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Potent and broad neutralization of HIV-1 by a llama antibody elicited by immunization

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Llamas (*Lama glama*) naturally produce heavy chain–only antibodies (Abs) in addition to conventional Abs. The variable regions (VHH) in these heavy chain–only Abs demonstrate comparable affinity and specificity for antigens to conventional immunoglobulins despite their much smaller size. To date, immunizations in humans and animal models have yielded only Abs with limited ability to neutralize HIV-1. In this study, a VHH phagemid library generated from a llama that was multiply immunized with recombinant trimeric HIV-1 envelope proteins (Envs) was screened directly for HIV-1 neutralization. One VHH, L8CJ3 (J3), neutralized 96 of 100 tested HIV-1 strains, encompassing subtypes A, B, C, D, BC, AE, AG, AC, ACD, CD, and G. J3 also potently neutralized chimeric simian-HIV strains with HIV subtypes B and C Env. The sequence of J3 is highly divergent from previous anti–HIV-1 VHH and its own germline sequence. J3 achieves broad and potent neutralization of HIV-1 via interaction with the CD4-binding site of HIV-1 Env. This study may represent a new benchmark for immunogens to be included in B cell–based vaccines and supports the development of VHH as anti–HIV-1 microbicides.

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Abbreviations used: Ab, antibody; Fab, antigen-binding fragment; IC50, 50% inhibitory concentration; nAb, neutralizing Ab; NHP, nonhuman primate; RLU, relative light unit; sCD4, soluble CD4; SHIV, chimeric simian-HIV.

More than 30 yr ago, the first cases of AIDS were reported, followed 2 yr later by the identification of the causative agent of this disease, HIV. There were ~2.6 million new infections in 2009, of which 1.8 million occurred in sub-Saharan Africa where the majority of new infections continue to occur (http://www.unaids.org). The need for a safe and efficacious HIV preventive vaccine is as urgent as 30 yr ago and remains the best long-term strategy to prevent the transmission of HIV/AIDS (Cohen, 2008). In addition to the lack of a prophylactic vaccine, as yet, no strong correlate of protective immunity has been directly established. However, considering classical vaccinology and the principles underlying the partial immune control of HIV-1 infection, it has been postulated that an effective vaccine will need to produce coordinated B and T cell responses (Burton et al., 2004; Pantophlet and Burton, 2006; McElrath and Haynes, 2010). Notably, passive immunization with monoclonal antibodies (Abs [mAbs]) derived from HIV-1–infected individuals has demonstrated the potential for neutralizing Abs (nAbs) as a protective component of a vaccine-induced immunological memory response (Mascola et al., 1999; Shibata et al., 1999; Baba et al., 2000; Parren et al., 2001; Watkins et al., 2011). Consequently, demonstrating, as we do here, that immunization can induce Abs that can broadly neutralize HIV-1 is a major goal of HIV-1 vaccine development (Stamatatos et al., 2009; Mascola and Montefiori, 2010).

Potent nAbs, which neutralize up to 90% of virus strains tested, have been recently isolated (Walker et al., 2009, 2011; Burton and

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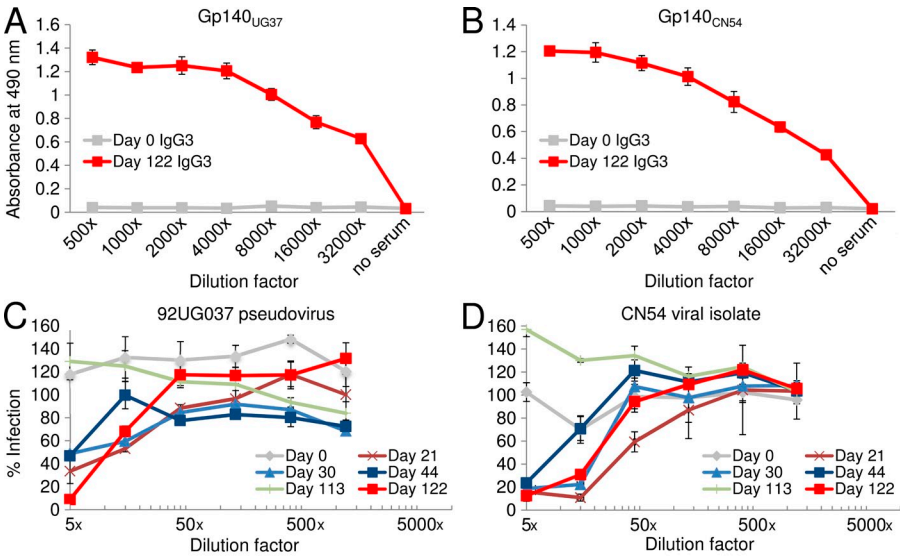
Weiss, 2010; Wu et al., 2010; Scheid et al., 2011) from patients, substantially raising the benchmark for nAbs elicited by immunization. Notably, many of these nAbs neutralize HIV-1 via interaction with the CD4-binding site of HIV-1 envelope protein (Env), and a considerable number of such nAbs have now been isolated from multiple donors (Corti et al., 2010), validating the CD4-binding site as target for protective Abs. Furthermore, the neutralization activity in many broadly neutralizing human sera is reported to be mediated by CD4-binding site–targeting Abs (Li et al., 2007). However, immunization in a variety of animal models has rarely produced any mAbs with potency or breadth comparable even with the long-established human nAbs such as b12 (Burton et al., 1991). Moreover, the potency and breadth of immunization-elicited nAbs (Zhang et al., 2007; Forsell et al., 2009) have not by any means approached the standard of the newly described human mAbs obtained during HIV-1 infection (Walker et al., 2009, 2011; Wu et al., 2010), although recently robust Env-specific memory B cell responses have been seen in immunized nonhuman primates (NHPs; Sundling et al., 2010). Neutralization with plasma samples was predominantly restricted to clade B tier 1 viruses with one representative from clades A and C neutralized and detectable and less potent neutralization against some tier 2 viruses (Sundling et al., 2010). It should be noted that antigen-binding fragments (Fabs) of CD4-induced epitope mAbs, such as 17b (Thali et al., 1993), exhibit the ability to neutralize viruses that the corresponding full-length IgG cannot (Moulard et al., 2002) and that it has been possible to induce 17b-like mAb after immunization (Vaine et al., 2010). However, the breadth of the 17b Fab was evaluated as neutralizing HIV-1 with comparable efficiency to b12 IgG (Moulard et al., 2002), which in contrast was less effective in Fab form (Moulard et al., 2002; Labrijn et al., 2003), as are the majority of Fabs compared with their corresponding whole Ab molecules (Ugolini et al., 1997).

The natural occurrence of llama heavy chain–only Abs (Hamers-Casterman et al., 1993) and the independent neutralization properties of their variable domains, i.e., the single-domain VHH, enabled screening of the VHH without the need for random recombination of heavy and light chains. Preliminary immunization of llamas with monomeric Gp120 previously led to the isolation of neutralizing VHH against HIV-1 (Forsman et al., 2008; Hinz et al., 2010; Koh et al., 2010). Given the limitations of the previous anti-HIV VHH, a new neutralization screening process was developed to isolate the extremely broad and potent VHH J3 described herein. The power of the screening process was increased by removing the biopanning stage, which favors VHH that bind to recombinant HIV-1 Env but does not distinguish between virus neutralizing and nonneutralizing binding properties. Consequently, in this study, the direct neutralization screening of a phagemid VHH library, generated from a llama immunized with two recombinant HIV Gp140 proteins, yielded a llama VHH that neutralizes 96% of HIV-1 isolates tested via interaction with a CD4-binding site epitope. This is a marked improvement on anti–HIV-1 mAbs or Ab fragments previously isolated after immunization, including but not limited to other anti–HIV-1 VHH, and demonstrates for the first time that a mammalian immune response to HIV-1 Env can elicit an Ab that as a VHH fragment is as broadly cross-reactive against a large panel of HIV-1 variants as the best full-length conventional human mAbs produced as a result of natural infection.

RESULTS

Llama immunization and phagemid library generation

A *Lama glama* (designated llama 8) was injected intramuscularly with a mixture of Gp140 trimers derived from a subtype BC HIV-1 strain, CN54 ($Gp140_{CN54}$), and a subtype A strain, 92UG037 ($Gp140_{UG37}$). Llama 8 was immunized in parallel with another llama, and the library generated from the other llama is described elsewhere (Strokappe et al., 2012). Previously, llamas immunized with monomeric HIV-1 proteins had been shown to produce anti–HIV-1 VHH with limited breadth and neutralization potency (Forsman et al., 2008; Hinz et al., 2010; Koh et al., 2010). In addition, the neutralization activity induced by human immunization trials (VAX04) with monomeric Env has been reported to be inferior to that induced by trimer immunization of NHPs (Sundling et al., 2010). Thus, this subsequent immunization schedule included trimeric Env with the aim that the functional glycoprotein spike of infectious HIV-1 would be more accurately mimicked and presented to the llama immune system. The protein immunogens were administered with Stimune adjuvant seven times as described in the immunization schedule depicted in Table S1. The llama immune response was satisfactory, as indicated by an increase in the ability of postimmunization sera, relative to preimmunization sera, to bind to the immunogen Env proteins $Gp140_{UG37}$ and $Gp140_{CN54}$ in ELISA (Fig. 1, A and B). Retrospectively, sera from llama 8 were assessed for neutralization abilities against pseudovirus with 92UG037 or the CN54 primary isolate. Neutralization against both viruses was seen with sera taken 122 d after immunization compared with sera taken on day 0 (Fig. 1, C and D). For the neutralization-sensitive viruses IIIB and 93MW956, appreciably higher ID50 values were seen with the day 122 sera compared with the ID50 values of the homologous viruses (Fig. 1 E). In contrast, the ID50 value obtained for Bal26 (tier 1b neutralization sensitivity) was only slightly higher than those calculated for the homologous viruses, and the ID50 for 96ZM956 (tier 2) was similar to the ID50 values for the homologous viruses (Fig. 1 E). Immune phagemid libraries were constructed using blood collected from llama 8 on day 122, and library construction followed. In brief, RNA was extracted from purified peripheral blood lymphocytes (Chomczynski and Sacchi, 2006) from postimmunization blood at day 122 and cDNA generated to enable the amplification of the conventional and heavy chain IgG repertoire. The heavy chain–only Ab cDNAs were separated by gel electrophoresis and used as a template in a nested PCR, which enabled the isolation of the VHH repertoire via the insertion of restriction sites. The resulting cDNA fragments were ligated



E

Virus:	92UG037	CN54	IIIB	Bal.26	93MW956.26	96ZM965.01
Subtype:	A	BC	B	B	C	C
Day 122 ID50:	1:11.5	1:12.5	1:169	1:45	1:>10000	1:77

Figure 1. Llama 8 immune response evaluation. (A and B) Serial dilutions of llama sera obtained on days 0 and 122 were incubated on ELISA plates preimmobilized with $Gp140_{UG37}$ (A) or $Gp140_{CN54}$ (B) recombinant ENV. Binding was assessed as described in Materials and methods. All samples were assayed in triplicate. (C and D) Sera taken on days 0 and 122 were preincubated with the 92UG037 pseudovirus (C) or CN54 viral isolate (D), and neutralization activity was assessed as described in Materials and methods. All samples were assayed in duplicate. Error bars represent standard deviation from the mean. (E) ID50 titers (µg/ml) for sera taken on day 122 against both homologous viruses and the indicated heterologous viruses. Experiments were repeated in duplicate and on two independent occasions, except for the heterologous virus neutralization assays which were performed once in duplicate.

into a phagemid vector for display on filamentous bacteriophage M13 (De Haard et al., 2005; Joosten et al., 2005) and electroporated in *Escherichia coli* TG1 cells. Rescue with helper phage VCS-M13 and polyethylene glycol precipitation was performed as described previously (Marks et al., 1991), and a phage stock containing 5×10^{11} pfu/ml was generated. The library from llama 8 had a diversity of $>10^7$ and VHH inserts in >90% of the phagemids.

Direct neutralization screening of the phagemid library 8

Previously, VHH from immunized llamas were isolated from phagemid libraries via sequential rounds of biopanning on immobilized proteins to enrich the libraries for VHH that bind specifically to the protein target under investigation. For example, the previously described anti–HIV-1 VHH (Forsman et al., 2008) were isolated from fractions of a phagemid library that had been previously enriched for the ability to bind to $Gp120_{IIIB}$ and then compete with soluble CD4 (sCD4) . However, it is well established that some anti–HIV-1 mAbs that can bind efficiently to recombinant Env do not neutralize functional virus and are thus termed non-nAbs (Mascola and Montefiori, 2010). Indeed, one of the means by which HIV-1 evades a protective human immune response is by eliciting the production of non-nAbs or strain-specific nAbs (Willey and Aasa-Chapman, 2008). Therefore, it was hypothesized that enriching the phagemid library for VHH that bind most efficiently could result in the preferential selection of strongly binding VHH over strongly neutralizing VHH and thus that a direct neutralization screen would be preferable to isolate broad and potently neutralizing VHH. This hypothesis was supported by the successful isolation of two broad and potent human mAbs, PG9 and PG16, via a high-throughput neutralization screen of human B cells (Walker et al., 2009). Consequently, a new screening method was developed whereby the VHH were assessed directly for their ability to neutralize HIV-1 pseudovirus without any prior selection for the ability to bind to Env or compete with characterized anti–HIV-1 mAb or sCD4.

The direct neutralization screening method involved robotically isolating 2,816 VHH clones plated out onto agar from the phagemid library into 384-well master plates. The VHH clones were expressed in 96-well plates and targeted to the periplasm of the *E. coli*. Subsequently, the VHH-containing periplasmic extract was harvested by freeze-thawing followed by filtration and assessed for the ability to neutralize the subtype A MS208.A1 and subtype C 93MW956.1 HIV-1 pseudoviruses in parallel via the TZM-bl cell-based assay, whereby infection by HIV-1 elicits the expression of firefly luciferase and can be quantitatively measured by light emission. Neutralization was defined as reduction in relative light units (RLUs) to <40% of non-HIV–specific VHH (Derdeyn et al., 2000; Wei et al., 2002; Li et al., 2005) and stratified as strong if the RLUs were reduced to <20% and intermediate if reduced to <40%. As both MS208.A1 and 93MW956.1 are classified as tier 1 pseudoviruses (Mascola et al., 2005), and thus relatively easy to neutralize, the VHH identified as neutralizing to both a strong and intermediate level in the initial screen were reexpressed and assessed for their ability to neutralize four tier 2/3 viruses: subtype C ZM214M.PL15, subtype B THRO4156.18, subtype A 92UG037, and CRF_AG (circulating recombinant form AG) T257-31. Individual sequences were obtained for 8 of the 2,816 VHH clones screened that were able to neutralize one or more of these tier 2 pseudoviruses. The level of antigen specificity in VHH that did not neutralize in this screen was not comprehensively investigated, although to date, 48 unique sequences

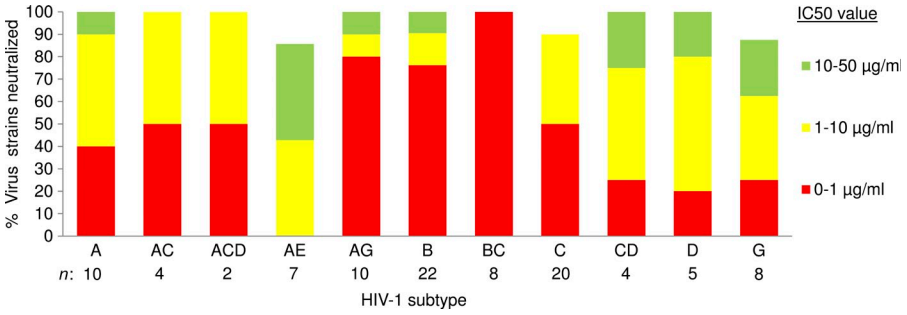


Figure 2. Breadth of J3 HIV-1 neutralization. Percentage of virus strains neutralized by J3 stratified by subtype as described in Materials and methods. To aid comprehension, the columns have been subdivided to show the percentage of strains from each subtype that were neutralized with IC50 values falling into the intervals indicated in the key. The neutralization activity of each VHH was assayed in duplicate or triplicate, and negative controls included mouse leukemia and rabies pseudoviruses. The majority of the panel of viruses were assayed once.

have been obtained from llama 8 via a combination of neutralization screening and panning selection. Interestingly, periplasmic extract containing one VHH, designated J3, neutralized five of the six screening viruses. Therefore, J3 was subcloned into an expression vector to allow improved protein production with a C-terminal His tag to enable purification via affinity chromatography and quantification of both Ab yield and neutralization potency.

J3 neutralizes 96% of HIV-1 strains tested

Initially,VHH J3 was titrated from a starting concentration of 50 µg/ml against the pseudoviruses used in the direct neutralization screen. For the first two strains tested, MS208.A1 and 93MW956.1, 50% inhibitory concentration (IC50) values were calculated as 6.5 µg/ml and <0.023 µg/ml, respectively (Table S2). Furthermore, all four pseudoviruses in the second screening panel were also neutralized to <50% of total virus (Table S2). Thus, J3 neutralized representatives from subtypes A, B, C, and CRF_02AG. Further neutralization assays undertaken in two laboratories revealed that J3 also neutralizes pseudoviruses, replication-competent molecular clones, and T cell–grown isolates from subtypes D, G, and CRF AE, AC, ACD, and BC (Table S2). Notably, among both the B (Keele et al., 2008) and C viruses tested are pseudoviruses derived from transmitter/founder isolates, which are all neutralized with IC50 values of <1 µg/ml and 2 µg/ml for subtypes B and C, respectively.

When the neutralization data are stratified by subtype, J3 exhibits 100% neutralization of all viruses from subtypes A, B, D, and CRF_02AG, AC, ACD, BC, and CD (Fig. 2 and Table S2). The median IC50 values are 2.77, 0.74, 3.09, 0.6, 0.99, 4.42, 0.32, and 2.34 µg/ml for these subtypes, respectively, and interestingly, all CRF_BC strains were neutralized with IC50 values of <1 µg/ml. Furthermore, J3 neutralized 90, 88, and 86% of viruses from the other three remaining subtypes C, G, and CRF_01AE, respectively. In total, J3 neutralized 96% of 100 strains tested (Fig. 2 and Table S2), significantly more than any other anti-HIV mAbs elicited after immunization in an animal model. This includes previously characterized anti-HIV VHH that are either limited in terms of breadth (Forsman et al., 2008; Hinz et al., 2010; Koh et al., 2010) and/or potency (Strokappe et al., 2012). The breadth of J3 neutralization compares favorably to the 78–93% neutralization seen with the recently isolated human mAbs that arose during HIV-1 infection (Walker et al., 2009, 2011; Burton and Weiss, 2010; Wu et al., 2010).

Neutralization data for a substantial subset of the viruses tested in this study have previously been reported for other human nAbs that target the CD4-binding site (Wu et al., 2010). Thus, the neutralization profile of J3 for this subset of 69 viruses was compared with that for VRC01/2, b12, and CD4-Ig (Wu et al., 2010), which were previously tested in the same laboratory (Table S3). Within this subset, neutralization (defined as an IC50 value <50 µg/ml) was observed for 88.4, 85.5, 47.8, 92.8, and 94.2% of viruses for VRCO1, VRC02, b12, CD4-Ig, and J3, respectively. VRCO1/2 and J3 neutralized all viruses from subtypes A, B, D, and BC, whereas b12 neutralized 33.3, 72.2, 80, and 60%, respectively, for these subtypes (Fig. 3). In addition, J3 neutralized all of the AG viruses, whereas VRCO1 neutralized 50% and b12 failed to neutralize any AG viruses (Fig. 3). Viruses from subtypes C, AE, and D were neutralized in a comparable manner by J3 and VRC01/2 and in both cases more effectively than by b12 (Fig. 3). However, it is important to note that these comparisons are based on IC50 values in microgram/milliliter and not molar values. Interestingly, there are two viruses (AE 620345.c1 and C Du172.17) out of the subset of 69 that both VRC01/2 and

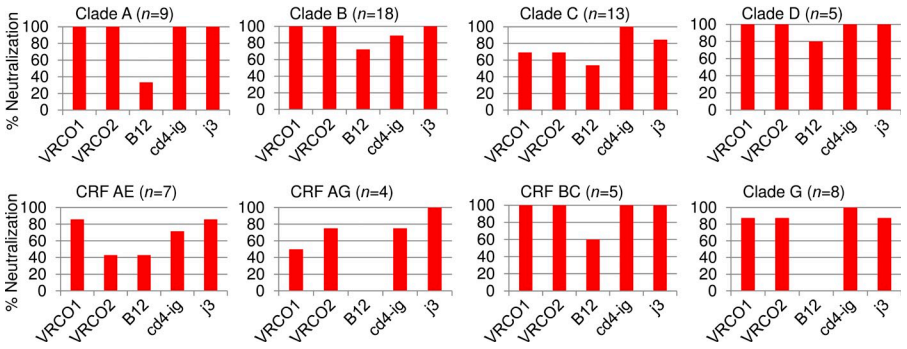


Figure 3. Comparison of breadth of neutralization achieved by VRCO1/2, b12, and CD4-Ig. Percent neutralization for the matched subset of viruses assayed against by J3, VRC01/2, b12, and CD4-Ig (using data published by Wu et al. [2010]) was stratified by clade.

J3 failed to neutralize (Table S3). Another clade C virus, CAP45.2.00.G3, was effectively neutralized by VRCO1/2 but not by J3, and within the subset of 69 viruses, there are 6 viruses that are resistant to VRCO1/2 but sensitive to J3. A total of four viruses in this study were resistant to neutralization by J3, and as described previously (Wu et al., 2010), two of these are also resistant to VRCO1/2-mediated neutralization, whereas the other two, X2160_c2 and 620345.c1, are susceptible. Unfortunately, viral sequence data for the latter have yet to be established, so sequence analysis of the other three resistant viruses was undertaken, and it was found that no single amino acid mutation is present in all three resistant viruses that is not present in susceptible viruses. However, in all three cases, there are multiple mutations relative to the sequences of closely related virus sequences. Therefore, it is hypothesized that multiple clusters of mutations contribute to the J3 resistance as has been described for naturally occurring viral resistance to VRCO1/2 (Li et al., 2011), albeit in a distinct way as indicated by the different susceptibilities of distinct viruses to neutralization by VRC01/2 or J3.

J3 neutralizes all chimeric simian-HIV (SHIV) strains tested

To further characterize J3 and to evaluate its potential as an anti-HIV microbicide, its ability to neutralize SHIV pseudoviruses was investigated. J3 was found to potently neutralize six SHIV pseudoviruses from subtypes B and C, with IC50 values all <0.5 μg/ml. The clade C SHIVs were in fact potently neutralized with IC50 values of <0.02 μg/ml. The strains assayed included one derived from SHIV1157IP EL-p, a clade C SHIV strain which has been used in recent mucosal challenges in NHPs (Humbert et al., 2008), and SHIV1157IPD3N4 (Table 1), a highly replication-competent, mucosally transmissible clade C R5 SHIV which rapidly induces abnormalities in immune parameters and could therefore be used to assess post–acute viremia levels as readout parameters of vaccine or microbicide efficacy (Song et al., 2006).

Table 1. SHIV neutralization by J3

SHIV pseudovirus	Clade	IC50 in TZM-bl cells
		J3 VHH
		μg/ml
SHIVsf162p4	B	0.0685
SHIVsf162p3	B	0.2154
SHIV89.6p	B	0.4215
SHIV89.6	B	0.1528
SHIV1157IPD3N4	C	0.01976
SHIV1157IP EL-p	C	0.006205

VHH J3 neutralization activity was assessed in the indicated SHIV pseudoviruses, as described in Materials and methods. The derivation of SHIV1157IPD3N4 and SHIV1157IP EL-p is described in detail in Humbert et al. (2008). The SHIV neutralization activity of J3 was assayed in duplicate on TZM-bl cells as described in Materials and methods, the subtype B SHIVs assays were undertaken once, and the subtype C SHIVs were assayed in two independent experiments.

Thus, the potent neutralization of these strains by J3 indicates this VHH is a suitable candidate for inclusion in microbicide formulations to be tested in NHP models.

J3 binds to immunogen HIV Env trimers and competes for the CD4-binding site

J3 binding specificities were examined in ELISA (see Materials and methods) where it bound to both the llama immunogen trimer proteins $Gp140_{CN54}$ and $Gp140_{UG37}$ (Fig. 4, A and B). Sigmoidal concentration–dependent binding was observed in a similar fashion to that seen with the previously isolated, but less broadly neutralizing VHH, A12 (Forsman et al., 2008; Hinz et al., 2010; Koh et al., 2010). However, the binding curves are not identical for both VHH as their interactions with Env are distinct from one another, indicating that their interactions with Env are qualitatively different as reflected in the strikingly superior neutralization profile of J3. In addition, J3 binds to monomeric $Gp120_{IIIB}$ derived from HIV-1 strain IIIB, demonstrating that it achieves such a wide breadth of neutralization by interacting with HIV-1 Env in a monomer- rather than trimer-specific fashion (Fig. 4 C). Thus, J3 binds to Env from subtypes A, B, and CRF_BC in a comparable way with the less broadly neutralizing VHH A12. However, the nature and consequences of the two distinct VHH binding to Env are clearly diverse as A12 binds to $Gp140_{UG37}$ but is unable to neutralize the 92UG037 HIV-1 pseudovirus. Therefore, the difference seen in neutralization breadth could not have been easily predicted by binding ability. This in turn confirms the rationale behind the direct neutralization screen undertaken, that VHH binding to recombinant Env is not functionally equivalent to HIV-1 neutralization as has already been established for human mAbs (Poignard et al., 2003).

To assess whether J3 binds, as does A12 (Forsman et al., 2008) to a CD4-binding site–related epitope, the interaction of J3 with a mutant Env derived from HIV-1 strain YU2 ($Gp120_{YU2}$) was evaluated compared with wild-type protein. The mutant Env contains a single residue mutation at position 368 of an aspartic acid to an arginine that abolishes CD4 binding (Li et al., 2007). The binding of J3 was evaluated by ELISA, which revealed that the D368R mutation in Env prevents J3 binding as compared with interaction seen with the wild-type Env (Fig. 4 D). Many CD4-binding site Abs have previously been characterized from infected patients (Corti et al., 2010; Wu et al., 2010; Zhou et al., 2010; Diskin et al., 2011; Scheid et al., 2011) and their epitopes characterized via structural and mutational methods, including the use of the D368R mutant Env in binding experiments. Thus, from this preliminary epitope analysis, it can be concluded that the J3 epitope is similarly affected by mutation of residue 368 as are VRCO1 and b12 but not HJ16, which can bind to the D368R Env (Corti et al., 2010; Wu et al., 2010). However, the observation that distinct viruses are resistant to neutralization by J3 from those that are resistant to the highly characterized broadly neutralizing VRC01 implies there is also variation in the residues precisely targeted by VRC01 and J3, and structural studies are underway to define the key contacts of J3 within the CD4-binding site.

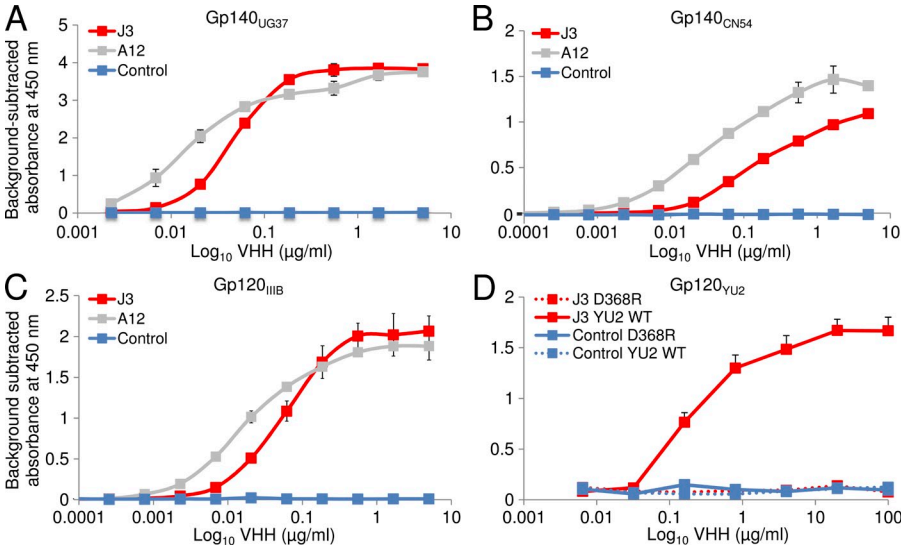


Figure 4. J3 binding to trimeric and monomeric Env in ELISA. (A and B) VHH binding to subtype A $Gp140_{UG37}$ (A) and CRF BC $Gp140_{CN54}$ (B) was assessed by ELISA. Recombinant Envs were immobilized, triplicate serial dilutions of VHH A12, J3, and a negative control VHH were added, and binding was detected as described in the Materials and methods. (C and D) Monomeric subtype B $Gp120_{IIIB}$ (C) and D368R or wild-type monomeric subtype B $Gp120_{YU2}$ (D). All error bars represent standard deviation from the mean. All data are representative of at least two independent experiments

Competition ELISA experiments further showed that J3 preincubation with Gp140 competed with sCD4 for binding to the HIV-1 Env (Fig. 5 A), as did the previously described VHH A12 (Forsman et al., 2008), in contrast to the negative control (an anti–HIV-1 VHH which was not specific for the CD4-binding site). In addition, preincubating J3 with the HIV-1 Env competed with the anti-CD4 human mAb b12, as did preincubation with the VHH A12, whereas the control VHH did not compete with b12 (Fig. 5 B). Furthermore, preincubation of A12 with HIV-1 Env competed with biotinylated J3 for binding to Gp140, although unlabeled J3 itself appeared to bind with a higher affinity than A12 and thus competed more efficiently with biotinylated J3. This implies that J3 and A12 bind partially overlapping epitopes within the CD4-binding site but that J3 probably has a higher affinity for its epitope within the CD4-binding site than does A12, whereas the control in this case, the Gp41-specific mAb 2F5, did not compete with biotinylated J3 binding (Fig. 5 C). Thus, J3 competes with both sCD4 and a previously characterized human mAb, b12 (Burton et al., 1994), indicating that J3 targets the CD4-binding site effectively to achieve its neutralization breadth across a wide range of HIV-1 subtypes by interacting with invariant residues that are conserved in Env. The precise nature of these interactions will be defined by structural studies.

Affinity maturation of J3 resulted in a shortened CDR2 (complementarity determining region 2)

Analysis of the amino acid sequence (Fig. 6 A) of J3 revealed it is part of a distinct phylogenetic family as compared with previously described anti–HIV-1 VHH (Fig. S1; Forsman et al., 2008; Hinz et al., 2010; Koh et al., 2010). The most striking aspect of the

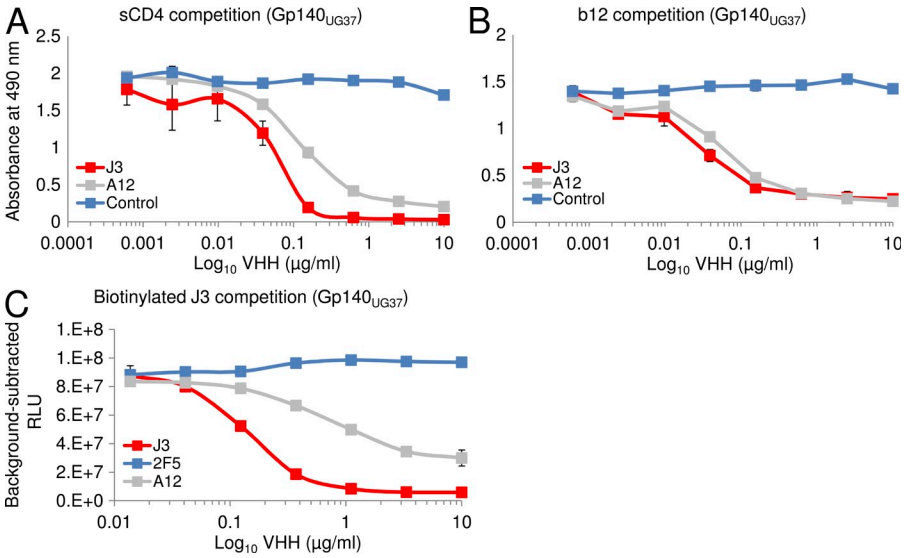


Figure 5. J3 competition for the CD4-binding site of HIV-1 Env in ELISA. (A) Dose-dependent competition of VHH A12, J3, and a negative control VHH with sCD4 for binding to $Gp140_{UG37}$. Recombinant Env was immobilized, and duplicate 10-fold serial dilutions of the indicated VHH were added, followed by 0.1 µg/ml sCD4. sCD4 binding to Env was detected as described in Materials and methods. (B) Dose-dependent competition of VHH A12, J3, and a negative control VHH with mAb b12 for binding to $Gp140_{UG37}$. Recombinant Env was immobilized, and duplicate 10-fold serial dilutions of the indicated VHH were added, followed by 1 µg/ml b12. Binding to Env was detected as described in Materials and methods. (C) Dose-dependent competition of VHH J3 and A12 with biotinylated J3 for binding to $Gp140_{UG37}$. Recombinant Env was immobilized, and triplicate fivefold serial dilutions of the indicated mAb or VHH were added (including a negative control mAb 2F5), followed by 0.5 µg/ml of biotinylated J3. Biotinylated J3 binding was detected as described in Materials and methods. All error bars represent standard deviation from the mean. All data are representative of at least two independent experiments.

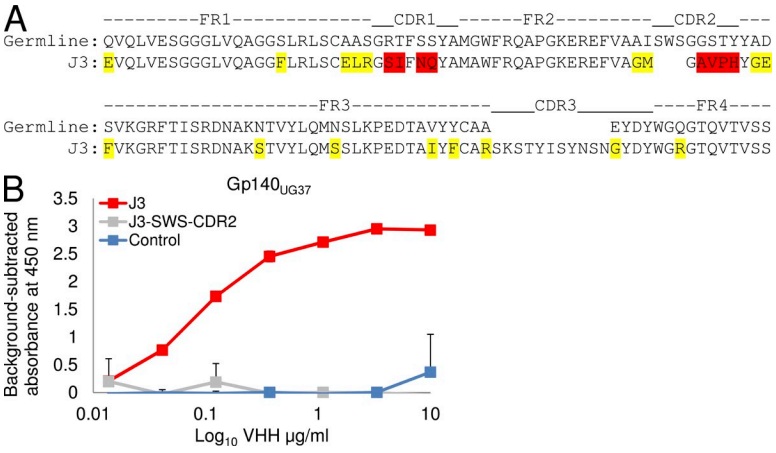


Figure 6. J3 amino acid and germline sequences. (A) J3 amino acid sequence as determined by purified pCAD51 A12 DNA and J3 germline determined based on sequence data (unpublished data) and DNA analysis of the area on which the V and J genes encoding VHH are located on the *L. glama* and *L. pacos* genomes. Residues that deviate from germline outside the CDRs are highlighted in yellow, and those inside CDR1 and CDR2 are highlighted in red. Note that the portion of the VHH sequence arising from the D genes (mapping to CDR3) is too small to enable alignment via ClustalW or BLAST with D genes from *L. glama* or *L. pacos*, and no significant association is found, thus the gap in the germline depicted. (B) VHH binding to subtype A $Gp140_{UG37}$ was assessed by ELISA. Recombinant Envs were immobilized, triplicate serial dilutions of mutant, wild-type J3, or a negative control VHH were added, and binding was detected as described in Materials and methods. All error bars represent standard deviation from the mean. All data are representative of at least two independent experiments.

sequence is a deletion in CDR2, reducing its size from 8 to 5 aa. Insertions and substitutions in both CDR2 and CDR3 of human anti–CD4-binding site mAbs have been shown to be of critical importance for potency by altering the interaction with the inner domain and bridging sheet of Env (Diskin et al., 2011). The deletion in the CDR2 of J3 may be required to enable the precise binding of this extremely broad neutralizing anti–HIV-1 VHH to its epitope, and a mutant J3 in which the corresponding three germline residues have been reinserted can no longer bind to Env (Fig. 6 B). The role of CDR2 in the J3–Env interaction will be further clarified by structural studies. Notably, such a deletion can be considered rare as a comparison of sequences of 1,014 different VHH showed only one CDR2 deletion of 3 aa (not depicted).

This deletion in CDR2 is the result of affinity maturation as it is not present in the germline sequence (Fig. 6 A) determined for J3 (determined using 23 V genes from *L. glama* and 7 J genes from *Lama pacos* [not depicted]). The 23 unique V genes encoding VHH (based on analysis of >180 clones) can be separated in several families based on the presence of the motifs KEREG, KQREL, and KEREF. J3 is derived from the family typified by the KEREF motif. Within this family, there are two very similar germlines, designated Vt and Vs. Both of these germline sequences encode the same amino acid sequence. However, aligning the J3 DNA sequence with that of both Vs and Vt germlines using both Blast and ClustalX programs shows Vt to be the most closely aligned germline to J3. However, it should be noted that in the absence of the sequencing of the full *L. glama* genome, it is possible that additional V genes exist that are more closely related than Vt to J3. A family-specific approach (Koh et al., 2010) will be used to isolate further VHH clones related to J3 and enable estimation of the frequency of J3-like clones in the library. However, there is some disparity between the low level of neutralization seen with the postimmune sera compared with the breadth and potency of J3. Given the previous isolation of multiple VHH from the same V gene germline, it is unlikely this disparity is caused by a rare germline precursor but rather suboptimal stimulation and proliferation of the J3-secreting B cell clone. In addition, no J3-inhibitory activity was present in the postimmune sera, which may have masked the J3 neutralization activity (not depicted). Based on the screening protocol used in this study, the frequency of J3-like clones is <0.04% of the VHH repertoire and 0.012% of the total Ab repertoire. Therefore, future immunogens aiming to elicit a J3-like response will need substantial improvement to increase the amount of specific stimulation.

In addition to the shortened CDR2, there are 25 separate sites where residues deviate from their germline sequence counterparts, including residues located both in the CDRs and in framework regions (Fig. 6 A). These residues have been altered during the maturation of the J3 heavy chain–only Ab and may play important roles in the function of the VHH. This hypermutation from the germline confirms that J3 was indeed the result of affinity maturation in response to an antigen that the llama immune system encountered, in this case the two subtype HIV-1 Env immunogens used in immunization. Analysis of nAbs isolated from HIV-1–infected patients has implied that a substantial degree of affinity maturation is necessary to produce effective neutralization (Burton et al., 1994; Walker et al., 2009; Scheid et al., 2011), and the divergence of J3 from its germline sequence is characteristic of such affinity maturation. Recently, a convergence of residues found in the heavy chains of patient-derived broadly neutralizing anti–HIV-1 mAbs has been reported (Scheid et al., 2011). To establish how similar llama heavy chain–only Abs are to human heavy chains, the nucleotide sequences of both J3 and A12 were compared with available human heavy chain sequences (Fig. 7). The human VH3-23*04 sequence shares 79.57% homology with the V gene segment of J3 and 84.38% homology with that of A12. Notably, this level of homology was not greatly different from the 85.60% homology that J3 shares with its precursor llama germline V gene sequence indicated in Fig. 6. Furthermore, the human JH*02 sequence shares 83.33% homology with the J gene segment of J3 and 78.72% homology with A12 (Fig. 7).

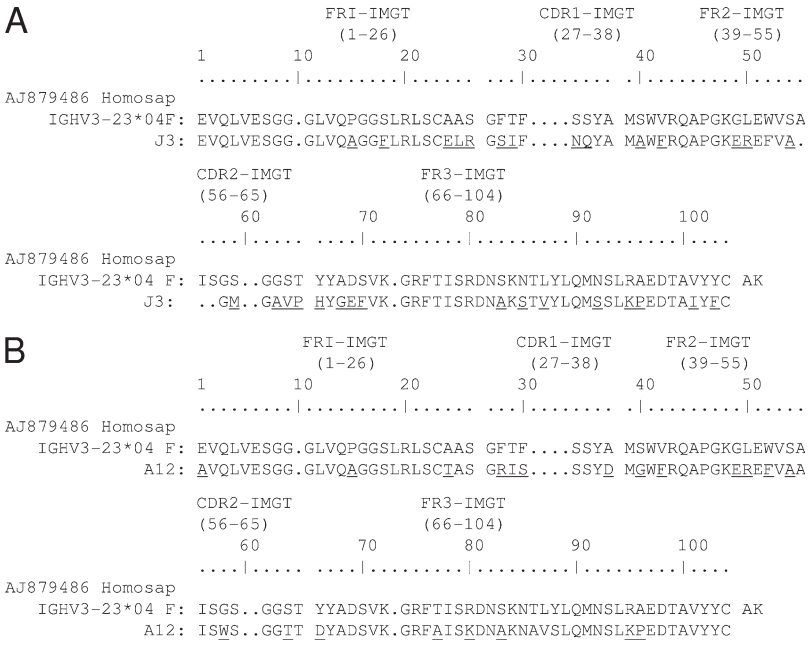


Figure 7. Phylogenetic relationship of heavy chain V genes from llamas and humans. (A) J3 and A12 sequences were aligned with human germline IgVH sequences through the VQUEST tool of IMGT (http://www.IMGT.org; GenBank/EMBL/DDBJ accession no. AJ879486). (B) J3 sequence was aligned with human germline IgVH sequences through the VQUEST tool of IMGT (GenBank accession no. AJ879486). Residues that differ in the VHH sequence as compared with the human germline are underlined.

DISCUSSION

The isolation of J3 represents a significant improvement on previous nAbs derived from immunized animals as in single-domain VHH form it has a comparable breadth and potency to the best nAbs obtained from a limited number of natural human infections. In contrast, previous nAb clones characterized from immunized animals have only exhibited limited breadth (Forsman et al., 2008; Sundling et al., 2010). A caveat to this is the observation that sera with 17b-like binding specificity can be induced after immunization of humans (Vaine et al., 2010), and it should be noted that 17b and other Abs to CD4-induced epitopes are less broadly neutralizing as full-length mAbs than in Fab form (Labrijn et al., 2003). However, given the previously reported decrease in neutralization ability seen with the Fab of b12 (Labrijn et al., 2003), it appears the CD4-binding site of Env is not per se more easily targeted for neutralization by small Ab fragments as is the CD4-induced binding site, presumably because of the required presence of the coreceptor interacting with Env in the latter case. As J3 neutralizes via interaction with the CD4-binding site, it is unlikely that its breadth is a function solely of the smaller size of VHH. Therefore, whether the breadth and potency of J3 are conserved in a full-length heavy chain–only llama Ab will be investigated. Thus, this study reports for the first time an nAb response after experimental immunization with a breadth equivalent to that seen in the best Ab responses in natural infection.

Llama 8 was multiply immunized with trimeric Gp140 Env derived from a subtype A and CRF BC viruses in the presence of the adjuvant Stimune. The resulting phagemid VHH library was screened via a selection method based on neutralization function rather than pre-enriching a polyclonal mixture of VHH for the ability to bind to recombinant HIV-1 Env and/or compete with nAbs. However, to isolate a VHH specific for a particular binding site on Env, the original biopanning method remains advantageous, as it enables targeted selection of VHH via enriching for those VHH that compete with a known nAb to a specific region. As Env glycoproteins were used to elicit J3 and given that patient-derived nAbs have been shown to provide protection from infection when passively infused into NHPs (Mascola et al., 1999, 2000; Burton et al., 2011; Watkins et al., 2011), these recombinant immunogens represent valid vaccine candidates for HIV. However, given the weak level of serum neutralization observed, they will require further modification to induce high titer J3-like broad cross-neutralizing serum responses.

J3 neutralizes 96% of all strains tested in this study, which included 100 representatives from a wide range of subtypes and CRFs. In fact, J3 neutralizes 100% of subtype A, B, D, and CRF AC, ACD, AG, BC, and CD and >85% of the remaining subtypes tested, C, G, and CRF AE. In an analysis of viruses previously tested against VRCO1/2 and b12, J3 neutralized 94.2% of viruses (this study) compared with VRCO1, which neutralized 88.4% of the subset of 69 viruses (Wu et al., 2010). Sensitivity to the full-length human nAb and the llama VHH were similarly distributed across different subtypes in this subset. However, at the level of individual viruses, resistance to VRCO1 did not confer resistance to J3 and vice versa. No clear pattern of residues associated with J3 resistance was discernible from analysis of the available sequences for viruses that were not neutralized by J3. Although the evaluation of J3 against additional viruses may provide further insight into key residues for J3-mediated neutralization, it may be more thorough to evaluate the neutralization sensitivity of a panel of engineered viral mutants to define the functional J3 epitope in combination with escape mutant studies.

J3 binds to both the trimeric immunogens that llama 8 received as well as monomeric $Gp120_{IIIB}$ and $Gp120_{YU2}$. J3 targets the binding site of Env for the HIV-1 cellular receptor CD4 as confirmed by the loss of binding seen with the CD4-binding site D368R $Gp120_{YU2}$ mutant relative to wild-type $Gp120_{YU2}$. In addition, J3 competes with sCD4 itself as well as with the well-characterized human mAb b12. Competition experiments with A12, a previously isolated anti–CD4-binding site VHH, showed that binding assays, while demonstrating a

level of difference between the two VHH, were not able to detect the disparity between these two VHH in terms of neutralization ability. Together with the level of neutralization breadth observed, these findings indicate that J3 recognizes part of the Env CD4-binding site and very few if any of the adjacent amino acids, although this remains to be defined in detail.

This study describes the isolation of an extremely broad and potent HIV-1–neutralizing VHH, J3, from a screen of almost 3,000 clones. This method provides a relatively quick screening process compared, for example, with the 30,000 human B cell clones screened to isolate the PG9 and PG16 nAbs (Walker et al., 2009). Thus, this method could be used alongside testing the immune sera for neutralization to evaluate new HIV-1 immunogens in the established llama model, accelerated by the advantage of heavy chain–only Abs that they do not require heavy and light chain random recombination. Furthermore, the independently functional nature of the VHH Ab fragments (Vanlandschoot et al., 2011) coupled with their intrinsic stability (Gorlani et al., 2012) makes them suitable candidates for anti–HIV-1 microbicide development. Thus, broad and potent neutralizing VHH isolated in this way, such as J3, can both aid progress toward an HIV-1 vaccine and simultaneously provide a useful reagent for anti-HIV therapeutics and/or prophylaxis.

Recently, the precedent for an anti–HIV-1 microbicide has been established by the successful phase III trial of an antiretroviral-containing gel (Abdool Karim et al., 2010). Furthermore, immunological microbicides, such as mAbgel (Brinckmann et al., 2011), are under investigation. A VHH with the breadth of J3 has potential as the active component of such a microbicide because of the intrinsic temperature and pH stability of VHH (Gorlani et al., 2012) and the ease and low cost with which they can be manufactured as compared with full-length human nAbs. In addition, J3 neutralizes a range of SHIV strains as well, making it an ideal candidate for SHIV mucosal challenge experiments either via passive immunization or in a microbicide formulation. The potential use of microbicides is not mutually exclusive with that of a vaccine, as the two could be complementary in a situation whereby an effective microbicide could limit the probability infection and thus support a vaccine-induced immune response.

In conclusion, the isolation of such a potent and broad VHH fragment from an animal immunized with a relatively simple combination of recombinant protein immunogens argues for the potential of vaccination to elicit cross-reactive protective anti-HIV Abs, and further study of this VHH will provide insight into how to recapitulate the elicitation of such Abs, hopefully in conventional IgG format. VHH J3 neutralizes 96% of all strains tested to date, and these include a great variety of HIV-1 subtypes. This highly cross-reactive anti–HIV-1 VHH differs from previous anti–HIV-1 VHH not only in its breadth but in terms of the immunization procedure undertaken, the screening method by which it was isolated and its phylogenetically distinct and affinity-matured sequence. The breadth of neutralization achieved via targeting the CD4-binding site demonstrated by J3 has only previously been seen as a result of natural infection (Corti et al., 2010; Wu et al., 2010; Scheid et al., 2011). The definition of the precise CD4-binding site epitope targeted by J3 will be clarified by structural studies and may be used to optimize future immunogens, which can be easily and efficiently evaluated using this redefined screening process for llama VHH libraries.

MATERIALS AND METHODS

mAbs. mAb b12 (Burton et al., 1991) and 2F5 (Buchacher et al., 1994) were obtained through the Centralized Facility for AIDS Reagents (CFAR), National Institute for Biological Standards and Controls (NIBSC), supported by the EC FP6/7 EUROPRISE Network of Excellence, Next Generation HIV-1 Immunogens inducing broadly reactive Neutralising antibodies (NGIN) Consortium, and the Bill and Melinda Gates Global HIV Vaccine Research Cryorepository–Collaboration for Aids Vaccine Discovery (GHRC-CAVD) Project, and were donated by D. Burton (The Scripps Research Institute, La Jolla, CA) and H. Katinger (Polymun Scientific, Vienna, Austria), respectively.

Recombinant HIV-1 Env proteins. Recombinant trimeric Gp140 from HIV-1 92UG037 (subtype A) for immunizations and ELISAs was provided by S. Jeffs (Imperial College London, London, England, UK). Recombinant trimeric Gp140 derived from HIV-1 CN54 for immunizations was provided by Polymun Scientific. Recombinant D368R and wild-type monomeric Gp120 from HIV-1 YU2 (subtype B) for ELISAs were provided by J. Mascola (National Institutes of Health [NIH], Bethesda, MD). Recombinant Gp120 from HIV-1 IIIB (EVA657) and recombinant trimeric Gp140 from HIV-1 CN54 (ARP699) for ELISAs were obtained from the CFAR, NIBSC and were donated by Immunodiagnostics and Polymun Scientific, respectively.

Viruses. HIV-1 IIIB (ARP101) and YU2 were obtained from the CFAR, NIBSC. IIIB was donated by R. Gallo and M. Popovic (University of Maryland School of Medicine, Baltimore, MD). HIV-1 CRF07_BC primary isolate CN54 was obtained from EMPRO. HIV-1 IIIB was propagated in H9 cells, and CN54 was propagated in peripheral blood mononuclear cells. All other replication-competent virus stocks were prepared from HIV-1 molecular clones by transfection of 293T cells. The 92BR025.C1 (C111) molecular clone was obtained by amplifying Gp120 from cells infected with the WHO panel peripheral blood mononuclear cell–grown isolate and inserting it into the pHXB2 backbone (Forsman et al., 2008). HIV-1 Env pseudotyped viruses were produced in 293T cells by cotransfection with the pSG3Δ*env* plasmid (Kirchherr et al., 2007). The subtype B and C HIV-1 Reference Panels of Env Clones (Li et al., 2005, 2006) were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), NIH. HIV-1 subtype CRF07_BC Gp160 clones, subtype CRF02_AG Gp160 clones (263-8, T278-50, and T266-60), and the 92UG037, 93MW965.26, and 96ZM651.02 Gp160 clones were provided by D. Montefiori (Duke University Medical Center, Durham, NC) through the Comprehensive Antibody Vaccine Immune Monitoring Consortium (CA2 VIMC) as part of the CAVD. All additional pseudoviruses were produced at the CAVD Preclinical Neutralizing Antibody Core laboratory. Virus CVS-11 pseudotyped with rabies virus G protein (Wright et al., 2008) was provided by E. Wright (University of Westminster, London, England, UK).

Cells. TZM-bl cells (Derdeyn et al., 2000; Wei et al., 2002; Li et al., 2005) were obtained through the NIH AIDS Research and Reference Reagent Program from J.C. Kappes (University of Alabama at Birmingham, Birmingham, AL), X. Wu (NIAID, NIH), and Tranzyme, Inc. and cultured in Dulbecco's modified Eagle medium (Invitrogen) containing 10% (vol/vol) FCS.

Immunization of *L. glama* and construction of the VHH phage library. Llama 8 was immunized with recombinant Gp140 derived from HIV-1 CN54 and 92UG037. Immunizations and VHH library construction were performed as described previously (De Haard et al., 2005). In brief, the llamas received seven intramuscular injections as per Table S1. Each injection consisted of a freshly prepared 4.5-ml water in oil emulsion prepared by vigorously mixing 2 vol U of antigen (50 or 100 μg) with 2.5 vol U of the adjuvant Stimune (CEDI Diagnostics). This study was performed in strict accordance with the Dutch Experiments on Animals Act 1997. In accordance with article 18 of the Act, the protocol was assessed and approved by the Animal Ethics Committee of Utrecht University (permit number: DEC#2007.III.01.013). All efforts were made to minimize discomfort related to immunizations and blood sampling. The animal welfare officers of Utrecht University checked the mandatory administration and supervised the proper conduct of procedures and the well-being of the llamas that were used. The anti-Env immune response in sera was verified in an ELISA against immobilized recombinant $Gp120_{IIIB}$. Total RNA was isolated from peripheral blood lymphocytes collected after immunization (on day 122), and cDNA was prepared. The VHH repertoire was amplified and cloned into the pCAD50 phagemid vector. To obtain recombinant bacteriophages expressing the VHH as fusion proteins with the M13 bacteriophage gene III 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.

Isolation of anti–HIV-1 VHH through direct HIV-1 neutralization screening. Phages expressing the cloned VHH repertoire were plated onto agar containing 100 μg/ml ampicillin and 2% syncytial stain (1 g methylene blue and 0.33 g basic fuchsin in 200 ml methanol). Individual clones were picked using a Norgren CP7200 colony picker (RapidPick; Hudson Robotics) into 384-well master plates. 2,816 individual clones were expressed in TG1 *E. coli* cells in a 96-well plate format. Each clone was expressed in 1 ml of 2× TY medium containing 100 μg/ml ampicillin and 0.1% glucose, followed by induction of VHH production with 0.1 mM isopropyl-β-D-thiogalactopyranoside. Bacterial pellets were frozen at −80°C for a minimum of 1 h and then thawed and resuspended in PBS. The periplasmic extract from each well was separated from bacterial debris by filtration through a 0.2-μM polyvinylidene fluoride membrane and screened for the ability to neutralize HIV-1. To enable semi–high-throughput screening and characterization of VHH, neutralization was measured using 200 50% tissue culture infective doses of virus in the TZM-bl cell-based assay developed by Derdeyn et al. (2000), Wei et al. (2002), and Li et al. (2005), with Bright-Glo luciferase reagent (Promega) using a Glomax plate reader (Promega). DNA from the individual VHH that neutralized all viruses to <20% seen with control was purified, sequenced, and recloned into the pCAD51 expression vector followed by transformation into TG1 cells for purification and further characterization. The HIV-1 neutralization potencies of llama 8 sera were also evaluated in TZM-bl cells as described above. Serum samples were heat-inactivated to destroy complement by incubation at 56°C for 1 h before use in neutralization assays. Threefold serial dilutions of llama sera were then tested, starting at a 1:5 dilution.

VHH purification and neutralization profiling. Expression from the pCAD51 vector incorporates a 6-His and a c-Myc tag to the C terminus of the VHH and removes the bacteriophage gene III product. The VHH were purified by means of the attached His tag using TALON Metal Affinity Resin (Takara Bio Inc.). The neutralization activity of the VHH was assayed in duplicate/triplicate at either University College London or VIMC laboratories. No virus inactivation was observed with a negative control VHH or with a pseudovirus bearing a rabies virus G-protein Env or mouse leukemia virus Env. VHH IC50 titers were calculated using the XLFit4 software (UCL-ID Business Solutions) or the Labkey Neutralizing Antibody Tool (Piehler et al., 2011).

SHIV. Molecularly cloned SHIV 89.6p and HIV-1 89.6 were obtained from J. Sodroski (Dana-Farber Cancer Institute, Boston, MA) through the NIH AIDS Research and Reference Reagent Program. SHIV1157ipEL-p and a molecular clone of SHIV1157ipd3N4 were provided by R. Ruprecht (Dana-Farber Cancer Institute), and SHIVSF162p3 was a gift of C. Cheng-Mayer (Aaron Diamond AIDS Research Center, New York, NY). SHIVSF162P4 was obtained from the Division of AIDS, NIAID, NIH.

Pseudotyped SHIV viruses were prepared by E.J. Verschoor and Z. Fagrouch (Biomedical Primate Research Centre [BPRC], Rijswijk, Netherlands) essentially as described by Wei et al. (2002). In short, the full-length *env* genes were amplified from molecularly cloned viruses or from viral RNA, and the PCR products were cloned into the expression plasmid pcDNA3.1 (Life Technologies). Individual clones were sequenced and selected for their suitability to produce pseudoviruses in a small-scale infection assay on TZM-bl indicator cells before performing neutralization assays (Montefiori, 2005). For this purpose, small stocks of pseudotyped viruses were prepared by transfection of 293T cells with a mixture of the pcDNA-*env* plasmid and the pSG3Δ*env* plasmid, which contains an Env-deficient molecular clone of HIV-1 SG3 (Kirchherr et al., 2007). After incubation, cell-free virus stocks were produced by low-speed centrifugation, followed by filtration through a 45-μm filter, and used to infect TZM-bl cells. Viruses that induced luciferase activity were selected for the pseudovirus neutralization assay.

The neutralization activity of the VHH was assayed in duplicate at the BPRC laboratory in the TZM-bl cell-based assay developed by Derdeyn et al. (2000), Wei et al. (2002), and Li et al. (2005), containing 15 μg/ml DEAE-Dextran, and assayed with Britelite Plus Reagent (PerkinElmer) according to manufacturer's instructions using a Victor light plate reader (PerkinElmer). VHH IC50 titers were calculated using the Luc5Samples02-NotProtected.xls program (courtesy of D. Montefiori).

ELISAs. Clear 96-well MaxiSorp plates (Thermo Fisher Scientific) were coated overnight with 2 μg/ml HIV-1 $Gp120_{IIIB}$ or $Gp140_{UG37}$. Plates were blocked using 5% milk powder in TBS. Serial dilutions in TBS supplemented with 0.05% Tween (TBS-T) containing 1% milk powder (TMT) of the VHH to be assayed and of a negative control VHH were then added to the plates in triplicate wells, and the plates were incubated at room temperature for 1 h and subsequently washed four times with TBS-T. The wells were then incubated with 0.5 μg/ml mouse anti–c-Myc–horseradish peroxidase–conjugated Ab (Roche) in TMT for 1 h at room temperature. After six washes with TBS-T, TMB ELISA substrate (Thermo Fisher Scientific) was added, and the plates were incubated at 37°C for 0.5 h. Absorbance at 450 nm was detected, and background-subtracted data were plotted against VHH concentration.

Immune response ELISAs. 50 μl of 2 μg/ml $Gp140_{CN54}$ and $Gp140_{UG37}$ was coated on MaxiSorp plates overnight at 4°C. After blocking with 4% milk powder in PBS, serial dilutions of preimmune serum (day 0) and immune plasma (day 122) were incubated for 1 h in triplicate. The serum and plasma were diluted in 1% milk powder in PBS by serial dilution from 500- to 32,000-fold, with twofold dilution steps in between. Detection of bound llama single chain Abs was performed by incubation with mAb 8E1 followed by an incubation with peroxidase-conjugated donkey anti–mouse Ig (1:5,000 in 1% milk powder in PBS). Anti–llama IgG3 mAb 8E1 was provided by BAC BV. 50 μl *O*-phenylenediamine containing 0.03% H_2O_2 was added to the wells. After ~5 min, the reaction was stopped with 1 M H_2SO_4. The absorbance of the reaction was measured at 490 nm.

Competition ELISAs. Clear 96-well MaxiSorp plates were coated overnight with 2 μg/ml $Gp140_{UG37}$ and washed three times with PBS with 0.05% Tween (PBS-T). Plates were blocked using 4% milk powder in PBS and then washed three times in PBS-T. Serial dilutions in PBS-T supplemented with 1% milk powder (PBS-M) of the VHH to be assayed and of a negative control VHH were then added to the plates in duplicate/triplicate wells as indicated in the figure legends, and the plates were incubated at room temperature for 1 h. The wells were then incubated with either 1 μg/ml b12 or 0.1 μg/ml sCD4 in PBS-M for 1 h at room temperature. After three washes with PBS-T, the wells were incubated with either 1:5,000 goat anti–human peroxidase or 1:10,000 L120 in PBS-M for the b12 and sCD4 wells,

respectively, and incubated at room temperature for 1 h. After three washes, the sCD4 wells were incubated for an additional hour with 1:5,000 and donkey anti–mouse peroxidase in PBS-M at room temperature. Binding was detected for both b12 (Fig. 5 C) and sCD4 (Fig. 5 A) competition assays by washing the wells six times with PBS-T and adding *O*-phenylenediamine supplemented with 0.03% hydrogen peroxide for 45 min at room temperature. Absorbance at 490 nm was detected, and background-subtracted data were plotted against VHH concentration.

Alternatively, white 96-well MaxiSorp plates were coated overnight with 1 μg/ml HIV-1 $Gp140_{UG37}$ (Fig. 5 B). Plates were blocked using 5% milk powder in TBS. Serial dilutions in TMT of the mAb/VHH to be assayed and of a negative control mAb/VHH were then added to the plates in triplicate wells, and the plates were incubated at room temperature for 1 h and subsequently washed four times with 0.05% TBS-T. The wells were then incubated with 0.5 μg/ml of biotinylated VHH J3 in TMT for 1 h at room temperature. J3 was prebiotinylated using an EZ-Link Micro Sulfo-NHS-Biotinylation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. After four washes with TBS-T, conjugated streptavidin alkaline phosphatase was added at 0.125 μg/ml, and the plates were incubated at room temperature for 1 h. After six washes with TBS-T, Lumi-phos substrate (Thermo Fisher Scientific) was added, and the plates were incubated at 37°C for 0.5 h. RLUs were detected, and background-subtracted data were plotted against VHH concentration.

Online supplemental material. Fig. S1 illustrates the phylogenetic relationship of the J3 and A12 VHH families. Table S1 shows the llama immunization protocol with HIV-1 Env. Table S2, included as an Excel file, contains the individual IC50 (μg/ml) titers obtained for J3 against 100 viruses. Table S3, included as an Excel file, reiterates the IC50 titers (μg/ml) for VRC01/2, b12, and CD4-Ig as published previously (Wu et al., 2010) for a subset of 69 matched viruses against which J3 was also tested. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20112655/DC1.

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