Llama antibody fragments with cross-subtype HIV-1 neutralizing properties and high affinity for HIV-1 gp120

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ABSTRACT

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 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 subtype 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 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. Anti-envelope VHH might also prove useful in defining neutralizing and non-neutralizing epitopes on HIV-1 envelope proteins, with implications for HIV-1 vaccine design.

INTRODUCTION

During 2007, there were an estimated 2.5 million new HIV-1 infections, 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, non-covalently bound to the transmembrane glycoprotein, gp41 (89, 91, 95). In addition to the functional spikes, there is also evidence for the presence of non-functional derivates, 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 co-receptor, 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 post-immunization and from humans following HIV-1 infection. Out of these, only a handful has been found to be broadly neutralizing across HIV-1 subtypes (8) and all are a 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 group M, N and O, 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-D, F-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 defining neutralizing and non-neutralizing epitopes on HIV-1 envelope proteins of various subtypes. We employed the non-conventional immune system of camelids to generate novel anti-envelope 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 or yeast (26, 27). This notion, together with advantageous characteristics in terms of stability, solubility and production yield in fermentation processes (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 subtype B and C. These VHH may be useful as tools for HIV-1 vaccine design and may 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 Dr D. Burton, Scripps Institute, La Jolla, USA. MAb GP68 (74) was obtained through the Centralised Facility for AIDS Reagents (CFAR), NIBSC, Potters Bar, Herts, UK (original source Dr A. Osterhaus and Dr M. Schutten). MAbs 654-D (29, 37, 40) and 447-52D (30, 31) were obtained through the CFAR, NIBSC (original source Dr S. Zolla-Pazner). MAb 17b (79) was obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA (original source Dr. James 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). QC 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 Dr I. Jones, Reading University, UK, through the European Microbicides Project (EMPRO). Recombinant gp140 from HIV-1 92UG037 (subtype A) was kindly provided by Dr S. Jeffs, Imperial College London, UK. Recombinant gp120 from HIV-1 IIIB (EVA607) was obtained from the CFAR, NIBSC. Recombinant gp120 derived from HIV-1 92UG037 and 92BR025 (subtype C) were expressed and purified as detailed in supplemental file 1.

Viruses

The sources of HIV-1 PBMC isolates, HIV-1 replication-competent molecular clones and *env* clones used to prepare pseudotyped viruses and to express recombinant gp120 are detailed in supplemental file 1.

Cells

TZM-bl cells (18, 65, 86) were obtained through the NIH AIDS Research and Reference Reagent Program, from Dr. J. C. Kappes, Dr. X. Wu and Tranzyme Inc, and cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Paisley, UK) containing 10% (v/v) fetal calf serum (FCS). NP2 glioma cells (76), expressing the HIV-1 cellular receptor CD4 and either of the co-receptors CXCR4 (NP2/CD4/CXCR4) or CCR5 (NP2/CD4/CCR5), were cultured in DMEM containing 5% (v/v) FCS. H9 cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) FCS. Phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMC) were obtained from blood donors and cultured in RPMI 1640 supplemented with 10% (v/v) 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 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 anti-envelope immune response in sera was verified in an enzyme-linked immunosorbent assay (ELISA) against immobilized recombinant gp120. The neutralization activity of serum or plasma samples from day 0 (pre-immunization) and days 28, 39 and 43 (post-immunization) was evaluated in TZM-bl cells. Total RNA was isolated from peripheral blood lymphocytes and lymph node biopsies collected post-immunization (on day 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 geneIII product, transformed TG1 *E. 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

Phages expressing the cloned VHH repertoire were incubated with immobilized gp120 and eluted with sCD4 to enrich for VHH targeting the CD4bs in two subsequent rounds of selections. 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 supplemental file 1, 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 TCID50 of virus in the TZM-bl cell-based assay developed by Derdeyn *et al*. (18), Wei *et al*. (86) and Li *et al*. (43), with Bright-Glo™ Luciferase Reagent (Promega, Southampton, UK). 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 IC_{50} and IC_{90} titers were calculated using the XLFit4 software (ID Business Solutions, Guildford, UK).

In addition, VHH neutralization of HIV-1 was assayed in phytohemagglutinin-stimulated PBMC. 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 hours at 37°C the cells were washed twice in growth medium. Cell culture supernatant was collected on day 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, U.S.A.) according to the manufacturer's instructions. Neutralization was measured as reduction in p24 content in test wells compared to in virus control wells. All VHH, including a negative control VHH (and MAb b12), were assayed in triplicate and in PBMC from two separate donors.

VHH binding to recombinant envelope proteins in ELISA, competition ELISA and surface plasmon resonance assays

VHH binding to recombinant envelope proteins and ability to compete with sCD4, anti-gp120 MAbs and each other was assayed in ELISA, as detailed in supplemental file 1. VHH binding to gp120 and competition with sCD4 was assayed in surface plasmon resonance experiments carried out using BIAcore (Uppsala, Sweden), as detailed in supplemental file 1.

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, anti-envelope 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 post-immunization serum and plasma samples from llama L44 (supplemental file 2, Fig. S1). 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 length of elution. Selections where a larger number of clones were eluted by sCD4, compared to elution with 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 ELISA and/or *in vitro* neutralization 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 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 ELISA. VHH clones with confirmed specificity for gp120 were also tested for ability to neutralize the corresponding virus and a limited panel of heterologous isolates of subtype A, B and C.

Only one out of 96 clones selected using HIV-1 CN54 gp120 was found to bind to CN54 gp120 in 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 no or less than 50% neutralization of 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 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 eight different clones that could be separated into two distinct groups. The first group consisted of three clones with eight to fourteen amino acid differences in the framework 1, 2 and 3 regions as well as in the CDR1, CDR2 and CDR3. They displayed a CDR3 of 18 amino acids. Based on superior neutralizing activity against the limited panel of HIV-1 isolates (data not shown), two of these VHH, designated A12 and D7, were chosen for

1 further characterization. The second group of VHH consisted of five clones with one to three
2 amino acid differences only, in framework regions 1 and 3. These displayed a shorter CDR3 of
3 10 amino acids. All five clones were shown to have similar neutralization profiles (data not
4 shown), and one representative clone, designated C8, was chosen for further characterization.
5 Thus, in total, three VHH (A12, D7 and C8) were selected for further characterization
6 (supplemental file 2, Table S1). The amino acid sequences of these VHH are shown in
7 supplemental file 2, Fig. S1.
8
9 In order to allow for selection of VHH recognizing motifs conserved among HIV-1 subtypes, the
10 phage libraries were also panned against recombinant gp120 derived from subtype A (92UG037)
11 and subtype C (92BR025) gp120, in addition to IIIB gp120, alternating the antigen in different
12 combinations in two subsequent rounds of panning. Enrichment of clones eluted by sCD4 was
13 only observed in selections using the phage library from llama L44. Approximately 700
14 individual VHH were isolated, expressed and screened for neutralization of HIV-1 as well as for
15 binding to gp120 in ELISA. VHH clones selected using 92BR025 gp120 were screened for
16 ability to neutralize HIV-1 92BR025, whereas VHH selected using 92UG037 gp120 were
17 screened against HIV-1 92UG037. In addition, all VHH were screened for ability to neutralize
18 HIV-1 IIIB. Out of the 700 clones isolated, 43 were observed to bind to gp120 in ELISA and to
19 neutralize HIV-1. The results from the neutralization and binding screening showed complete
20 correlation, with the exception that the clones selected using subtype A 92UG037 gp120 were not
21 able to neutralize 92UG037 virus, despite being able to neutralize HIV-1 IIIB. The relatively low
22 frequency of VHH able to bind to gp120 and to neutralize HIV-1 (43 out of 700, corresponding
23 to 6%) probably reflects the nature of the competitive elution method, as a significant number of
24 clones were non-specifically 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 one 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 concentration required to achieve 50% and 90% reduction of infectivity (IC_{50} and IC_{90}) compared to a virus control was determined. A negative control VHH was tested in parallel, as well as a pseudovirus bearing the rabies virus G protein (87). No non-specific virus inactivation was observed.

The results of the neutralization characterization are summarized in Table 1 and in Fig. 2. 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 subtype B, C and CRF07_BC, but not other subtypes tested. Overall, it neutralized 27 out of 65 viruses (42%), with IC_{50} values in the range of <0.003-38 μg/ml (Table 1 and Fig. 2). VHH D7 showed a similar but slightly less cross-

reactive neutralization profile 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 (Table 1 and Fig. 2).

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 (Table 1 and Fig. 2). 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. 2). Moreover, MAb b12 neutralized 56% of the subtype C viruses, compared to 48% for VHH A12. As expected, fewer isolates were neutralized at IC_{90} level by the VHH and MAb b12 (Fig. 2). Like the VHH, MAb b12 did not neutralize (to ≥50%) the subtype A, A/G or D viruses included in the study (Table 1). It should be noted that the VHH are approximately ten times smaller than MAb b12, but that MAb b12, on the other hand, has two antigen-recognizing domains per molecule, making accurate estimates of relative neutralization potencies impossible. To be able to make a direct comparison, the VHH would need to be presented in the context of a complete camelid immunoglobulin.

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 PBMC from two different donors, scored on day 5 post-infection, are shown in Table 2. VHH A12 was able to inhibit infection of SF162 in PBMC from both donors, with and IC_{75} 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 PBMC 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. Furthermore, 90% neutralization was only observed in PBMC from one of the two donors, and it required a 6-fold higher concentration of A12 compared to in TZM-bl cells (Table 2). MAb b12 was more than 20-fold more potent than VHH A12 at 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).

The 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 3. 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 22-35 nM (12, 59, 88). Another study reported K_D values in the range of 2.2-16 nM for 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).

The VHH bind to recombinant envelope proteins of subtype A, B and C in ELISA

VHH binding to HIV-1 gp120 was assayed in 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. 3A. 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 subtype B and C, none of VHH A12, D7 and C8 was found to bind well to the protein against which they had been raised, CN54 gp120, in 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 the VHH epitopes

The VHH compete with sCD4 for binding to recombinant envelope proteins

Since the VHH were specifically selected for their ability to compete with sCD4, their potency to inhibit binding of sCD4 to recombinant gp120 and gp140 was evaluated in ELISA. Titrations of VHH were pre-incubated 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. 3B). 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 ELISA, which is why the ability of the selected VHH to inhibit sCD4 binding to HIV-1 CN54 gp120 could not be evaluated. The ability of the neutralizing VHH to compete with sCD4 for binding to recombinant gp120 and gp140 indicates 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 over-lapping 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. Using surface plasmon resonance enables testing whether the VHH can inhibit binding of sCD4

to CN54 gp120 (the immunogen), since binding of fluid-phase (only) CN54 gp120 to immobilized sCD4, could be observed in BIAcore but not in ELISA. Recombinant sCD4 was captured by an anti-CD4 antibody immobilized on the sensor chip. Titrations of VHH were pre-incubated 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 (supplemental file 2, Fig. S3). Both VHH also inhibited sCD4 binding to IIIB gp120 (data not shown). This finding confirms the observations made in the ELISA experiments, 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 biding 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.

The 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 (TCLA) 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. 4A). 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 (Table 1), 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. 4A).

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. 4B). 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 TCLA 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 pre-incubated with IIIB gp120, followed by subsequent inhibition with immobilized VHH pre-coated onto plates and detection of IIIB gp120 binding to VHH. Anti-CD4bs MAb b12 could clearly inhibit VHH binding to IIIB gp120 (Fig. 4B), which is consistent with the results described in Fig. 3B. 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, binding to the very C-terminal region of gp120, as it has been used both to capture and detect gp120 throughput 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 the 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.

The 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 ability to compete with each other for binding to gp120 (Fig. 4C). 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 the VHH either bind to epitopes that overlap, or that steric hindrance inhibits simultaneous binding. VHH C8 required higher concentrations than VHH A12 and 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 for VHH C8 (Table 3). 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. 3B, 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. 4C).

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 of phage libraries displaying the VHH repertoires of the immunized animals on recombinant gp120, and by introducing a competitive elution step using sCD4, we could 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 subtype 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 subtype 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 twelve 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 subtype 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 neutralize 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), where a small panel of MAbs was tested against an extensive panel of 90 viruses using a high-throughput pseudovirus-based neutralization assay and where 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 twelve 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 over-lapping epitopes on gp120, despite competing with each other for binding to gp120 in 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 anti-retroviral 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*. Anti-envelope 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 reports that extensively cross-reactive antibodies (albeit at low titer) have been 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 allows 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, is ongoing, but has 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 ability to neutralize virus (25, 35, 50, 54, 62, 66, 71, 73), as the structure of recombinant envelope glycoproteins is likely to be different from the structure 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 and for panning of VHH libraries. Interestingly, VHH binding to recombinant 92BR025 gp120 seems to correlate with ability to neutralize 92BR025 virus. VHH A12 and C8 were able to bind well to recombinant 92BR025 gp120 in ELISA, whereas D7 bound with weaker signal (Fig. 3A). 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 (Table 1). 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 when 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 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, using this method of selection, we could select for VHH that could bind to envelope proteins of subtype A, B, and C, regardless of which of the above antigens that were used in the panning. However, when selecting 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 subtype B and C. To our knowledge, this is the first description of broadly neutralizing MAbs to HIV-1 envelope, which are 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. Anti-envelope VHH might also prove of use in defining neutralizing and non-neutralizing 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:**371-81.

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:**580-4.

3. **Barbas, C. F., 3rd, E. Bjorling, F. Chiodi, N. Dunlop, D. Cababa, T. M. Jones, S. L. Zebedee, M. A. Persson, P. L. Nara, E. Norrby, and et al.** 1992. Recombinant human Fab fragments neutralize human type 1 immunodeficiency virus in vitro. Proc Natl Acad Sci U S A **89:**9339-43.

4. **Barbas, C. F., 3rd, 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 U S A **91:**3809-13.

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:**751-9.

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:**329-40.

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:**8812-27.

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:**13232-52.

9. **Buchacher, A., R. Predl, K. Strutzenberger, W. Steinfellner, A. Trkola, M. Purtscher, G. Gruber, C. Tauer, F. Steindl, A. Jungbauer, and 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 Retroviruses **10:**359-69.

10. **Burton, D. R., C. F. Barbas, 3rd, 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 U S A **88:**10134-7.

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, and et al.** 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science **266:**1024-7.

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 IgG1kappa 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:**1856-66.

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:**2853-7.

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:**763-7.

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 U S A **103:**4586-91.

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:**4531-41.

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:**8358-67.

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:**442-50.

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:**500-15.

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:**783-8.

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:**645-53.

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:**7346-50.

24. **Foote, J., and H. N. Eisen.** 1995. Kinetic and affinity limits on antibodies produced during immune responses. Proc Natl Acad Sci U S A **92:**1254-6.

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:**2779-85.

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:**589-99.

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:**9753-64.

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:**8312-20.

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:**9035-45.

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:**635-43.

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:**446-8.

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:**W13-23.

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:**1084-91.

36. **Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO).** 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 Retroviruses **8:**1099-106.

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 Retroviruses **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:**767-8.

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:**4001-8.

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:**3512-20.

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:**371-82.

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:**10108-25.

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:**11776-90.

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:**226-38.

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:**10103-7.

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 (Pt 12):**3327-33.

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 Retroviruses **13:**19-27 "[

50. **Moore, J. P., Y. Cao, L. Qing, Q. J. Sattentau, J. Pyati, R. Koduri, J. Robinson, C. F. Barbas, 3rd, 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:**101-9.

51. **Moore, J. P., and R. W. Doms.** 2003. The entry of entry inhibitors: a fusion of science and medicine. Proc Natl Acad Sci U S A **100:**10598-602.

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:**1863-72.

53. **Moore, J. P., A. Trkola, and T. Dragic.** 1997. Co-receptors for HIV-1 entry. Curr Opin Immunol **9:**551-62.

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:**2515-28.

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 U S A **99:**6913-8.

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:**6642-7.

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:**230-5.

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 U S A **97:**9026-31.

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:**1580-8.

61. **Pantophlet, R., and D. R. Burton.** 2006. GP120: target for neutralizing HIV-1 antibodies. Annu Rev Immunol **24:**739-69.

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:**3512-9.

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:**S137-62.

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 macrophagetropic isolates of human immunodeficiency virus type 1. J Virol **72:**2855-64.

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:**353-65.

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 U S A **99:**16249-54.

68. **Roben, P., J. P. Moore, M. Thali, J. Sodroski, C. F. Barbas, 3rd, 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:**4821-8.

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:**7293-305.

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:**185-96.

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 alpha1-->2 mannose residues on the outer face of gp120. J Virol **76:**7306-21.

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 Retroviruses **14:**1451-6.

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:**919-23.

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 Retroviruses **18:**957-67.

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:**313-21.

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 Retroviruses **17:**1757-65.

78. **Sundberg, E. J., and R. A. Mariuzza.** 2002. Molecular recognition in antibody-antigen complexes. Adv Protein Chem **61:**119-60.

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:**3978-88.

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:**1100-8.

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:**343-6.

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:**105-11.

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:**21-8.

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:**1121-31.

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:**1896-905.

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:**2204-13.

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 U S A **93:**15030-5.

89. **Wyatt, R., and J. Sodroski.** 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science **280:**1884-8.

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:**1165-71.

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:**9233-42.

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 U S A **104:**10193-8.

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:**732-7.

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:**847-52.

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:**10892-905.

FIGURE LEGENDS

FIGURE 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. Phages bound to gp120 were 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.

FIGURE 2. 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 less than or equal to 50 μg/ml.

FIGURE 3. (A) VHH binding to recombinant envelope proteins derived from HIV-1 92UG037 (subtype A), IIIB (subtype B) and 92BR025 (subtype C) in 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 ELISA. Three-fold serial dilutions of VHH were pre-incubated with IIIB gp120 or 92UG037 gp140 and subsequently incubated with sCD4 pre-coated 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 mean and bars the standard deviation of duplicate reactions.

FIGURE 4. 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 ELISA. Serial dilutions of VHH were pre-incubated 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), 447-52D (V3) as well as MAb b12 (CD4bs) for binding to recombinant gp120 in ELISA. VHH A12, D7 and C8 were coated onto the wells of microtiter plates, as indicated above each graph. Serial dilutions of each MAb were pre-incubated 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 ELISA. VHH A12, D7 and C8 were coated onto the wells of microtiter plates, as indicated above each graph. Serial dilutions of each VHH and sCD4 were pre-incubated 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 mean and bars the standard deviation of duplicate reactions.

TABLES

TABLE 1. VHH and MAb b12 IC_{50} titers against HIV-1 in TZM-bl cells[a].

Virus[b]	Type[c]	Subtype	Tier[d]	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

[a]VHH and MAb b12 neutralization activity was assessed in TZM-bl cells, as described in the text, • indicates >50 μg/ml. To aid comprehension, the titers have been color-coded so that the darker the color, the more potent the neutralization; nd, not determined; n/a, not applicable.

[b]Viruses are described in the text. Rabies CVS-11 is pseudotyped with rabies virus G-protein from strain CVS-11 (87).

[c]TCLA, T-cell line adapted isolate; PBMC, PBMC-propagated primary isolate; MC, molecular clone; PV, envelope pseudotyped virus.

[d]Classified as suitable for tier 1, 2 or 3 assessment of neutralizing antibodies (46).

TABLE 2. VHH and MAb b12 neutralization of HIV-1 SF162 in PBMC[a].

	IC_{50} (μg/ml)		**IC_{75} (μg/ml)**		**IC_{90} (μg/ml)**		**IC_{90} in TZM-bl cells (μg/ml)**
	PBMC donor 1	PBMC donor 2	PBMC donor 1	PBMC donor 2	PBMC donor 1	PBMC 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, C8 and #3 (negative control VHH) as well as MAb b12 was assayed in triplicate for ability to neutralize HIV-1 SF162 in PBMC from two different donors, as described in the text. HIV-1 p24 content on day 5 post infection in test wells compared to in virus only control wells was determined and the lowest concentration giving rise to 50%, 75% and 90% reduction in p24 content was calculated using XLfit4 software, • indicates >50 μg/ml.

TABLE 3. VHH kinetic constants and affinities for IIIB gp120[a].

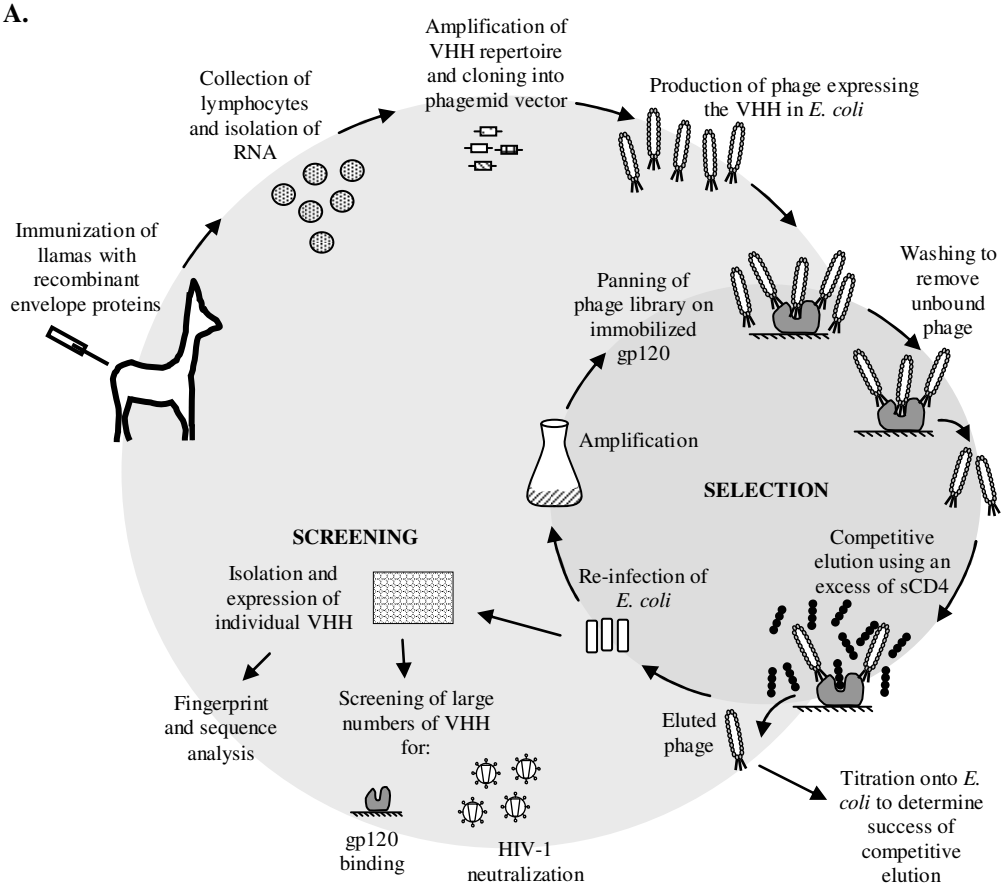
VHH	K_D (nM)	k_a (1/(Ms))	k_d (1/s)
A12	0.1	2.73×10^5	2.98×10^{-5}
D7	0.097	4.00×10^5	3.89×10^{-5}
C8	0.85	2.05×10^5	1.74×10^{-4}

[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 by surface plasmon resonance studies.

FIGURES

FIGURE 1

A.



B.

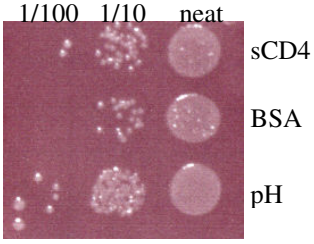


FIGURE 2

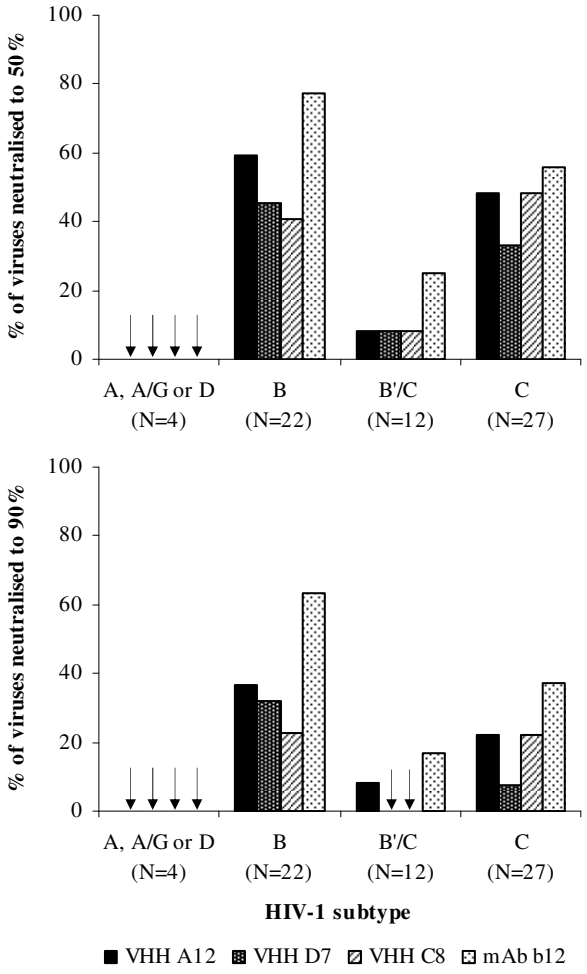
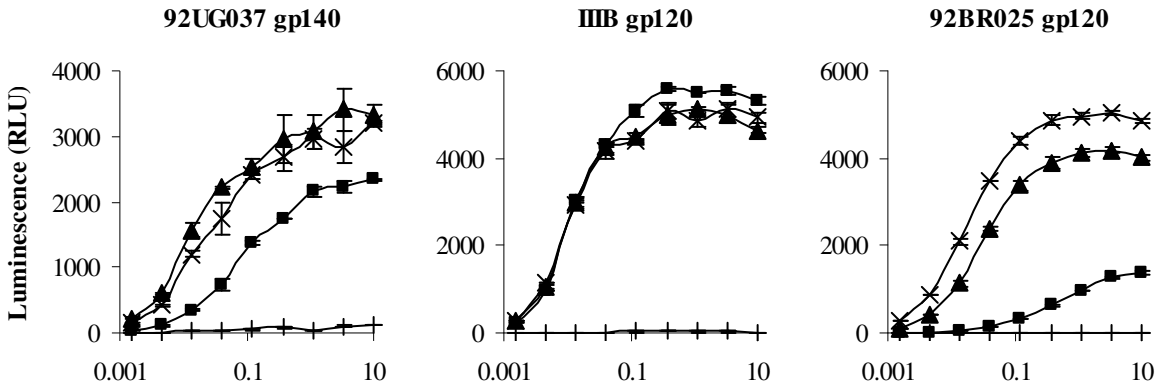

% of viruses neutralised to 50%
100
80
60
40
20
0
A, A/G or D (N=4)
B (N=22)
B'/C (N=12)
C (N=27)
% of viruses neutralised to 90%
100
80
60
40
20
0
A, A/G or D (N=4)
B (N=22)
B'/C (N=12)
C (N=27)
HIV-1 subtype
VHH A12
VHH D7
VHH C8
mAb b12

FIGURE 3

A. VHH binding to envelope proteins



B. VHH inhibition of sCD4 binding to envelope proteins

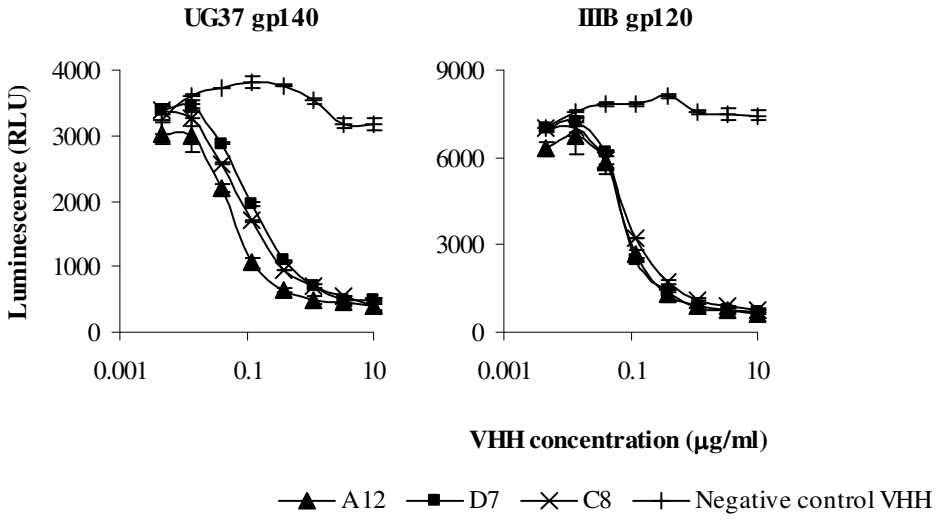
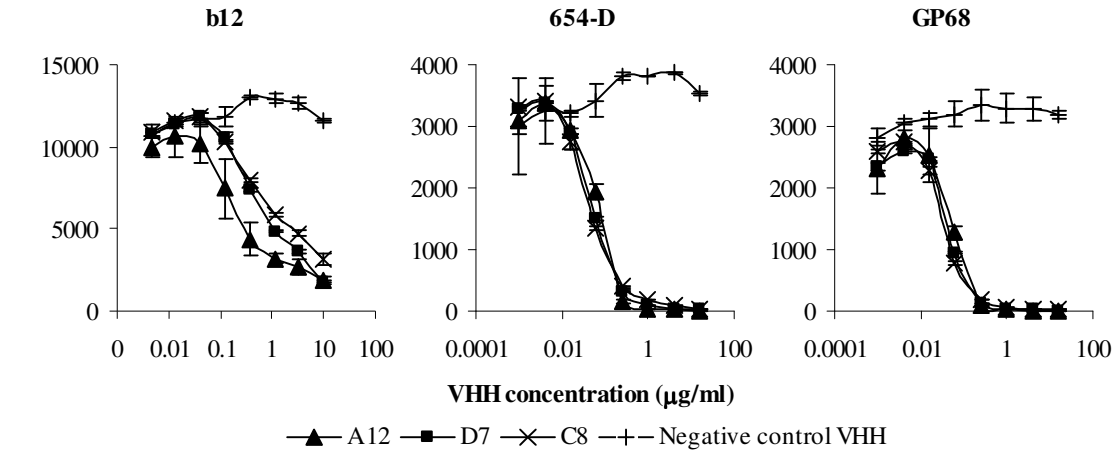
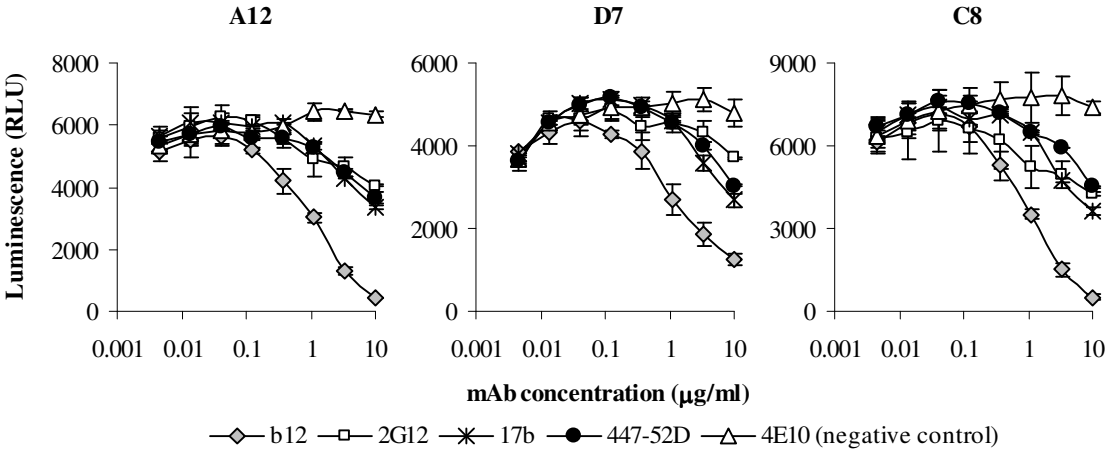


FIGURE 4

A. VHH inhibition of anti-CD4bs mAbs binding to gp120



B. Anti-gp120 mAb inhibition of VHH binding to gp120



C. VHH inhibition of each other binding to gp120