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# Llama Antibody Fragments with Cross-Subtype Human Immunodeficiency Virus Type 1 (HIV-1)-Neutralizing Properties and High Affinity for HIV-1 gp120▽†

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**Members of the *Camelidae* family produce immunoglobulins devoid of light chains. We have characterized variable domains of these heavy chain antibodies, the VHH, from llamas immunized with human immunodeficiency virus type 1 (HIV-1) envelope protein gp120 in order to identify VHH that can inhibit HIV-1 infection. To increase the chances of isolating neutralizing VHH, we employed a functional selection approach, involving panning of phage libraries expressing the VHH repertoire on recombinant gp120, followed by a competitive elution with soluble CD4. By immunizing with gp120 derived from an HIV-1 subtype B′/C primary isolate, followed by panning on gp120 from HIV-1 isolates of subtypes A, B, and C, we could select for VHH with cross-subtype neutralizing activity. Three VHH able to neutralize HIV-1 primary isolates of subtypes B and C were characterized. These bound to recombinant gp120 with affinities close to the suggested affinity ceiling for in vivo-maturated antibodies and competed with soluble CD4 for this binding, indicating that their mechanism of neutralization involves interacting with the functional envelope spike prior to binding to CD4. The most potent VHH in terms of low 50% inhibitory concentration ($IC_{50}$) and $IC_{90}$ values and cross-subtype reactivity was A12. These results indicate that camelid VHH can be potent HIV-1 entry inhibitors. Since VHH are stable and can be produced at a relatively low cost, they may be considered for applications such as HIV-1 microbicide development. Antienvelope VHH might also prove useful in defining neutralizing and nonneutralizing epitopes on HIV-1 envelope proteins, with implications for HIV-1 vaccine design.**

During 2007, there were an estimated 2.5 million new human immunodeficiency virus type 1 (HIV-1) infections, with the majority of these acquired through heterosexual transmission (36). Even though antiretroviral therapy has proven effective in slowing disease progression, these drugs are expensive and not readily available to the majority of HIV-1-infected individuals. Thus, there is a need for effective preventive methods to control the HIV-1 pandemic, such as an HIV-1 vaccine or a topically applied HIV-1 microbicide. Agents that inhibit HIV-1 entry have potential use as microbicides, antiretroviral drugs, or prophylactics (42, 51). Furthermore, they may be useful tools in HIV-1 vaccine design in that they can help characterization of HIV-1 envelope proteins.

HIV-1 entry into target cells is mediated by the viral envelope spike, which consists of homotrimers of the surface glycoprotein, gp120, noncovalently bound to the transmembrane glycoprotein, gp41 (89, 91, 95). In addition to the functional spikes, there is also evidence for the presence of nonfunctional derivatives, such as gp41 stumps and gp120/gp41 monomers, on the viral surface (54, 66). Most variants of HIV-1 enter cells through attachment of the envelope spike to the main cellular receptor CD4 (15, 39), which triggers a conformational change allowing interaction with a cellular coreceptor, typically CCR5 or CXCR4 (53), eventually leading to fusion of virus and cell membranes. Potent entry inhibitors can target various stages of this process (51). Neutralizing monoclonal antibodies (MAbs) can act as HIV-1 entry inhibitors by targeting epitopes on the functional spike (61). HIV-1 has, however, evolved a number of ways to evade the humoral immune response, including variable regions, carbohydrate shields, extreme diversity, and conformational and entropic masking, and the neutralizing antibody response in HIV-1 infection is therefore in general rather weak and narrow (63, 89).

Many MAbs to HIV-1 envelope have been isolated from animals such as mice postimmunization and from humans fol-

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lowing HIV-1 infection. Of these, only a handful have been found to be broadly neutralizing across HIV-1 subtypes (8), and have been the result of HIV-1 infection rather than immunization. Two of these are directed against gp120: MAb b12, which binds to an epitope that overlaps a subset of the CD4-binding site (CD4bs) of gp120 (3, 10, 11, 68, 94), and MAb 2G12, which recognizes a carbohydrate motif (9, 70, 72, 80). Two broadly neutralizing MAbs, 4E10 and 2F5, recognize gp41 (9, 56, 77, 96). MAbs X5 (55), which recognizes an epitope on gp120 that is better-exposed after CD4 binding, and m14 (92), which competes with CD4 for binding to gp120, also display some neutralizing activity across HIV-1 subtypes, as do a minority of MAbs to the V3 region of gp120 (30, 31).

All of the broadly neutralizing MAbs reported to date are from individuals infected with HIV-1 of subtype B, which is dominant in Europe and North America. Phylogenetically, HIV-1 is classified into groups M, N, and O, with group M accounting for over 99% of infections and being the most variable, with extraordinary diversity in the envelope sequence between isolates. Group M is divided into subtypes A to D, F to H, J, and K, plus a number of circulating recombinant forms (CRFs), with subtype C currently infecting more people than any other subtype (34, 47). MAbs to HIV-1 envelope, whether obtained from natural HIV-1 infection or from immunization, can help in defining neutralizing and nonneutralizing epitopes on HIV-1 envelope proteins of various subtypes. We employed the nonconventional immune system of camelids to generate novel antienvelope antibodies.

In addition to conventional antibodies, members of the *Camelidae* family (camels, dromedaries, and llamas) produce antibodies without light chains, so-called heavy chain antibodies (33). The antigen-binding properties of these heavy chain antibodies are provided by one single fragment, the variable region of the heavy chain, which has been termed the VHH, or nanobody. Despite their small size of approximately 14 kDa, these VHH have characteristics in terms of affinity and specificity similar to those of conventional antibodies (81). They display complementarity-determining regions (CDRs) of which on average the CDR2 and CDR3 are longer than the corresponding CDRs of conventional antibodies (85). Furthermore, they have been shown to have a preference for cleft recognition and for binding into active sites (16, 41). The VHH domain can be easily cloned and expressed to high levels in bacteria and yeast (26, 27). This notion, together with advantageous characteristics in terms of stability and solubility (20, 64, 81), has led to successful development of camelid VHH in a number of applications against a range of biological targets (2, 13, 14, 19, 21, 57, 58, 69, 83, 84), including neutralization of rotavirus (28, 60). We hypothesized that the small size of VHH in combination with their protruding CDR3 loops and their preference for cleft recognition may allow them to recognize conserved motifs on gp120 that are occluded from conventional antibodies.

Here, we describe the identification of a set of llama VHH that can inhibit binding of soluble CD4 (sCD4) to HIV-1 envelope proteins and neutralize HIV-1 primary isolates of subtypes B and C. These VHH may be useful as tools for HIV-1 vaccine design and could possibly be developed as candidate HIV-1 microbicides.

## MATERIALS AND METHODS

**Monoclonal antibodies and sera from HIV-1-seropositive individuals.** MAb b12 (10) was kindly provided by D. Burton, Scripps Institute, La Jolla, CA. MAb GP68 (74) was obtained through the Centralised Facility for AIDS Reagents (CFAR), National Institute for Biological Standards and Controls (NIBSC), Potters Bar, Herts, United Kingdom (original source, A. Osterhaus and M. Schutten). MAbs 654-D (29, 37, 40) and 447-52D (30, 31) were obtained through the CFAR, NIBSC (original source, S. Zolla-Pazner). MAb 17b (79) was obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (original source, J. E. Robinson). MAbs 2G12 (9) and 4E10 (77) were obtained from Polymun Scientific GmbH, Vienna, Austria, as part of the Collaboration for AIDS Vaccine Discovery (CAVD). Quality control sera 2, 5, and 6 from HIV-1-seropositive individuals have been described previously (48).

**Recombinant HIV-1 envelope proteins.** Recombinant gp120 from HIV-1 CN54 (subtype B′/C; CRF07_BC) was kindly provided by I. Jones, Reading University, Reading, United Kingdom, through the European Microbicides Project. Recombinant gp140 from HIV-1 92UG037 (subtype A) was kindly provided by S. Jeffs, Imperial College London, London, United Kingdom. Recombinant gp120 from HIV-1 IIIB (EVA607) was obtained from the CFAR, NIBSC. Recombinant gp120 derived from HIV-1 92UG037 and 92BR025 (subtype C) was expressed and purified as detailed in File S1 in the supplemental material.

**Viruses.** The sources of HIV-1 peripheral blood mononuclear cell (PBMC) isolates, HIV-1 replication-competent molecular clones, and *env* clones used to prepare pseudotyped viruses and to express recombinant gp120 are detailed in File S1 of the supplemental material.

**Cells.** TZM-bl cells (18, 65, 86) were obtained through the NIH AIDS Research and Reference Reagent Program from J. C. Kappes, X. Wu, and Tranzyme, Inc., and cultured in Dulbecco's modified Eagle medium (Invitrogen, Paisley, United Kingdom) containing 10% (vol/vol) fetal calf serum (FCS). NP2 glioma cells (76), expressing the HIV-1 cellular receptor CD4 and either of the coreceptors CXCR4 (NP2/CD4/CXCR4) or CCR5 (NP2/CD4/CCR5), were cultured in Dulbecco's modified Eagle's medium containing 5% (vol/vol) FCS. Phytohemagglutinin-stimulated PBMCs were obtained from blood donors and cultured in RPMI 1640 supplemented with 10% (vol/vol) FCS and 20 U of interleukin-2 (Roche, Lewes, United Kingdom) per ml.

**Immunization of *Llama glama*, evaluation of antibody response, and construction of VHH phage libraries.** Two llamas (numbered L40 and L44) were immunized with recombinant gp120 derived from HIV-1 CN54. Immunizations and VHH library construction were carried out as described previously (17). In brief, the llamas received six intramuscular injections at weekly intervals. Each injection consisted of a freshly prepared 4.5-ml water-in-oil emulsion prepared by vigorously mixing 2 volume units of antigen (50 or 100 μg) with 2.5 volume units of the adjuvant Stimune (CEDI Diagnostics, Lelystad, The Netherlands). The antienvelope immune response in sera was verified in an enzyme-linked immunosorbent assay (ELISA) against immobilized recombinant gp120. The neutralization activities of serum or plasma samples from day zero (preimmunization) and days 28, 39, and 43 (postimmunization) were evaluated in TZM-bl cells. Total RNA was isolated from peripheral blood lymphocytes and lymph node biopsies collected postimmunization (on days 39 and 43), and cDNA was prepared. The VHH repertoire was amplified and cloned into the pAX050 phagemid vector. To obtain recombinant bacteriophages expressing the VHH as fusion proteins with the bacteriophage gene III product, transformed TG1 *Escherichia coli* cells were grown to logarithmic phase and then infected with helper phage M13KO7. The phage particles were precipitated with polyethylene glycol to remove free VHH.

**Selection of anti-CD4bs VHH through panning on gp120 followed by competitive elution with sCD4 and subsequent isolation and screening of individual VHH.** Phage expressing the cloned VHH repertoire was incubated with immobilized gp120 and eluted with sCD4 to enrich for VHH targeting the CD4bs in two subsequent rounds of selection. Individual VHH were isolated and screened for binding to recombinant gp120 and ability to neutralize HIV-1. The selection and screening procedures are detailed in File S1 of the supplemental material, as are the procedures for expression and purification of selected VHH.

**HIV-1 neutralization assays.** VHH neutralization activities were evaluated in three different neutralization assays. In the initial screening of VHH, their neutralization ability was assessed in NP2/CD4/CCR5 (or CXCR4) glioma cells against concentration-matched irrelevant VHH, followed by detection of infection by HIV-1 p24 immunostaining, as described previously (1). In order to enable high-throughput neutralization screening and characterization of VHH, neutralization was subsequently measured using 200 50% tissue culture infective doses of virus in the TZM-bl cell-based assay developed by Derdeyn et al. (18),

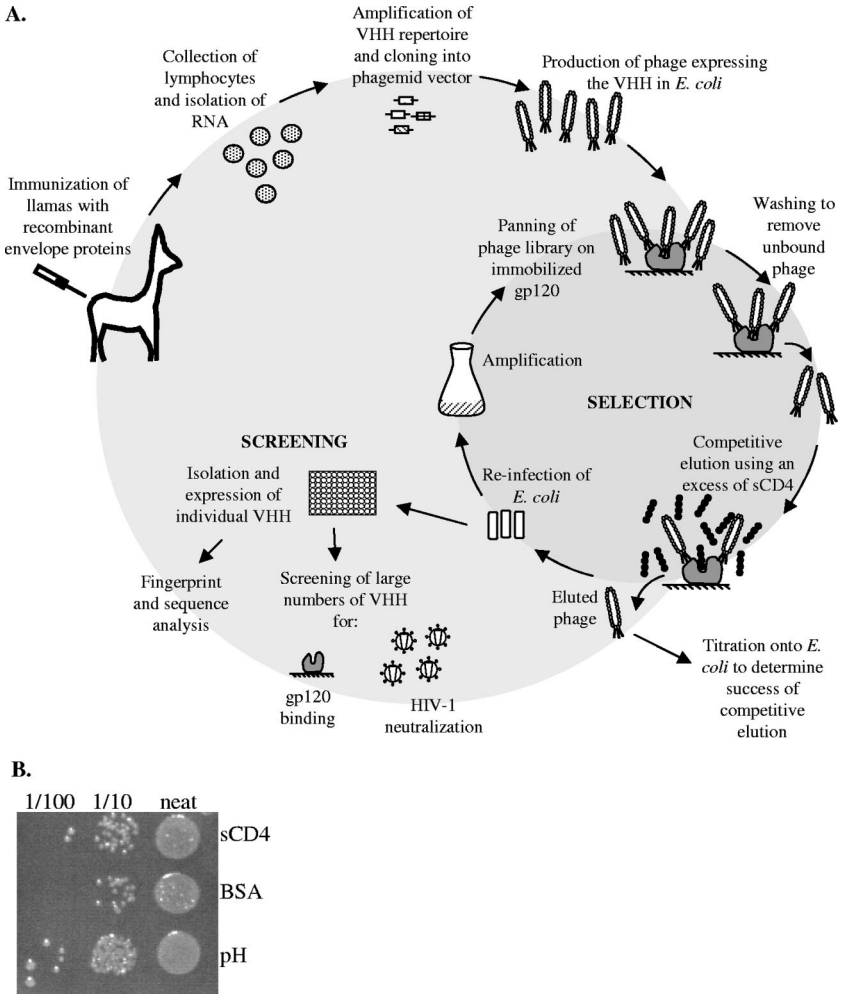


FIG. 1. (A) Schematic overview of the strategy for isolation of llama VHH targeting the CD4bs of gp120. (B) Titration of eluted phage onto *E. coli* TG1 cells. Phage bound to gp120 was eluted using sCD4. As a control, elution with BSA was performed in parallel, as was a general elution by low-pH shock using glycine. Shown is a representative titration of eluted phage, where more clones were eluted by sCD4 than by BSA and from which individual VHH were isolated and expressed.

Wei et al. (86), and Li et al. (43), with Bright-Glo luciferase reagent (Promega, Southampton, United Kingdom). The neutralization activity of each VHH and MAb b12 was assayed in duplicate and on a minimum of two separate occasions, apart from the CRF07_BC pseudovirus panel, against which the VHH and MAb b12 were assayed only once, in duplicate. No virus inactivation was observed with a negative control VHH or with a pseudovirus bearing a rabies virus G-protein envelope (87). VHH and MAb b12 50% inhibitory concentration ($IC_{50}$) and $IC_{90}$ titers were calculated using the XLFit4 software (ID Business Solutions, Guildford, United Kingdom).

In addition, VHH neutralization of HIV-1 was assayed in phytohemagglutinin-stimulated PBMCs. Serial dilutions of VHH (and MAb b12 in parallel) were incubated with virus for 1 h at 37°C and added to $10^5$ cells in 96-well round-well plates. After 24 h at 37°C the cells were washed twice in growth medium. Cell culture supernatant was collected on days 0, 1, 3, 5, and 8 after infection and assayed for HIV-1 p24 antigen using an antigen capture assay kit (SAIC-Frederick, Frederick, MD) according to the manufacturer's instructions. Neutralization was measured as the reduction in p24 content in test wells compared to that in virus control wells. All VHH, including a negative control VHH (and MAb b12), were assayed in triplicate and in PBMCs from two separate donors.

**VHH binding to recombinant envelope proteins in an ELISA, competition ELISA, and surface plasmon resonance assay.** VHH binding to recombinant envelope proteins and ability to compete with sCD4, anti-gp120 MAbs, and each other was assayed in an ELISA, as detailed in File S1 of the supplemental material. VHH binding to gp120 and competition with sCD4 was assayed in surface plasmon resonance experiments carried out using BIAcore (Uppsala, Sweden), as detailed in File S1 of the supplemental material.

## RESULTS

**Immunization of llamas and selection and screening of VHH targeting the CD4bs of gp120.** Two llamas (L40 and L44) were immunized with recombinant gp120 derived from HIV-1 CRF07_BC primary isolate CN54, according to current animal welfare regulations. Following the immunization schedule, antienvelope antibodies were detectable by ELISA in serum samples from both animals (data not shown). Weak neutralization activity against HIV-1 of subtype C was observed in postimmunization serum and plasma samples from llama L44 (see File S2 and Fig. S1 in the supplemental material). The VHH repertoires from blood and lymph node lymphocytes were cloned into a phagemid vector, and phage libraries were generated.

In order to select VHH targeting the CD4bs, we employed a directed selection strategy, as outlined in Fig. 1A. VHH were

selected by panning the phage display libraries on immobilized gp120, either directly coated onto the plate or captured by the D7324 antibody, followed by a competitive elution using excess sCD4, at two different concentrations and with varied lengths of elution. Selections where a larger number of clones were eluted by sCD4, compared to elution with bovine serum albumin (BSA), were taken forward to a second round of CD4bs-targeted selection (Fig. 1B). In general, no correlation was observed between the number of clones eluted by sCD4 and the concentration of sCD4 or length of incubation with sCD4. After two rounds of panning, selections where enrichment of clones eluted by sCD4 could be observed were chosen and individual VHH were expressed and screened for binding to recombinant gp120 in an ELISA and/or in vitro neutralization assay of HIV-1.

The phage libraries were first panned against the immunogen gp120 from HIV-1 CN54 (CRF07_BC) and in parallel against gp120 from HIV-1 IIIB (subtype B). The IIIB gp120 was included in the selection protocol as a control due to doubts regarding the functionality of the CN54-derived immunogen in terms of CD4 binding and ability to mediate infection. CN54 gp120 displayed poor binding to sCD4 in the ELISA, and no infectious virus was obtained when the same envelope clone was slotted into HIV-1 vectors (data not shown). Furthermore, including a subtype B envelope in the panning may promote selection of VHH with cross-subtype recognition properties. Selections where specific enrichment was observed, i.e., in which a larger number of clones were eluted by sCD4 than by BSA, were chosen, and 144 individual VHH were expressed and screened for binding to CN54 or IIIB gp120 in the ELISA. VHH clones with confirmed specificity for gp120 were also tested for the ability to neutralize the corresponding virus and a limited panel of heterologous isolates of subtypes A, B, and C.

Only 1 out of 96 clones selected using HIV-1 CN54 gp120 was found to bind to CN54 gp120 in the ELISA (data not shown). This VHH was derived from llama L40. It reduced infection of HIV-1 CN54 by approximately 50% at 100 μg/ml and displayed 0 or less than 50% neutralization with other strains (data not shown). Due to the limited neutralizing activity, this VHH was not further characterized.

Panning on IIIB gp120 was more successful. Out of 48 clones picked for analysis, 30 were confirmed to bind to IIIB gp120 in the ELISA, and 24 out of the 30 were observed to neutralize HIV-1 IIIB (>90% reduction of infection using VHH in *E. coli* periplasmic extracts). These VHH were all derived from llama L44. Fingerprint analysis and sequencing of the 24 clones revealed some identical VHH, leaving 8 different clones that could be separated into two distinct groups. The first group consisted of three clones with 8 to 14 amino acid differences in the framework 1, 2, and 3 regions as well as in CDR1, CDR2, and CDR3. They displayed a CDR3 of 18 amino acids. Based on superior neutralizing activities against the limited panel of HIV-1 isolates (data not shown), two of these VHH, designated A12 and D7, were chosen for further characterization. The second group of VHH consisted of five clones with one to three amino acid differences only, in framework regions 1 and 3. These displayed a shorter CDR3 of 10 amino acids. All five clones were shown to have similar neutralization profiles (data not shown), and one representative clone, designated C8, was chosen for further characterization. Thus, in total, three VHH (A12, D7, and C8) were selected for further characterization (see File S2 and Table S1 in the supplemental material). The amino acid sequences of these VHH are shown in File S2 and Fig. S1 of the supplemental material.

In order to allow for selection of VHH recognizing motifs conserved among HIV-1 subtypes, the phage libraries were also panned against recombinant gp120 derived from subtype A (92UG037) and subtype C (92BR025) gp120, in addition to IIIB gp120, alternating the antigen in different combinations in two subsequent rounds of panning. Enrichment of clones eluted by sCD4 was only observed in selections using the phage library from llama L44. Approximately 700 individual VHH were isolated, expressed, and screened for neutralization of HIV-1 as well as for binding to gp120 in the ELISA. VHH clones selected using 92BR025 gp120 were screened for the ability to neutralize HIV-1 92BR025, whereas VHH selected using 92UG037 gp120 were screened against HIV-1 92UG037. In addition, all VHH were screened for the ability to neutralize HIV-1 IIIB. Out of the 700 clones isolated, 43 were observed to bind to gp120 in the ELISA and to neutralize HIV-1. The results from the neutralization and binding screening showed complete correlation, with the exception that the clones selected using subtype A 92UG037 gp120 were not able to neutralize 92UG037 virus, despite being able to neutralize HIV-1 IIIB. The relatively low frequency of VHH able to bind to gp120 and to neutralize HIV-1 (43 out of 700, corresponding to 6%) probably reflects the nature of the competitive elution method, as a significant number of clones were nonspecifically eluted by BSA, as can be seen in Fig. 1B. Sequence analysis of the 43 neutralizing clones revealed that they were all either identical to VHH A12 (21 clones), which had already been selected from the same library through panning on HIV-1 IIIB gp120 alone, as described above, or identical to A12 with 1 amino acid difference in the framework 1 region (22 clones). Further characterization of the latter VHH revealed a neutralization profile similar to that of A12 (data not shown).

**Characterization of VHH neutralization properties in TZM-bl cells.** For high-throughput characterization of the neutralization activities of VHH A12, D7, and C8, we employed the TZM-bl cell-based neutralization assay (18, 43, 86). The neutralization potencies were assayed against HIV-1 of subtypes A, B, C, D, CRF07_BC, and CRF02_AG using either PBMC-propagated primary isolates, T-cell-line-adapted viruses, or recombinant replication-competent chimeric viruses as well as envelope-pseudotyped viruses expressing envelopes cloned straight from plasma of infected individuals or from PBMC-propagated primary isolates. The lowest VHH concentrations required to achieve 50% and 90% reductions of infectivity ($IC_{50}$ and $IC_{90}$) compared to a virus control were determined. A negative control VHH was tested in parallel, as well as a pseudovirus bearing the rabies virus G-protein (87). No nonspecific virus inactivation was observed.

The results of the neutralization characterization are summarized in Fig. 2 and 3. In summary, VHH A12 generally showed the most broad and potent neutralizing activity of the VHH, being able to neutralize HIV-1 primary isolates of subtypes B, C, and CRF07_BC, but not the other subtypes tested. Overall, it neutralized 27 out of 65 viruses (42%), with $IC_{50}$s in the range of <0.003 to 38 μg/ml (Fig. 2 and 3). VHH D7

| Virus | Type | Subtype | Tier | $IC_{50}$ in TZM-bl cells (μg/ml) VHH A12 | VHH D7 | VHH C8 | MAb b12 |
|---|---|---|---|---|---|---|---|
| 92UG037.A9 | MC | A | nd | • | • | • | • |
| T257-31 | PV | A/G | 2 | • | • | • | • |
| T33-7 | PV | A/G | 2 | • | • | • | • |
| IIIB | TCLA | B | 1 | 0.07 | 0.1 | 0.8 | 0.07 |
| MN | TCLA | B | 1 | 0.004 | 0.06 | 1 | 0.02 |
| SF162 | PBMC | B | 1 | 1.3 | 2.3 | • | 0.4 |
| BaL | PBMC | B | nd | 3.6 | 8.1 | 3.9 | 0.4 |
| JRFL | MC | B | nd | • | • | • | <1.9 |
| YU2 | MC | B | nd | 34 | • | • | 8.6 |
| 23.8.12 | MC | B | nd | 0.02 | 0.03 | 0.1 | 0.1 |
| 4.10.3 | MC | B | nd | <0.003 | <0.003 | 0.07 | • |
| 8.8.8 | MC | B | nd | 0.003 | 0.1 | • | 0.03 |
| 23.2.E | MC | B | nd | • | • | • | 7.4 |
| 6535.3 | PV | B | 2 | 0.1 | 0.2 | 28 | 2.5 |
| QH0692.42 | PV | B | 2 | 13 | 17 | 21 | 0.7 |
| SC422661.8 | PV | B | 2 | • | • | • | <1.9 |
| PVO.4 | PV | B | 2 | • | • | • | • |
| TRO.11 | PV | B | 2 | • | • | • | • |
| AC10.0.29 | PV | B | 2 | • | • | • | 2.2 |
| RHPA4259.7 | PV | B | 2 | • | • | • | <1.9 |
| THRO4156.18 | PV | B | 2 | 6.2 | 7.2 | 18 | 0.5 |
| REJO4541.67 | PV | B | 2 | 27 | • | 32 | 32 |
| TRJO4551.58 | PV | B | 2 | 16 | • | • | • |
| WITO4160.33 | PV | B | 2 | • | • | • | 11 |
| CAAN5342.A2 | PV | B | 2 | • | • | • | • |
| CN54 | PBMC | B′/C | nd | 1.4 | 5.1 | 9.9 | • |
| CH181.12 | PV | B′/C | 2 | • | • | • | <1.9 |
| CH064.20 | PV | B′/C | 2 | • | • | • | • |
| CH091.9 | PV | B′/C | 2 | • | • | • | • |
| CH117.4 | PV | B′/C | 2 | • | • | • | • |
| CH119.10 | PV | B′/C | 2 | • | • | • | • |
| CH110.2 | PV | B′/C | 2 | • | • | • | • |
| CH114.8 | PV | B′/C | 2 | • | • | • | • |
| CH120.6 | PV | B′/C | 2 | • | • | • | • |
| CH115.12 | PV | B′/C | 2 | • | • | • | 36 |
| CH070.1 | PV | B′/C | 2 | • | • | • | • |
| CH038.12 | PV | B′/C | 2 | • | • | • | <1.9 |
| ZA97001 | PBMC | C | nd | • | • | • | • |
| 97IN003 | PBMC | C | nd | • | • | • | • |
| 92BR025.C1 | MC | C | nd | 0.2 | • | 2 | • |
| CA6 | MC | C | nd | • | • | • | <1.9 |
| CB7 | MC | C | nd | 0.7 | 4 | 36 | 0.17 |
| C37.4.2 | MC | C | nd | • | • | • | 36 |
| C38.2.2 | MC | C | nd | 5.4 | 22 | 33 | • |
| C27b | MC | C | nd | 38 | • | • | 13 |
| C27d | MC | C | nd | 0.02 | 0.03 | 0.7 | 0.02 |
| C222 | MC | C | nd | 0.03 | 49 | 3 | • |
| C261 | MC | C | nd | <0.003 | 0.004 | 32 | • |
| ZA97001.1 | MC | C | nd | 0.05 | 0.05 | 1.1 | 0.02 |
| 97IN003.4.2 | MC | C | nd | • | • | • | • |
| 93MW965.26 | PV | C | 1 | • | • | 0.3 | 0.02 |
| 96ZM651.02 | PV | C | 2 | 0.1 | • | 4.3 | • |
| Du156.12 | PV | C | 2 | • | • | • | <1.9 |
| Du172.17 | PV | C | 2 | • | • | • | <1.9 |
| Du422.1 | PV | C | 2 | • | • | • | <1.9 |
| ZM197M.PB7 | PV | C | 2 | 6 | 22 | 24 | 7.4 |
| ZM214M.PL15 | PV | C | 2 | • | • | • | <1.9 |
| ZM233M.PB6 | PV | C | 2 | 7 | 34 | 38 | • |
| ZM249M.PL1 | PV | C | 2 | • | • | • | 5.6 |
| ZM53M.PB12 | PV | C | 2 | • | • | • | • |
| ZM109F.PB4 | PV | C | 2 | 0.8 | 6.6 | 38 | • |
| ZM135M.PL10a | PV | C | 2 | • | • | • | • |
| CAP45.2.00.G3 | PV | C | 2 | • | • | • | <1.9 |
| CAP210.2.00.E8 | PV | C | 2 | 1.2 | • | 11 | 6.7 |
| 92UG001.D8 | MC | D | nd | • | • | • | • |
| Rabies CVS-11 | PV | n/a | n/a | • | • | • | nd |

FIG. 2. VHH and MAb b12 $IC_{50}$ titers against HIV-1 in TZM-bl cells. VHH and MAb b12 neutralization activity was assessed in the indicated viruses, as described in the text. Rabies virus CVS-11 is pseudotyped with rabies virus G-protein from strain CVS-11 (87). Abbreviations for virus types: TCLA, T-cell-line-adapted isolate; PBMC, PBMC-propagated primary isolate; MC, molecular clone; PV, envelope pseudotyped virus. The column labeled Tier indicates whether the virus is classified as suitable for tier 1, 2, or 3 assessment of neutralizing antibodies (46). ●, $IC_{50}$ >50 μg/ml; nd, not determined; n/a, not applicable. To aid comprehension, the titers have been shaded, with darker colors indicating more potent neutralization.

showed a similar but slightly less cross-reactive neutralization profile compared to that of A12, neutralizing 31% of viruses on an $IC_{50}$ level. VHH C8 neutralized 35% of viruses ($IC_{50}$ level). It seemed less potent than A12 and D7 against subtype B viruses but was as reactive as VHH A12 against subtype C viruses, although it neutralized a different pattern of viruses (Fig. 2 and 3).

**Comparison of VHH and MAb b12 neutralization breadth.** Human anti-CD4bs MAb b12 is one of the most extensively studied MAbs to HIV-1 envelope and is one of only a handful MAbs that have been shown to neutralize a wide range of HIV-1 isolates of different subtypes (11). To gain further insight into the neutralization breadth of the VHH, their neutralization profiles were compared to that of MAb b12 (Fig. 2 and 3). Overall, MAb b12 was more reactive against the viruses included in this study, neutralizing 54% of viruses at an $IC_{50}$ level, compared to 42% for A12, the most potent and broadly reactive of the VHH. More specifically, MAb b12 was found to be more reactive against HIV-1 of subtype B, neutralizing 77% of the viruses on an $IC_{50}$ level compared to 59% for VHH A12 (Fig. 3). Moreover, MAb b12 neutralized 56% of the subtype C viruses, compared to 48% for VHH A12. As expected, fewer isolates were neutralized at the $IC_{90}$ level by the VHH and MAb b12 (Fig. 3). Like the VHH, MAb b12 did not neutralize (to ≥50%) the subtype A, A/G, or D viruses included in the study (Fig. 2). It should be noted that the VHH are approximately 10 times smaller than MAb b12 but that MAb b12, on the other hand, has two antigen-recognizing domains per molecule, making it impossible to accurately estimate relative neutralization potencies. To be able to make a direct comparison, the VHH would need to be presented in the context of a complete camelid immunoglobulin.

TABLE 1. VHH and MAb b12 neutralization of HIV-1 SF162 in PBMCs[a]

| Antibody | $IC_{50}$ (μg/ml) | | $IC_{75}$ (μg/ml) | | $IC_{90}$ (μg/ml) | | $IC_{90}$ in TZM-bl cells (μg/ml) |
|---|---|---|---|---|---|---|---|
| | Donor 1 | Donor 2 | Donor 1 | Donor 2 | Donor 1 | Donor 2 | |
| A12 | 24 | 16 | 35 | 44 | 50 | ● | 7.8 |
| D7 | ● | <1.9 | ● | 46 | ● | ● | 9.3 |
| C8 | ● | ● | ● | ● | ● | ● | ● |
| 3 | ● | ● | ● | ● | ● | ● | ● |
| b12 | <1.9 | 1.9 | <1.9 | 2 | <1.9 | 2.1 | 1.2 |

[a] VHH A12, D7, and C8 and 3 (negative control VHH), as well as MAb b12, were assayed in triplicate for the ability to neutralize HIV-1 SF162 in PBMCs from two different donors, as described in the text. HIV-1 p24 content on day 5 postinfection in test wells was determined and compared to virus-only control wells, and the lowest concentration giving rise to a 50%, 75%, and 90% reduction in p24 content was calculated using XLfit4 software. ●, >50 μg/ml.

**VHH neutralization of HIV-1 in primary cells.** The ability of the VHH to inhibit HIV-1 SF162 infection of primary cells was tested in a PBMC-based assay. VHH and MAb b12 $IC_{50}$, $IC_{75}$, and $IC_{90}$ titers against SF162 in PBMCs from two different donors, scored on day 5 postinfection, are shown in Table 1. VHH A12 was able to inhibit infection of SF162 in PBMCs from both donors, with $IC_{75}$s of 35 and 44 μg/ml, respectively. In contrast, VHH C8 did not inhibit SF162 infection in primary cells, which is concordant with results obtained in the TZM-bl assay. For VHH D7, the results were less clear, as this VHH inhibited infection in PBMCs from only one of the two donors.

Thus, unlike in the TZM-bl cell-based neutralization assay, only VHH A12 was able to clearly neutralize SF162 in PBMCs.

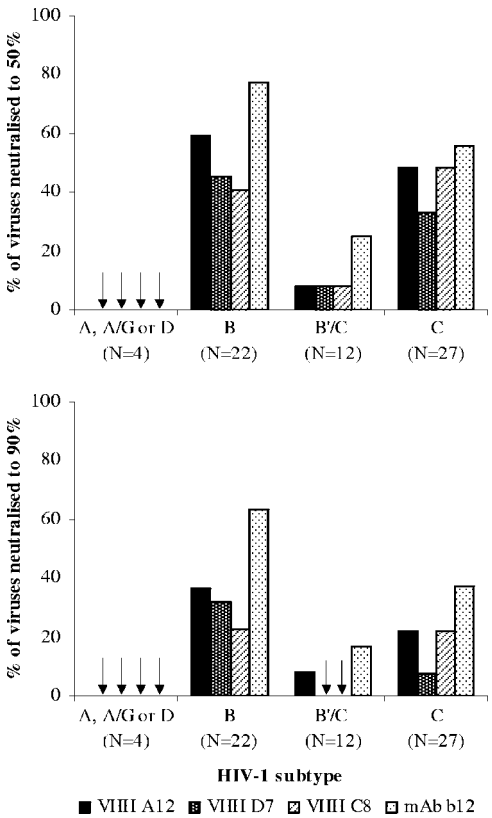


FIG. 3. Percentage of HIV-1 isolates neutralized by the VHH and by MAb b12, according to HIV-1 subtype. Virus neutralization was assayed in TZM-bl cells as described in the text. Shown is the percentage of viruses neutralized with an $IC_{50}$ and $IC_{90}$ of ≤50 μg/ml.

Furthermore, 90% neutralization was only observed in PBMCs from one of the two donors, and it required a sixfold-higher concentration of A12 than in TZM-bl cells (Table 1). MAb b12 was more than 20-fold more potent than VHH A12 at the $IC_{90}$ level in the PBMC assay, whereas the difference between MAb IgG1 b12 and VHH A12 was only 6-fold in TZM-bl cells. Further studies will be needed to explain this observed discrepancy between the results obtained in the PBMC and the TZM-bl assays. Such discrepancies have, however, been reported previously. The broadly neutralizing MAb 4E10 has, for example, been shown to be more broadly cross-subtype reactive and potent in engineered cell lines than in PBMCs (8). It has been suggested that the choice of target cell affects HIV-1 neutralization, and neutralization assays using engineered cell lines have been shown to be more sensitive than PBMC assays (8, 45, 49, 67, 75).

**VHH bind to recombinant IIIB gp120 with high affinities.** VHH affinity for gp120 was determined using surface plasmon resonance techniques. The kinetic data are summarized in Table 2. In summary, VHH A12 and D7 showed affinities in the high picomolar range for recombinant IIIB gp120, with equilibrium dissociation constants of 100 and 97 pM, respectively, approaching the affinity ceiling that has been suggested for antibodies generated through in vivo maturation (5, 24, 78). VHH C8 had a faster off-rate, leading to a more-than-eight-fold-higher $K_D$.

For comparison, Fab b12 has been reported to bind to IIIB gp120 with a $K_D$ of 6.3 nM (4). Furthermore, sCD4 has been reported to bind to envelope proteins, including IIIB gp120, with $K_D$ values of 22 to 35 nM (12, 59, 88). Another study reported $K_D$ values in the range of 2.2 to 16 nM for a range of anti-CD4bs Fab fragments (including Fab b12) to MN gp120 (62). Another human anti-CD4bs MAb, F105, has been shown to bind to IIIB gp120 with a $K_D$ of 0.62 nM (12).

**VHH bid to recombinant envelope proteins of subtypes A, B, and C in the ELISA.** VHH binding to HIV-1 gp120 was eval-

TABLE 2. VHH kinetic constants and affinities for IIIB gp120[a]

| VHH | $K_D$ (nM) | $k_a$ [1/(M · s)] | $k_D$ (1/s) |
|---|---|---|---|
| A12 | 0.1 | $2.73 \times 10^5$ | $2.98 \times 10^{-5}$ |
| D7 | 0.097 | $4.00 \times 10^5$ | $3.89 \times 10^{-5}$ |
| C8 | 0.85 | $2.05 \times 10^5$ | $1.74 \times 10^{-4}$ |

[a] VHH A12, D7, and C8 association rate constants ($k_a$), dissociation rate constants ($k_D$), and equilibrium dissociation constants ($K_D = k_D/k_a$) for recombinant IIIB gp120 were determined in surface plasmon resonance studies.

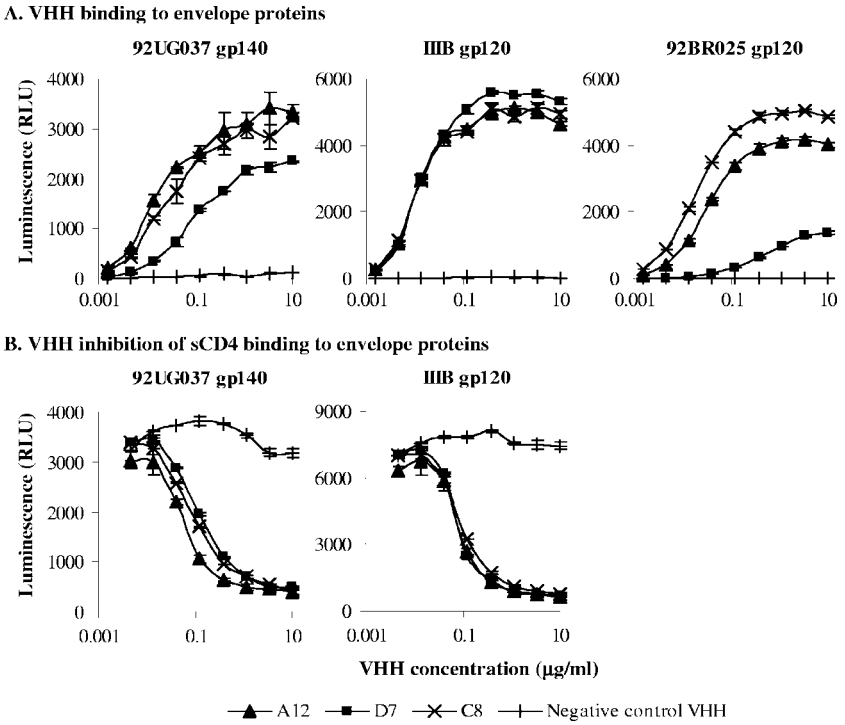


FIG. 4. (A) VHH binding to recombinant envelope proteins derived from HIV-1 92UG037 (subtype A), IIIB (subtype B), and 92BR025 (subtype C) in the ELISA. Recombinant envelope proteins were captured by immobilized antibody D7324. Serial dilutions of VHH A12, D7, C8, and a negative control VHH were then added, and binding was detected as described in the text. (B) Dose-dependent competition of VHH A12, D7, and C8 with sCD4 for binding to recombinant envelope proteins in the ELISA. Threefold serial dilutions of VHH were preincubated with IIIB gp120 or 92UG037 gp140 and subsequently incubated with sCD4 precoated on microtiter plates. Envelope protein binding to sCD4 was detected as described in the text. Chemiluminescence was measured, and background subtracted luminescence readings (in relative light units [RLU]) were plotted against VHH concentration. Data points represent the means and bars show the standard deviations of duplicate reactions.

uated in an ELISA. Cross-subtype reactivity was determined using gp120 or gp140 from HIV-1 of subtypes A, B, and C. Concentration-dependent binding of the selected VHH to gp120 and gp140 is shown in Fig. 4A. VHH A12, D7, and C8 bound equally well to HIV-1 IIIB gp120. In contrast, A12 and C8 showed stronger binding to HIV-1 92UG037 gp140 than did D7. VHH A12 and C8 were also able to bind well to recombinant HIV-1 92BR025 gp120, whereas D7 bound with a weaker signal. In spite of their ability to neutralize HIV-1 strains of subtypes B and C, none of VHH A12, D7, and C8 was found to bind well to the protein against which it had been raised, CN54 gp120, in the ELISA (data not shown). This contradictory observation might to some extent be explained by doubts regarding the structural integrity of the immunogen, as it did not show good binding to sCD4, nor was it able to mediate infection when expressed on the surface of virus particles (data not shown); however, these VHH had been selected through panning on IIIB gp120, as opposed to on CN54 gp120.

**Characterization of VHH epitopes. (i) VHH compete with sCD4 for binding to recombinant envelope proteins.** Since the VHH were specifically selected for their ability to compete with sCD4, their potencies to inhibit binding of sCD4 to recombinant gp120 and gp140 were evaluated in an ELISA. Titrations of VHH were preincubated with gp120 or gp140 followed by subsequent incubation with solid-phase-coated sCD4, after which gp120 or gp140 binding to sCD4 was detected. VHH A12, D7, and C8 all inhibited binding of sCD4 to HIV-1 IIIB gp120 and 92UG037 gp140 in a dose-dependent manner (Fig. 4B). VHH A12 was able to inhibit sCD4 binding to recombinant 92UG037 gp140 at slightly lower concentrations than the remaining VHH. No significant binding of sCD4 to CN54 gp120 could be observed in the ELISA, which is why the ability of the selected VHH to inhibit sCD4 binding to HIV-1 CN54 gp120 could not be evaluated. The abilities of the neutralizing VHH to compete with sCD4 for binding to recombinant gp120 and gp140 indicate that they inhibit HIV-1 infection by binding to the functional envelope spike prior to interaction with CD4.

To further confirm that the selected VHH recognize an epitope overlapping the CD4bs, or at least bind in a way that sterically hinders binding of sCD4, VHH D7 and C8 were assessed for their ability to inhibit binding of sCD4 to gp120 in a BIAcore surface plasmon resonance assay. Surface plasmon resonance enables testing whether or not the VHH can inhibit binding of sCD4 to CN54 gp120 (the immunogen), since binding of fluid-phase (only) CN54 gp120 to immobilized sCD4 can be observed in the BIAcore assay but not in the ELISA. Recombinant sCD4 was captured by an anti-CD4 antibody immobilized on the sensor chip. Titrations of VHH were preincubated with gp120 and then injected onto the chip. Both VHH D7 and C8 could completely inhibit binding of sCD4 to CN54 gp120, even at equimolar concentrations of VHH and gp120 (see File S2 and Fig. S3 in the supplemental material).

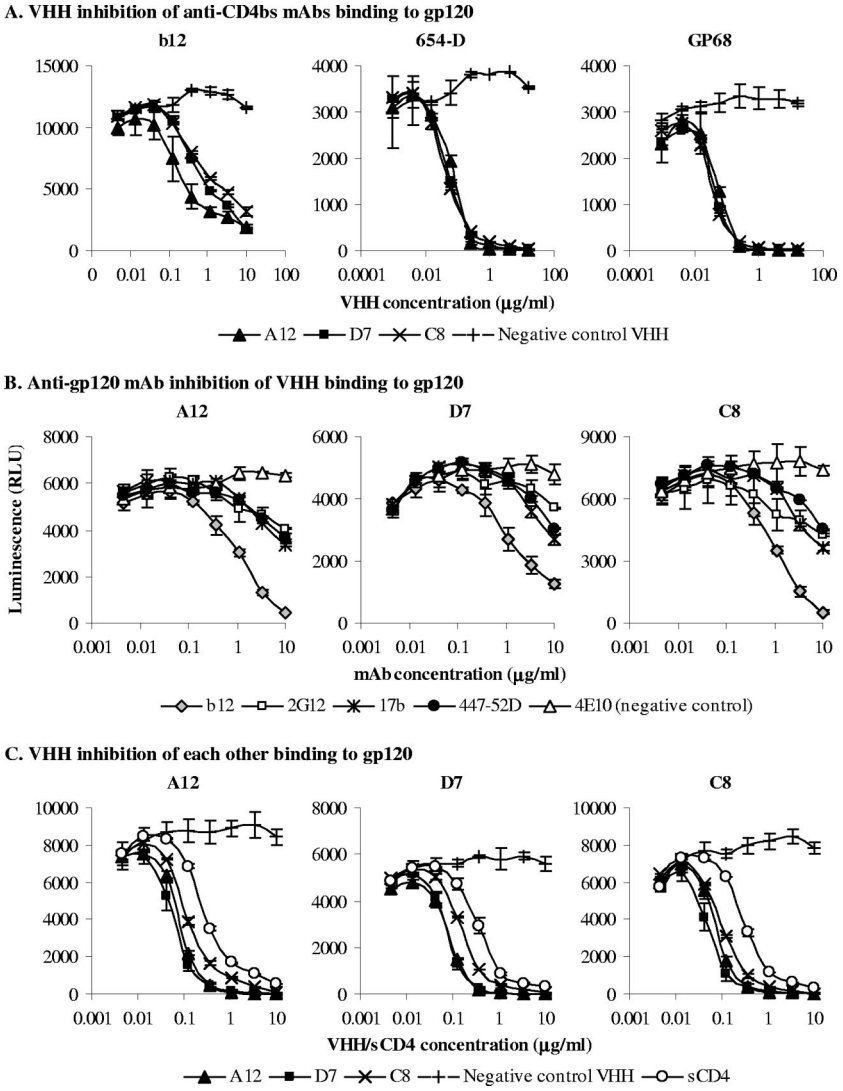


FIG. 5. VHH and anti-gp120 MAb cross-competition analysis. (A) Dose-dependent competition of VHH A12, D7, and C8 with anti-CD4bs MAbs b12, 654-D, and GP68 for binding to recombinant IIIB gp120 in the ELISA. Serial dilutions of VHH were preincubated with IIIB gp120. Envelope protein binding to human anti-CD4bs MAbs b12, 654-D, and GP68 was detected as described in the text. (B) VHH competition with anti-gp120 MAbs 2G12 (carbohydrate motif), 17b (CD4i), and 447-52D (V3), as well as MAb b12 (CD4bs), for binding to recombinant gp120 in the ELISA. Wells of microtiter plates were coated with VHH A12, D7, and C8, as indicated above each graph. Serial dilutions of each MAb were preincubated with IIIB gp120 and subsequently incubated with the immobilized VHH. Envelope protein binding to VHH was detected as described in the text. (C) VHH competition with each other and with sCD4 for binding to recombinant gp120 in the ELISA. Wells of microtiter plates were coated with VHH A12, D7, and C8, as indicated above each graph. Serial dilutions of each VHH and sCD4 were preincubated with IIIB gp120 and subsequently incubated with the immobilized VHH. Envelope protein binding to VHH was detected as described in the text. Background-subtracted luminescence readings were plotted against MAb concentration. Data points represent the means and bars show the standard deviations of duplicate reactions.

Both VHH also inhibited sCD4 binding to IIIB gp120 (data not shown). This finding confirms the observations made in the ELISAs, i.e., that the VHH compete with sCD4 for binding to gp120, either through binding to an epitope that partly overlaps with the CD4bs or by binding in a way that sterically hinders binding of sCD4; alternatively, VHH binding locks the gp120 in a conformation that hampers interaction with sCD4. Again, this finding may indicate that the VHH inhibit HIV-1 infection by interacting with gp120 prior to its engagement to CD4.

**(ii) VHH compete with anti-CD4bs MAbs for binding to recombinant gp120.** To further map the epitopes of the selected VHH, they were tested for their ability to compete with anti-CD4bs MAbs b12, 654-D, and GP68. Antibody b12 has been shown to bind to an epitope that overlaps a subset of the CD4bs (94). Human monoclonal antibodies 654-D and GP68 have been shown to compete with sCD4 for binding to recombinant gp120 but can only neutralize some T-cell-line-adapted isolates and not primary isolates (29, 37, 40, 74).

VHH A12, D7, and C8 were all found to compete with MAb

b12 for binding to recombinant gp120 (Fig. 5A). VHH A12 inhibited MAb b12 binding to IIIB gp120 at slightly lower concentrations than the other VHH. This observation may indicate that VHH A12 recognizes an epitope that overlaps with the b12 epitope to a greater extent, which could be in line with the neutralization results, where VHH A12 showed a broader neutralizing ability compared to the other VHH (Fig. 2), indicating that it binds to an epitope that is more conserved. VHH A12, D7, and C8 were all found to compete with anti-CD4bs antibodies 654-D and GP68, further confirming that the VHH recognize CD4bs-related epitopes (Fig. 5A).

In order to gain further understanding of the VHH epitopes, their ability to compete with MAbs to non-CD4bs epitopes of gp120 was evaluated (Fig. 5B). Included in the study was the broadly neutralizing MAb 2G12, which recognizes a carbohydrate motif on gp120 (9, 70, 72, 80), the anti-V3 MAb 447-52D, which neutralizes primary isolates of subtype B (30, 31), and MAb 17b, which recognizes a CD4-induced epitope and which neutralizes mainly T-cell-line-adapted isolates (79). In addition, anti-CD4bs MAb b12 and the anti-gp41 MAb 4E10 (9, 56) were included. An inhibition ELISA was set up, where the anti-gp120 MAbs were preincubated with IIIB gp120, followed by subsequent inhibition with immobilized VHH precoated onto plates and detection of IIIB gp120 binding to VHH. Anti-CD4bs MAb b12 could clearly inhibit VHH binding to IIIB gp120 (Fig. 5B), which is consistent with the results described for Fig. 5A. Some inhibition of VHH-gp120 binding was also observed for the three remaining non-CD4bs anti-gp120 MAbs, 2G12 (carbohydrate motif), 17b (CD4i), and 447-52D (V3). It is possible that binding of these MAbs to gp120 imposes some steric hindrance, hence inhibiting VHH binding. The sheer bulk of an antibody binding to gp120 may reduce the ability of gp120 to bind to the immobilized VHH. This notion is, however, not true for the antibody D7324, which binds to the very-C-terminal region of gp120, as it was used to both capture and detect gp120 throughout this study. Including an irrelevant MAb in this competition ELISA, in this case the anti-gp41 MAb 4E10, did not lead to reduced VHH-gp120 binding, indicating that it is the binding and not just the presence of MAbs 2G12, 17b, and 447-52D that slightly inhibits VHH-gp120 binding. Antibodies to CD4-induced epitopes, such as 17b, have previously been reported to compete with antibodies to CD4bs-related epitopes (52). In summary, the competition profiles of the VHH were found to be similar to competition profiles previously reported for anti-CD4bs MAbs (52, 92), suggesting that the VHH do recognize CD4bs-related epitopes.

**(iii) VHH compete with each other for binding to recombinant gp120.** To investigate whether the VHH bind to epitopes that are related, VHH A12, D7, and C8 were tested for their abilities to compete with each other for binding to gp120 (Fig. 5C). Soluble CD4 was also included in the experiment. Each of the VHH was able to inhibit binding of IIIB gp120 to itself and to the other two VHH, indicating that either the VHH bind to epitopes that overlap or that steric hindrance inhibits simultaneous binding. VHH C8 required higher concentrations than VHH A12 or D7 to completely inhibit gp120 binding to VHH A12 and D7, as well as to itself. This finding is in concordance with the higher affinity for IIIB gp120 observed for VHH A12 and D7 compared to VHH C8 (Table 2). VHH A12 and D7 showed similar inhibition curves, in line with their similar affinities for IIIB gp120. Soluble CD4 could also completely inhibit VHH-gp120 binding. This result supports the observation reported in Fig. 4B, where the VHH were found to potently inhibit sCD4 binding to gp120. On a molar level, sCD4 was as potent as VHH A12 and D7 at inhibiting VHH-gp120 binding (Fig. 5C).

## DISCUSSION

Our study shows that HIV-1-neutralizing VHH can be isolated from llamas immunized with recombinant gp120. To increase the chances of isolating neutralizing VHH, we employed a functional selection approach which enabled the identification of VHH that compete with CD4. By panning phage libraries displaying the VHH repertoires of the immunized animals on recombinant gp120 and by introducing a competitive elution step using sCD4, we were able to select for VHH targeting the CD4bs of gp120. Immunizing with gp120 derived from an HIV-1 CRF07_BC primary isolate, followed by panning on gp120 from either a subtype B virus or sequentially on gp120 from subtypes A, B, and C, allowed for selection of VHH with cross-subtype-neutralizing activity. Three VHH (A12, D7, and C8) able to neutralize HIV-1 primary isolates of subtypes B and C were identified. These were shown to bind to recombinant envelope proteins with affinities that are close to the suggested affinity ceiling for in vivo-maturated antibodies and to compete with sCD4 for this binding, indicating that they recognize epitopes that overlap or are in proximity to the CD4bs. Envelope binding competition analysis, using a set of anti-gp120 MAbs with defined epitopes, further confirmed that the VHH target the CD4bs. Hence, it is likely that the VHH neutralize HIV-1 by interacting with the envelope spike prior to its interaction with CD4.

The neutralizing activities of the VHH were evaluated against a large panel of HIV-1 isolates from subtypes A, B, C, D, CRF02_AG, and CRF07_BC. The most potent and broadly reactive VHH was A12. A12 was able to neutralize 59% of the subtype B viruses and 48% of the subtype C viruses included in the study on an $IC_{50}$ level, compared to 77% and 56% for the broadly neutralizing anti-CD4bs MAb b12. The difference in neutralization breadth between VHH A12 and MAb b12 was even more pronounced at an $IC_{90}$ level, indicating that VHH A12 is less broad in its cross-subtype neutralization ability than MAb b12. It should also be noted that MAb b12 was more reactive against the well-characterized subtype B and C reference panels of envelope pseudotyped viruses, which has been classified as suitable for tier 2 assessment of neutralizing antibodies.

Apart from the autologous virus HIV-1 CN54, the VHH were not able to neutralize any of the 12 CRF07_BC viruses tested, despite being derived from a llama immunized with the CRF07_BC isolate CN54. Taking the relatively broad neutralization properties of the VHH against viruses from subtypes B and C, this finding is a little surprising. Further studies are needed to elucidate the extent to which these VHH-resistant CRF07_BC envelope clones differ from the CN54 envelope.

The neutralization ability of MAb b12 has been tested in many studies. In this study, MAb b12 was tested in parallel to the VHH against a panel of 65 viruses and was able to neu-

tralize 54% of the viruses on an $IC_{50}$ level and 40% on an $IC_{90}$ level. These findings are in concordance with a previous study carried out by Binley et al. (8), in which a small panel of MAbs was tested against an extensive panel of 90 viruses in a high-throughput pseudovirus-based neutralization assay and in which MAb b12 was shown to neutralize 50% and 34% of viruses on an $IC_{50}$ and $IC_{90}$ level, respectively. Furthermore, MAb b12 was used in the characterization of the subtype B and C reference panels of envelope pseudotyped viruses described by Li et al. (43, 44), which were included in this study. Neutralization data obtained in this study for MAb b12 against these reference panels are concordant with the data previously published.

The VHH were found to neutralize a different spectrum of viruses compared to MAb b12. For example, MAb b12 was able to neutralize 12 subtype C or CRF07_BC viruses that VHH A12 was not able to neutralize ($IC_{50}$ level). At the same time, VHH A12 was able to neutralize eight subtype C or CRF07_BC viruses that b12 was unable to neutralize ($IC_{50}$ level). These differential neutralization reactivities may indicate that the VHH and MAb b12 recognize different but perhaps overlapping epitopes on gp120, despite competing with each other for binding to gp120 in the ELISA. Additional studies, such as alanine scanning of gp120, as well as structural studies are needed to fully characterize the precise epitopes to which the VHH bind and to determine whether the VHH and MAb b12 epitopes are related. Determining the epitopes of these VHH may provide additional information about vulnerable sites in proximity to the CD4bs of gp120, which may be of value for HIV-1 immunogen design.

Given the unique properties attributed to VHH, in terms of solubility, thermal and chemical stability, and high expression levels leading to a low production cost (20, 26, 64, 81), neutralizing VHH might prove useful in a number of applications, for example, as candidate HIV-1 microbicides as well as antiretroviral drugs or prophylactics. Topical application of MAb b12 has been shown to protect macaques from infection after vaginal challenge with SHIV, which supports the potential use of antibodies for topical prevention of HIV-1 transmission (82). Further studies are needed to establish whether the identified VHH would have preventive effects in vivo. Antienvelope VHH may also be of use in characterizing epitopes on HIV-1 envelope proteins. Attempts to design an HIV-1 immunogen that can elicit a broadly neutralizing humoral immune response have so far failed (61), although a recent study reported that extensively cross-reactive antibodies (albeit at a low titer) were induced by immunization of rabbits with a recombinant gp140 (93). It is possible that a set of neutralizing and non-neutralizing VHH may become useful in the design of such an immunogen.

The stability and solubility of VHH allow for engineering of multivalent and bispecific molecules (23). Engineered multivalent VHH to other antigens have previously been shown to exhibit better binding and neutralizing properties than their monovalent counterparts (13). It is possible that introduction of multivalency will increase the potency of the identified anti-gp120 VHH. Construction and evaluation of such multivalent VHH, using the VHH identified in this study, are ongoing but have so far not led to increased neutralization potency or breadth (data not shown). Selecting for VHH recognizing other epitopes and linking them to the CD4bs-targeted VHH described in this study, thus producing a bispecific VHH, might also result in more potent binding and neutralization properties.

The results obtained in this study confirm previous findings that antibody binding to recombinant envelope proteins does not necessarily correlate with the ability to neutralize virus (25, 35, 50, 54, 62, 66, 71, 73), as the structures of recombinant envelope glycoproteins are likely to be different from the structures of the envelope glycoproteins in the context of the functional spike (61, 91, 95). The ability of the VHH to bind to gp120 and gp140 from different subtypes indicates that they bind to a motif that is conserved and accessible on recombinant, soluble envelope proteins. The lack of ability of the VHH to neutralize some of the corresponding viruses indicates that the epitopes recognized by the VHH on recombinant envelope proteins are either not accessible or not present in the context of the functional envelope spike. The fact that the VHH can inhibit binding of sCD4 to recombinant 92UG037 gp140, despite not being able to neutralize 92UG037 virus, suggests that recombinant 92UG037 gp140 is not a good representative of the functional spike, and therefore such an antigen may not be suitable as an immunogen or for panning of VHH libraries. Interestingly, VHH binding to recombinant 92BR025 gp120 seems to correlate with the ability to neutralize 92BR025 virus. VHH A12 and C8 were able to bind well to recombinant 92BR025 gp120 in the ELISA, whereas D7 bound with a weaker signal (Fig. 4A). This observation correlates with the corresponding neutralization data, where A12 and C8 could neutralize HIV-1 92BR025 with $IC_{50}$ titers of 0.2 and 2 μg/ml, respectively, whereas the $IC_{50}$ for D7 was >50 μg/ml (Fig. 2). This finding may indicate that recombinant 92BR025 gp120 represents a good mimic of the 92BR025 gp120 structure in the functional spike. This notion is, however, speculative, and further studies are needed to evaluate the antigenic properties of this gp120. Immunizing llamas and selecting for anti-CD4bs VHH using an antigen that is a better representation of the functional envelope spike might result in VHH with broader cross-subtype neutralizing activities and lower inhibitory doses. Again, identification of such an envelope antigen is one of the major challenges in screening for agents that can act as HIV-1 entry inhibitors as well as in HIV-1 vaccine design (61).

The immunogen CN54 gp120 showed poor binding to sCD4 in biochemical assays and did not mediate infection when expressed on viruses. It was chosen as the immunogen due to its availability before these properties were known. It is possible that immunizing with an immunogen with better CD4-binding properties would result in more potent VHH. Previous studies have shown that purified trimeric envelope proteins are somewhat better at eliciting cross-subtype-neutralizing antibodies than monomeric recombinant gp120 (6, 7, 22, 32, 38, 90). We are currently screening llamas immunized with recombinant trimeric gp140.

The phage libraries were panned on HIV-1 IIIB gp120 alone, subtype A gp120 alone, or subtype C gp120 alone, as well as on the same antigens alternated in various combinations in two subsequent rounds of panning, always using the competitive elution with sCD4. Interestingly, with this method of selection, we could select for VHH that could bind to envelope proteins of subtypes A, B, and C, regardless of which of

the above antigens that were used in the panning. However, when selected using HIV-1 IIIB gp120 only, a range of VHH were isolated that could be grouped into two families, whereas when the subtype A and C envelopes were included in the panning, only the most potent and broadly reactive VHH in the set were selected. This finding suggests that alternating the antigen in the panning procedure enables selection of VHH with better cross-reactive properties.

In conclusion, we have characterized three cross-reactive VHH that can neutralize several HIV-1 primary isolates of subtypes B and C. To our knowledge, this is the first description of broadly neutralizing MAbs to HIV-1 envelope which were derived from an immunized animal, as all previously reported broadly neutralizing anti-HIV-1 MAbs have been a result of natural infection rather than immunization (61). These VHH compete with sCD4 and anti-CD4bs MAbs for binding to recombinant gp120 and gp140. The results indicate that heavy chain antibody fragments have a possible use as potent HIV-1 entry inhibitors. Since VHH are stable and can be produced at a relatively low cost, they may be considered for applications such as HIV-1 microbicide development. Antienvelope VHH might also prove of use in defining neutralizing and nonneutralizing epitopes on HIV-1 envelope proteins, with implications for HIV-1 vaccine design.

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## REFERENCES

1. **Aasa-Chapman, M. M., A. Hayman, P. Newton, D. Cornforth, I. Williams, P. Borrow, P. Balfe, and A. McKnight.** 2004. Development of the antibody response in acute HIV-1 infection. AIDS **18:**371–381.
2. **Baral, T. N., S. Magez, B. Stijlemans, K. Conrath, B. Vanhollebeke, E. Pays, S. Muyldermans, and P. De Baetselier.** 2006. Experimental therapy of African trypanosomiasis with a nanobody-conjugated human trypanolytic factor. Nat. Med. **12:**580–584.
3. **Barbas, C. F., III, E. Bjorling, F. Chiodi, N. Dunlop, D. Cababa, T. M. Jones, S. L. Zebedee, M. A. Persson, P. L. Nara, E. Norrby, et al.** 1992. Recombinant human Fab fragments neutralize human type 1 immunodeficiency virus in vitro. Proc. Natl. Acad. Sci. USA **89:**9339–9343.
4. **Barbas, C. F., III, D. Hu, N. Dunlop, L. Sawyer, D. Cababa, R. M. Hendry, P. L. Nara, and D. R. Burton.** 1994. In vitro evolution of a neutralizing human antibody to human immunodeficiency virus type 1 to enhance affinity and broaden strain cross-reactivity. Proc. Natl. Acad. Sci. USA **91:**3809–3813.
5. **Batista, F. D., and M. S. Neuberger.** 1998. Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. Immunity **8:**751–759.
6. **Beddows, S., M. Franti, A. K. Dey, M. Kirschner, S. P. Iyer, D. C. Fisch, T. Ketas, E. Yuste, R. C. Desrosiers, P. J. Klasse, P. J. Maddon, W. C. Olson, and J. P. Moore.** 2007. A comparative immunogenicity study in rabbits of disulfide-stabilized, proteolytically cleaved, soluble trimeric human immunodeficiency virus type 1 gp140, trimeric cleavage-defective gp140 and monomeric gp120. Virology **360:**329–340.
7. **Beddows, S., N. Schulke, M. Kirschner, K. Barnes, M. Franti, E. Michael, T. Ketas, R. W. Sanders, P. J. Maddon, W. C. Olson, and J. P. Moore.** 2005. Evaluating the immunogenicity of a disulfide-stabilized, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. J. Virol. **79:**8812–8827.
8. **Binley, J. M., T. Wrin, B. Korber, M. B. Zwick, M. Wang, C. Chappey, G. Stiegler, R. Kunert, S. Zolla-Pazner, H. Katinger, C. J. Petropoulos, and D. R. Burton.** 2004. Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. J. Virol. **78:**13232–13252.
9. **Buchacher, A., R. Predl, K. Strutzenberger, W. Steinfellner, A. Trkola, M. Purtscher, G. Gruber, C. Tauer, F. Steindl, A. Jungbauer, et al.** 1994. Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. AIDS Res. Hum. Retrovir. **10:**359–369.
10. **Burton, D. R., C. F. Barbas III, M. A. Persson, S. Koenig, R. M. Chanock, and R. A. Lerner.** 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. Proc. Natl. Acad. Sci. USA **88:**10134–10137.
11. **Burton, D. R., J. Pyati, R. Koduri, S. J. Sharp, G. B. Thornton, P. W. Parren, L. S. Sawyer, R. M. Hendry, N. Dunlop, P. L. Nara, et al.** 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science **266:**1024–1027.
12. **Clayton, R., A. Ohagen, O. Goethals, A. Smets, M. Van Loock, L. Michiels, E. Kennedy-Johnston, M. Cunningham, H. Jiang, S. Bola, L. Gutshall, G. Gunn, A. Del Vecchio, R. Sarisky, S. Hallenberger, and K. Hertogs.** 2007. Binding kinetics, uptake and intracellular accumulation of F105, an anti-gp120 human IgG1κ monoclonal antibody, in HIV-1 infected cells. J. Virol. Methods **139:**17–23.
13. **Coppieters, K., T. Dreier, K. Silence, H. de Haard, M. Lauwereys, P. Casteels, E. Beirnaert, H. Jonckheere, C. Van de Wiele, L. Staelens, J. Hostens, H. Revets, E. Remaut, D. Elewaut, and P. Rottiers.** 2006. Formatted anti-tumor necrosis factor alpha VHH proteins derived from camelids show superior potency and targeting to inflamed joints in a murine model of collagen-induced arthritis. Arthritis Rheum. **54:**1856–1866.
14. **Cortez-Retamozo, V., N. Backmann, P. D. Senter, U. Wernery, P. De Baetselier, S. Muyldermans, and H. Revets.** 2004. Efficient cancer therapy with a nanobody-based conjugate. Cancer Res. **64:**2853–2857.
15. **Dalgleish, A. G., P. C. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **312:**763–767.
16. **De Genst, E., K. Silence, K. Decanniere, K. Conrath, R. Loris, J. Kinne, S. Muyldermans, and L. Wyns.** 2006. Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. Proc. Natl. Acad. Sci. USA **103:**4586–4591.
17. **De Haard, H. J., S. Bezemer, A. M. Ledeboer, W. H. Muller, P. J. Boender, S. Moineau, M. C. Coppelmans, A. J. Verkleij, L. G. Frenken, and C. T. Verrips.** 2005. Llama antibodies against a lactococcal protein located at the tip of the phage tail prevent phage infection. J. Bacteriol. **187:**4531–4541.
18. **Derdeyn, C. A., J. M. Decker, J. N. Sfakianos, X. Wu, W. A. O'Brien, L. Ratner, J. C. Kappes, G. M. Shaw, and E. Hunter.** 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J. Virol. **74:**8358–8367.
19. **Dolk, E., M. van der Vaart, D. Lutje Hulsik, G. Vriend, H. de Haard, S. Spinelli, C. Cambillau, L. Frenken, and T. Verrips.** 2005. Isolation of llama antibody fragments for prevention of dandruff by phage display in shampoo. Appl. Environ. Microbiol. **71:**442–450.
20. **Dumoulin, M., K. Conrath, A. Van Meirhaeghe, F. Meersman, K. Heremans, L. G. Frenken, S. Muyldermans, L. Wyns, and A. Matagne.** 2002. Single-domain antibody fragments with high conformational stability. Protein Sci. **11:**500–515.
21. **Dumoulin, M., A. M. Last, A. Desmyter, K. Decanniere, D. Canet, G. Larsson, A. Spencer, D. B. Archer, J. Sasse, S. Muyldermans, L. Wyns, C. Redfield, A. Matagne, C. V. Robinson, and C. M. Dobson.** 2003. A camelid antibody fragment inhibits the formation of amyloid fibrils by human lysozyme. Nature **424:**783–788.
22. **Earl, P. L., W. Sugiura, D. C. Montefiori, C. C. Broder, S. A. Lee, C. Wild, J. Lifson, and B. Moss.** 2001. Immunogenicity and protective efficacy of oligomeric human immunodeficiency virus type 1 gp140. J. Virol. **75:**645–653.
23. **Els Conrath, K., M. Lauwereys, L. Wyns, and S. Muyldermans.** 2001. Camel single-domain antibodies as modular building units in bispecific and bivalent antibody constructs. J. Biol. Chem. **276:**7346–7350.
24. **Foote, J., and H. N. Eisen.** 1995. Kinetic and affinity limits on antibodies produced during immune responses. Proc. Natl. Acad. Sci. USA **92:**1254–1256.
25. **Fouts, T. R., J. M. Binley, A. Trkola, J. E. Robinson, and J. P. Moore.** 1997. Neutralization of the human immunodeficiency virus type 1 primary isolate JR-FL by human monoclonal antibodies correlates with antibody binding to

the oligomeric form of the envelope glycoprotein complex. J. Virol. **71:**2779–2785.
26. **Frenken, L. G., J. G. Hessing, C. A. Van den Hondel, and C. T. Verrips.** 1998. Recent advances in the large-scale production of antibody fragments using lower eukaryotic microorganisms. Res. Immunol. **149:**589–599.
27. **Frenken, L. G., R. H. van der Linden, P. W. Hermans, J. W. Bos, R. C. Ruuls, B. de Geus, and C. T. Verrips.** 2000. Isolation of antigen specific llama VHH antibody fragments and their high level secretion by Saccharomyces cerevisiae. J. Biotechnol. **78:**11–21.
28. **Garaicoechea, L., A. Olichon, G. Marcoppido, A. Wigdorovitz, M. Mozgovoj, L. Saif, T. Surrey, and V. Parreno.** 2008. Llama-derived single-chain antibody fragments directed to rotavirus VP6 protein possess broad neutralizing activity in vitro and confer protection against diarrhea in mice. J. Virol. **82:**9753–9764.
29. **Gorny, M. K., J. P. Moore, A. J. Conley, S. Karwowska, J. Sodroski, C. Williams, S. Burda, L. J. Boots, and S. Zolla-Pazner.** 1994. Human anti-V2 monoclonal antibody that neutralizes primary but not laboratory isolates of human immunodeficiency virus type 1. J. Virol. **68:**8312–8320.
30. **Gorny, M. K., C. Williams, B. Volsky, K. Revesz, S. Cohen, V. R. Polonis, W. J. Honnen, S. C. Kayman, C. Krachmarov, A. Pinter, and S. Zolla-Pazner.** 2002. Human monoclonal antibodies specific for conformation-sensitive epitopes of V3 neutralize human immunodeficiency virus type 1 primary isolates from various clades. J. Virol. **76:**9035–9045.
31. **Gorny, M. K., J. Y. Xu, S. Karwowska, A. Buchbinder, and S. Zolla-Pazner.** 1993. Repertoire of neutralizing human monoclonal antibodies specific for the V3 domain of HIV-1 gp120. J. Immunol. **150:**635–643.
32. **Grundner, C., Y. Li, M. Louder, J. Mascola, X. Yang, J. Sodroski, and R. Wyatt.** 2005. Analysis of the neutralizing antibody response elicited in rabbits by repeated inoculation with trimeric HIV-1 envelope glycoproteins. Virology **331:**33–46.
33. **Hamers-Casterman, C., T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E. B. Songa, N. Bendahman, and R. Hamers.** 1993. Naturally occurring antibodies devoid of light chains. Nature **363:**446–448.
34. **Hemelaar, J., E. Gouws, P. D. Ghys, and S. Osmanov.** 2006. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. AIDS **20:**W13–W23.
35. **Herrera, C., C. Spenlehauer, M. S. Fung, D. R. Burton, S. Beddows, and J. P. Moore.** 2003. Nonneutralizing antibodies to the CD4-binding site on the gp120 subunit of human immunodeficiency virus type 1 do not interfere with the activity of a neutralizing antibody against the same site. J. Virol. **77:**1084–1091.
36. **Joint United Nations Programme on HIV/AIDS and World Health Organization.** 2007. 2007 AIDS epidemic update. http://www.unaids.org/en/KnowledgeCentre/HIVData/EpiUpdate/EpiUpdArchive/2007/default.asp.
37. **Karwowska, S., M. K. Gorny, A. Buchbinder, V. Gianakakos, C. Williams, T. Fuerst, and S. Zolla-Pazner.** 1992. Production of human monoclonal antibodies specific for conformational and linear non-V3 epitopes of gp120. AIDS Res. Hum. Retrovir. **8:**1099–1106.
38. **Kim, M., Z. S. Qiao, D. C. Montefiori, B. F. Haynes, E. L. Reinherz, and H. X. Liao.** 2005. Comparison of HIV type 1 ADA gp120 monomers versus gp140 trimers as immunogens for the induction of neutralizing antibodies. AIDS Res. Hum. Retrovir. **21:**58–67.
39. **Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature **312:**767–768.
40. **Laal, S., S. Burda, M. K. Gorny, S. Karwowska, A. Buchbinder, and S. Zolla-Pazner.** 1994. Synergistic neutralization of human immunodeficiency virus type 1 by combinations of human monoclonal antibodies. J. Virol. **68:**4001–4008.
41. **Lauwereys, M., M. Arbabi Ghahroudi, A. Desmyter, J. Kinne, W. Holzer, E. De Genst, L. Wyns, and S. Muyldermans.** 1998. Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. EMBO J. **17:**3512–3520.
42. **Lederman, M. M., R. E. Offord, and O. Hartley.** 2006. Microbicides and other topical strategies to prevent vaginal transmission of HIV. Nat. Rev. Immunol. **6:**371–382.
43. **Li, M., F. Gao, J. R. Mascola, L. Stamatatos, V. R. Polonis, M. Koutsoukos, G. Voss, P. Goepfert, P. Gilbert, K. M. Greene, M. Bilska, D. L. Kothe, J. F. Salazar-Gonzalez, X. Wei, J. M. Decker, B. H. Hahn, and D. C. Montefiori.** 2005. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol. **79:**10108–10125.
44. **Li, M., J. F. Salazar-Gonzalez, C. A. Derdeyn, L. Morris, C. Williamson, J. E. Robinson, J. M. Decker, Y. Li, M. G. Salazar, V. R. Polonis, K. Mlisana, S. A. Karim, K. Hong, K. M. Greene, M. Bilska, J. Zhou, S. Allen, E. Chomba, J. Mulenga, C. Vwalika, F. Gao, M. Zhang, B. T. Korber, E. Hunter, B. H. Hahn, and D. C. Montefiori.** 2006. Genetic and neutralization properties of subtype C human immunodeficiency virus type 1 molecular env clones from acute and early heterosexually acquired infections in Southern Africa. J. Virol. **80:**11776–11790.
45. **Louder, M. K., A. Sambor, E. Chertova, T. Hunte, S. Barrett, F. Ojong, E. Sanders-Buell, S. Zolla-Pazner, F. E. McCutchan, J. D. Roser, D. Gabuzda, J. D. Lifson, and J. R. Mascola.** 2005. HIV-1 envelope pseudotyped viral vectors and infectious molecular clones expressing the same envelope glycoprotein have a similar neutralization phenotype, but culture in peripheral blood mononuclear cells is associated with decreased neutralization sensitivity. Virology **339:**226–238.
46. **Mascola, J. R., P. D'Souza, P. Gilbert, B. H. Hahn, N. L. Haigwood, L. Morris, C. J. Petropoulos, V. R. Polonis, M. Sarzotti, and D. C. Montefiori.** 2005. Recommendations for the design and use of standard virus panels to assess neutralizing antibody responses elicited by candidate human immunodeficiency virus type 1 vaccines. J. Virol. **79:**10103–10107.
47. **McCutchan, F. E.** 2006. Global epidemiology of HIV. J. Med. Virol. **78**(Suppl. 1)**:**S7–S12.
48. **McKeating, J. A., A. McKnight, K. McIntosh, P. R. Clapham, C. Mulder, and R. A. Weiss.** 1989. Evaluation of human and simian immunodeficiency virus plaque and neutralization assays. J. Gen. Virol. **70:**3327–3333.
49. **Moog, C., C. Spenlehauer, H. Fleury, F. Heshmati, S. Saragosti, F. Letourneur, A. Kirn, and A. M. Aubertin.** 1997. Neutralization of primary human immunodeficiency virus type 1 isolates: a study of parameters implicated in neutralization in vitro. AIDS Res. Hum. Retrovir. **13:**19–27.
50. **Moore, J. P., Y. Cao, L. Qing, Q. J. Sattentau, J. Pyati, R. Koduri, J. Robinson, C. F. Barbas III, D. R. Burton, and D. D. Ho.** 1995. Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. J. Virol. **69:**101–109.
51. **Moore, J. P., and R. W. Doms.** 2003. The entry of entry inhibitors: a fusion of science and medicine. Proc. Natl. Acad. Sci. USA **100:**10598–10602.
52. **Moore, J. P., and J. Sodroski.** 1996. Antibody cross-competition analysis of the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein. J. Virol. **70:**1863–1872.
53. **Moore, J. P., A. Trkola, and T. Dragic.** 1997. Co-receptors for HIV-1 entry. Curr. Opin. Immunol. **9:**551–562.
54. **Moore, P. L., E. T. Crooks, L. Porter, P. Zhu, C. S. Cayanan, H. Grise, P. Corcoran, M. B. Zwick, M. Franti, L. Morris, K. H. Roux, D. R. Burton, and J. M. Binley.** 2006. Nature of nonfunctional envelope proteins on the surface of human immunodeficiency virus type 1. J. Virol. **80:**2515–2528.
55. **Moulard, M., S. K. Phogat, Y. Shu, A. F. Labrijn, X. Xiao, J. M. Binley, M. Y. Zhang, I. A. Sidorov, C. C. Broder, J. Robinson, P. W. Parren, D. R. Burton, and D. S. Dimitrov.** 2002. Broadly cross-reactive HIV-1-neutralizing human monoclonal Fab selected for binding to gp120-CD4-CCR5 complexes. Proc. Natl. Acad. Sci. USA **99:**6913–6918.
56. **Muster, T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, F. Ruker, and H. Katinger.** 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J. Virol. **67:**6642–6647.
57. **Muyldermans, S.** 2001. Single domain camel antibodies: current status. J. Biotechnol. **74:**277–302.
58. **Muyldermans, S., C. Cambillau, and L. Wyns.** 2001. Recognition of antigens by single-domain antibody fragments: the superfluous luxury of paired domains. Trends Biochem. Sci. **26:**230–235.
59. **Myszka, D. G., R. W. Sweet, P. Hensley, M. Brigham-Burke, P. D. Kwong, W. A. Hendrickson, R. Wyatt, J. Sodroski, and M. L. Doyle.** 2000. Energetics of the HIV gp120-CD4 binding reaction. Proc. Natl. Acad. Sci. USA **97:**9026–9031.
60. **Pant, N., A. Hultberg, Y. Zhao, L. Svensson, Q. Pan-Hammarstrom, K. Johansen, P. H. Pouwels, F. M. Ruggeri, P. Hermans, L. Frenken, T. Boren, H. Marcotte, and L. Hammarstrom.** 2006. Lactobacilli expressing variable domain of llama heavy-chain antibody fragments (lactobodies) confer protection against rotavirus-induced diarrhea. J. Infect. Dis. **194:**1580–1588.
61. **Pantophlet, R., and D. R. Burton.** 2006. GP120: target for neutralizing HIV-1 antibodies. Annu. Rev. Immunol. **24:**739–769.
62. **Parren, P. W., I. Mondor, D. Naniche, H. J. Ditzel, P. J. Klasse, D. R. Burton, and Q. J. Sattentau.** 1998. Neutralization of human immunodeficiency virus type 1 by antibody to gp120 is determined primarily by occupancy of sites on the virion irrespective of epitope specificity. J. Virol. **72:**3512–3519.
63. **Parren, P. W., J. P. Moore, D. R. Burton, and Q. J. Sattentau.** 1999. The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity. AIDS **13**(Suppl. A)**:**S137–S162.
64. **Perez, J. M., J. G. Renisio, J. J. Prompers, C. J. van Platerink, C. Cambillau, H. Darbon, and L. G. Frenken.** 2001. Thermal unfolding of a llama antibody fragment: a two-state reversible process. Biochemistry **40:**74–83.
65. **Platt, E. J., K. Wehrly, S. E. Kuhmann, B. Chesebro, and D. Kabat.** 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophage-tropic isolates of human immunodeficiency virus type 1. J. Virol. **72:**2855–2864.
66. **Poignard, P., M. Moulard, E. Golez, V. Vivona, M. Franti, S. Venturini, M. Wang, P. W. Parren, and D. R. Burton.** 2003. Heterogeneity of envelope molecules expressed on primary human immunodeficiency virus type 1 particles as probed by the binding of neutralizing and nonneutralizing antibodies. J. Virol. **77:**353–365.
67. **Reeves, J. D., S. A. Gallo, N. Ahmad, J. L. Miamidian, P. E. Harvey, M. Sharron, S. Pohlmann, J. N. Sfakianos, C. A. Derdeyn, R. Blumenthal, E. Hunter, and R. W. Doms.** 2002. Sensitivity of HIV-1 to entry inhibitors

correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. Proc. Natl. Acad. Sci. USA **99:**16249–16254.
68. **Roben, P., J. P. Moore, M. Thali, J. Sodroski, C. F. Barbas III, and D. R. Burton.** 1994. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. J. Virol. **68:**4821–4828.
69. **Roovers, R. C., T. Laeremans, L. Huang, S. De Taeye, A. J. Verkleij, H. Revets, H. J. de Haard, and P. M. van Bergen en Henegouwen.** 2007. Efficient inhibition of EGFR signaling and of tumour growth by antagonistic anti-EFGR nanobodies. Cancer Immunol. Immunother. **56:**303–317.
70. **Sanders, R. W., M. Venturi, L. Schiffner, R. Kalyanaraman, H. Katinger, K. O. Lloyd, P. D. Kwong, and J. P. Moore.** 2002. The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. J. Virol. **76:**7293–7305.
71. **Sattentau, Q. J., and J. P. Moore.** 1995. Human immunodeficiency virus type 1 neutralization is determined by epitope exposure on the gp120 oligomer. J. Exp. Med. **182:**185–196.
72. **Scanlan, C. N., R. Pantophlet, M. R. Wormald, E. Ollmann Saphire, R. Stanfield, I. A. Wilson, H. Katinger, R. A. Dwek, P. M. Rudd, and D. R. Burton.** 2002. The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of $\alpha 1 \rightarrow 2$ mannose residues on the outer face of gp120. J. Virol. **76:**7306–7321.
73. **Schonning, K., A. Bolmstedt, J. Novotny, O. S. Lund, S. Olofsson, and J. E. Hansen.** 1998. Induction of antibodies against epitopes inaccessible on the HIV type 1 envelope oligomer by immunization with recombinant monomeric glycoprotein 120. AIDS Res. Hum. Retrovir. **14:**1451–1456.
74. **Schutten, M., A. McKnight, R. C. Huisman, M. Thali, J. A. McKeating, J. Sodroski, J. Goudsmit, and A. D. Osterhaus.** 1993. Further characterization of an antigenic site of HIV-1 gp120 recognized by virus neutralizing human monoclonal antibodies. AIDS **7:**919–923.
75. **Shi, Y., J. Albert, G. Francis, H. Holmes, and E. M. Fenyo.** 2002. A new cell line-based neutralization assay for primary HIV type 1 isolates. AIDS Res. Hum. Retrovir. **18:**957–967.
76. **Soda, Y., N. Shimizu, A. Jinno, H. Y. Liu, K. Kanbe, T. Kitamura, and H. Hoshino.** 1999. Establishment of a new system for determination of coreceptor usages of HIV based on the human glioma NP-2 cell line. Biochem. Biophys. Res. Commun. **258:**313–321.
77. **Stiegler, G., R. Kunert, M. Purtscher, S. Wolbank, R. Voglauer, F. Steindl, and H. Katinger.** 2001. A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. AIDS Res. Hum. Retrovir. **17:**1757–1765.
78. **Sundberg, E. J., and R. A. Mariuzza.** 2002. Molecular recognition in antibody-antigen complexes. Adv. Protein Chem. **61:**119–160.
79. **Thali, M., J. P. Moore, C. Furman, M. Charles, D. D. Ho, J. Robinson, and J. Sodroski.** 1993. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. J. Virol. **67:**3978–3988.
80. **Trkola, A., M. Purtscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, K. Srinivasan, J. Sodroski, J. P. Moore, and H. Katinger.** 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol. **70:**1100–1108.
81. **van der Linden, R. H., L. G. Frenken, B. de Geus, M. M. Harmsen, R. C. Ruuls, W. Stok, L. de Ron, S. Wilson, P. Davis, and C. T. Verrips.** 1999. Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies. Biochim. Biophys. Acta **1431:**37–46.
82. **Veazey, R. S., R. J. Shattock, M. Pope, J. C. Kirijan, J. Jones, Q. Hu, T. Ketas, P. A. Marx, P. J. Klasse, D. R. Burton, and J. P. Moore.** 2003. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat. Med. **9:**343–346.
83. **Verheesen, P., A. de Kluijver, S. van Koningsbruggen, M. de Brij, H. J. de Haard, G. J. van Ommen, S. M. van der Maarel, and C. T. Verrips.** 2006. Prevention of oculopharyngeal muscular dystrophy-associated aggregation of nuclear polyA-binding protein with a single-domain intracellular antibody. Hum. Mol. Genet. **15:**105–111.
84. **Verheesen, P., M. R. ten Haaft, N. Lindner, C. T. Verrips, and J. J. de Haard.** 2003. Beneficial properties of single-domain antibody fragments for application in immunoaffinity purification and immuno-perfusion chromatography. Biochim. Biophys. Acta **1624:**21–28.
85. **Vu, K. B., M. A. Ghahroudi, L. Wyns, and S. Muyldermans.** 1997. Comparison of llama VH sequences from conventional and heavy chain antibodies. Mol. Immunol. **34:**1121–1131.
86. **Wei, X., J. M. Decker, H. Liu, Z. Zhang, R. B. Arani, J. M. Kilby, M. S. Saag, X. Wu, G. M. Shaw, and J. C. Kappes.** 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob. Agents Chemother. **46:**1896–1905.
87. **Wright, E., N. J. Temperton, D. A. Marston, L. M. McElhinney, A. R. Fooks, and R. A. Weiss.** 2008. Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: a cross-species comparison. J. Gen. Virol. **89:**2204–2213.
88. **Wu, H., D. G. Myszka, S. W. Tendian, C. G. Brouillette, R. W. Sweet, I. M. Chaiken, and W. A. Hendrickson.** 1996. Kinetic and structural analysis of mutant CD4 receptors that are defective in HIV gp120 binding. Proc. Natl. Acad. Sci. USA **93:**15030–15035.
89. **Wyatt, R., and J. Sodroski.** 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science **280:**1884–1888.
90. **Yang, X., R. Wyatt, and J. Sodroski.** 2001. Improved elicitation of neutralizing antibodies against primary human immunodeficiency viruses by soluble stabilized envelope glycoprotein trimers. J. Virol. **75:**1165–1171.
91. **Zanetti, G., J. A. Briggs, K. Grunewald, Q. J. Sattentau, and S. D. Fuller.** 2006. Cryo-electron tomographic structure of an immunodeficiency virus envelope complex in situ. PLoS Pathog. **2:**e83.
92. **Zhang, M. Y., X. Xiao, I. A. Sidorov, V. Choudhry, F. Cham, P. F. Zhang, P. Bouma, M. Zwick, A. Choudhary, D. C. Montefiori, C. C. Broder, D. R. Burton, G. V. Quinnan, Jr., and D. S. Dimitrov.** 2004. Identification and characterization of a new cross-reactive human immunodeficiency virus type 1-neutralizing human monoclonal antibody. J. Virol. **78:**9233–9242.
93. **Zhang, P. F., F. Cham, M. Dong, A. Choudhary, P. Bouma, Z. Zhang, Y. Shao, Y. R. Feng, L. Wang, N. Mathy, G. Voss, C. C. Broder, and G. V. Quinnan, Jr.** 2007. Extensively cross-reactive anti-HIV-1 neutralizing antibodies induced by gp140 immunization. Proc. Natl. Acad. Sci. USA **104:**10193–10198.
94. **Zhou, T., L. Xu, B. Dey, A. J. Hessell, D. Van Ryk, S. H. Xiang, X. Yang, M. Y. Zhang, M. B. Zwick, J. Arthos, D. R. Burton, D. S. Dimitrov, J. Sodroski, R. Wyatt, G. J. Nabel, and P. D. Kwong.** 2007. Structural definition of a conserved neutralization epitope on HIV-1 gp120. Nature **445:**732–737.
95. **Zhu, P., J. Liu, J. Bess, Jr., E. Chertova, J. D. Lifson, H. Grise, G. A. Ofek, K. A. Taylor, and K. H. Roux.** 2006. Distribution and three-dimensional structure of AIDS virus envelope spikes. Nature **441:**847–852.
96. **Zwick, M. B., A. F. Labrijn, M. Wang, C. Spenlehauer, E. O. Saphire, J. M. Binley, J. P. Moore, G. Stiegler, H. Katinger, D. R. Burton, and P. W. Parren.** 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J. Virol. **75:**10892–10905.