (dark grey), respectively. Results from peptide inhibition without IgG as well as control neutralization using pre-immunization IgG in combination with the peptides (wk 0) did not show significant enhancement or neutralization of the virus. Significant values are indicated by *** p<0.001, **p<0.01 and *p<0.05 as well as ns=not significant using a two-way repeated measures ANOVA with a Bonferroni's Multiple Comparison Test.