Available online at www.sciencedirect.com
ScienceDirect
Virology 363 (2007) 79–90
VIROLOGY
www.elsevier.com/locate/yviro

Cross-reactive HIV-1 neutralizing monoclonal antibodies selected by screening of an immune human phage library against an envelope glycoprotein (gp140) isolated from a patient (R2) with broadly HIV-1 neutralizing antibodies

Vidita Choudhry [a,1], Mei-Yun Zhang [a,b,1], Igor A. Sidorov [a], John M. Louis [c], Ilia Harris [d], Antony S. Dimitrov [d], Peter Bouma [e], Fatim Cham [e], Anil Choudhary [e], Susanna M. Rybak [f], Timothy Fouts [d], David C. Montefiori [g], Christopher C. Broder [e], Gerald V. Quinnan Jr. [e], Dimiter S. Dimitrov [a,*]

[a] *Protein Interactions Group, CCRNP, NCI-Frederick, NIH, Frederick, MD 21702, USA*
[b] *BRP, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD 21702, USA*
[c] *Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD 20892, USA*
[d] *Profectus BioSciences, Inc., 1450 South Rolling Road, Baltimore, MD 21227, USA*
[e] *Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA*
[f] *Biological Testing Branch, National Cancer Institute-Frederick, NIH, Frederick, MD 21702, USA*
[g] *Department of Surgery, Laboratory for AIDS Vaccine Research and Development, Duke University Medical Center, Durham, NC 27710, USA*

Received 23 September 2006; returned to author for revision 8 October 2006; accepted 12 January 2007
Available online 15 February 2007

Abstract

Elicitation of broadly cross-reactive neutralizing antibodies (bcnAbs) in HIV infections is rare. To test the hypothesis that such antibodies could be elicited by HIV envelope glycoproteins (Envs) with unusual immunogenic properties and to identify novel bcnAbs, we used a soluble Env ectodomain (gp140) from a donor (R2) with high level of bcnAbs as an antigen for panning of an immune phage-displayed antibody library. The panning with the R2 Env resulted in significantly higher number of cross-reactive antibody clones than by using Envs from two other isolates (89.6 and IIIB). Two of the identified human monoclonal antibodies (hmAbs), m22 and m24, had sequences, neutralizing and binding activities similar or identical to those of the gp120-specific bcnAbs m18 and m14. The use of the R2 Env but not other Envs for panning resulted in the identification of a novel gp41-specific hmAb, m46. For several of the tested HIV-1 primary isolates its potency on molar basis was comparable to that of T20. It inhibited entry of primary isolates from different clades with an increased activity for cell lines with low CCR5 surface concentrations. The m46 neutralizing activity against a panel of clade C isolates was significantly higher in an assay based on peripheral blood mononuclear cells (4 out of 5 isolates were neutralized with an IC_{50} in the range from 1.5 to 25 μg/ml) than in an assay based on a cell line with relatively high concentration of cell-surface-associated CCR5. In contrast to 2F5 and Z13, this antibody did not bind to denatured gp140 and gp41-derived peptides indicating a conformational nature of its epitope. It bound to a 5-helix bundle but not to N-heptad repeat coiled coils and a 6-helix bundle construct indicating contribution of both gp41 heptad repeats to its epitope and to a possible mechanism of neutralization. These results indicate that the R2 Env may contain unique exposed conserved epitopes that could contribute to its ability to elicit broadly cross-reactive antibodies in animals and humans; the newly identified antibodies may help in the development of novel vaccine immunogens and therapeutics.

Keywords: HIV; Antibody; Phage display; gp140; gp41; Inhibitors; Vaccines

* Corresponding author. CCRNP, CCR, NCI-Frederick, NIH, Bldg. 469, Rm 105, PO Box B, Miller Drive, Frederick, MD 21702-1201, USA. Fax: +1 301 846 5598.
E-mail address: dimitrov@ncifcrf.gov (D.S. Dimitrov).
[1] These authors contributed equally.

0042-6822/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2007.01.015

Introduction

Elicitation of broadly cross-reactive HIV-1 neutralizing antibodies (bcnAbs) *in vivo* is rare (Burton and Montefiori, 1997) likely due to protection of conserved structures of the virus envelope glycoprotein (Env) by variable loops, extensive glycosylation, occlusion within the oligomer, and conformational masking, and the rapid generation of mutants that outpace the development of such antibodies (Poignard et al., 2001; Johnson and Desrosiers, 2002; Burton, 2002; Wei et al., 2003; Richman et al., 2003; Garber et al., 2004). A number of Env-specific hmAbs have been identified (Zolla-Pazner, 2004) but only several exhibited neutralizing activity to primary isolates from different clades (Ferrantelli and Ruprecht, 2002; Burton, 2002) including IgG b12 (Roben et al., 1994; Burton et al., 1994), IgG 2G12 (Trkola et al., 1996; Scanlan et al., 2002; Sanders et al., 2002), m14 (Zhang et al., 2004), m18 (Zhang et al., 2003), 447-52D (Gorny et al., 1992), IgG 2F5 (Muster et al., 1993), IgG 4E10 (Stiegler et al., 2001; Zwick et al., 2001), Fab X5 (Moulard et al., 2002) and Fab Z13 (Zwick et al., 2001). Identification and characterization of novel bcnAbs may provide additional insights into the closely guarded conserved structures that could serve as epitopes for neutralizing antibodies, as well as for understanding mechanisms of HIV entry and evasion of immune responses, and for development of vaccines or entry inhibitors.

Recently, it has been proposed that individuals possessing bcnAbs were infected with viruses encoding Envs with unusual immunogenic properties (Cham et al., 2006). We have hypothesized that mimicking immune responses by using such Envs as selecting antigens for screening of immune human antibody libraries could not only further test this proposition but also may lead to identification of novel bcnAbs with implications for development of vaccine immunogens, inhibitors and research tools.

The clade B, R2 Env was isolated from a donor (R2) with long-term nonprogressive HIV-1 infection and high level of bcnAbs (Vujcic and Quinnan, 1995; Quinnan et al., 1999; Zhang et al., 2002). It has been shown to mediate CD4-independent HIV-1 entry into cells and utilizes CCR5 but not CXCR4 as coreceptor. Immunization of small animals and macaques with the R2 Env resulted in induction of antibodies that neutralized heterologous primary HIV-1 strains (Dong et al., 2003; Quinnan et al., 2005). Here we describe the selection of bcnAbs by using an antigen based on the recombinant soluble form of the Env ectodomain ($gp140_{R2}$). The antibodies selected from an immune human Fab phage display library by panning against $gp140_{R2}$ bound to Envs from primary isolates and inhibited entry mediated by Envs of primary isolates from different clades. One unique gp41-specific bcnAb, m46, was identified which in contrast to 2F5, 4E10 and Z13 binds to a conformational epitope. These results indicate that as previously proposed, the Env from the R2 isolate may have unique exposed conserved epitopes targeted by broadly cross-reactive antibodies that may have potential as vaccine immunogens and as targets for inhibitors. The selected antibodies can be used for identification and characterization of these conserved epitopes that can serve as templates for design of new vaccine immunogens (a retrovaccinology approach for development of vaccines). These antibodies could also have potential as therapeutics in combination with other antiretroviral drugs including other antibodies, and as research reagents.

Results

Selection of phage-displayed Fabs by panning against $gp140_{R2}$ and control gp140s

The *R2 Env* gene was isolated from a donor (R2) with long-term nonprogressive HIV-1 infection whose serum contains broadly cross-reactive primary isolate neutralizing antibodies (bcnAbs) and the *R2 Env* can be neutralized cross-reactively by HIV-immune human sera; the *R2 Env* is competent for CD4-independent infection and a soluble oligomeric form ($gp140_{R2}$) elicited bcnAbs in monkeys indicating enhanced exposure of conserved epitopes (Quinnan et al., 1999, 2005; Zhang et al., 2002). Therefore, we have hypothesized that panning of phage-displayed antibody libraries against ($gp140_{R2}$) is likely to result in selection of bcnAbs. To test this hypothesis, and to begin to identify and characterize conserved epitopes on R2, we used $gp140_{R2}$ as an antigen for panning of an immune human antibody library derived from three long-term nonprogressors with high level of bcnAbs. Two strategies were used: (1) standard biopanning where gp140 alone was incubated with the library and (2) a procedure, we recently developed (Zhang et al., 2006), termed competitive antigen panning (CAP), where tagged gp140 is mixed with an excess of untagged gp120 and the library; thus the CAP methodology enhances selection of gp41-specific antibodies in the context of gp140.

With the first strategy four rounds of panning were performed using $gp140_{R2}$ as selecting antigen. The resulting libraries were screened with $gp140_{R2}$, $gp120_{89.6}$, $gp120_{JR\text{-}FL}$ and $gp140_{IIIB}$. Total of 30 clones from the third and fourth rounds were screened by phage ELISA and 14 highest affinity binders were sequenced. Table 1 shows the sequences of the heavy chain complementarity determining region 3 (H3) of selected antibody clones and their similarity to known hmAbs. Three clones had almost identical sequences between themselves and with m18, which was selected by SAP using $gp140_{89.6}$ and $gp140_{IIIB}$ (Zhang et al., 2003, 2006). One of these clones was identical to m18 and designated as m22—we wish to keep this designation to indicate that it was selected by using R2. Five clones were also similar to m18 (differed by two residues in the heavy chain), while one clone had the same heavy chain as m18 but different light chain. Two clones were similar to m14, which was also selected by using SAP with $gp140_{89.6}$ and $gp140_{IIIB}$ as selecting antigens (Zhang et al., 2004). One of these clones, designated as m24, differed from m14 by one residue in the CDRH3 and three other residues in the framework. Fabs m22 and m24 were expressed, purified and tested for neutralization.

To find out whether other Envs used as antigens can also lead to selection of cross-reactive antibodies including antibodies similar to m14 and m18, we performed four rounds of panning

Table 1
Sequences of the H3s of antibody clones selected by panning against $gp140_{R2}$

$gp140_{R2}$ Clones	H3	Sequence similarity
R-1 (m22)	A R Y H R H F I R G P L S F D Y	m18
R-2 (m24)	A R E R R V L R W L G F P R G G L D Y	m14
R-3	A R Y Y R H V I R G P L S F D Y	m18
R-4	A R Y H R H F I R G P L S F D Y	m18
R-5	A R Y Y R H V I R G P L S F D Y	m18
R-6	A R Y Y R H V I R G P L S F D Y	m18
R-7	A R Y Y R H V I R G P L S F D Y	m18
R-8	A R Y N R H M I R G P L S F D Y	m18
R-9	A R Y H R H F I R G P L S F D Y	m18
R-10	A R Y Y R H V I R G P L S F D Y	m18
R-11	A R E R R V L L W L G F P R G G L D Y	m14
R-12	A R L K L R G A F D F	Unique
R-13	A R L K L R G A F D F	Unique
R-14	A R A E R V L F W L G Q P R G G L D P	Unique

The antibody DNA was sequenced and the amino acid sequences of the complementarity determining region 3 (H3) were determined by using the GCG program. The differences of each clone are highlighted in comparison to the previously identified antibodies m14 and m18.

against Env glycoproteins from two other isolates, 89.6 ($gp140_{89.6}$) and IIIB ($gp120_{IIIB}$) using the same panning conditions as for $gp140_{R2}$, and screened 30 antibody clones selected by each panning antigen with $gp140_{R2}$, $gp120_{IIIB}$ and $gp140_{89.6}$ using phage ELISA. Most of the clones (94%) selected by using gp140 from R2 bound to the homologous selecting antigen ($gp140_{R2}$) but also significant number (about 30–40%) bound to the heterologous Envs IIIB and 89.6 (Fig. 1). Using Env glycoproteins from 89.6 and IIIB isolates as selecting antigens also lead to high number of clones that bound to their respective homologous antigens although that number (about 60% of all selected clones) was smaller than the respective percentage for R2 (94%); interestingly, in both cases about the same number of clones (about 60%) bound also to the heterologous Env (gp140 from R2) (Fig. 1). However, fewer clones (about 10%) selected by using Env glycoproteins either from IIIB or 89.6 cross-reacted with each other (Fig. 1). The highest binders in phage ELISA were sequenced and compared to the m14 and m18 sequences. Two independent panning experiments with $gp140_{R2}$ resulted in maximum number of antibody clones similar to either m14 or m18 (73% of sequenced clones), while significantly smaller number of clones from panning with $gp120_{IIIB}$ (33%) and $gp140_{89.6}$ (10%) were similar to these two cross-reactive antibodies (Table 2). These results suggest that gp140 from R2 contains conserved structures that serve as epitopes of bcnAbs like m14 and m18, and that they are more antigenic than those from Envs from IIIB and 89.6.

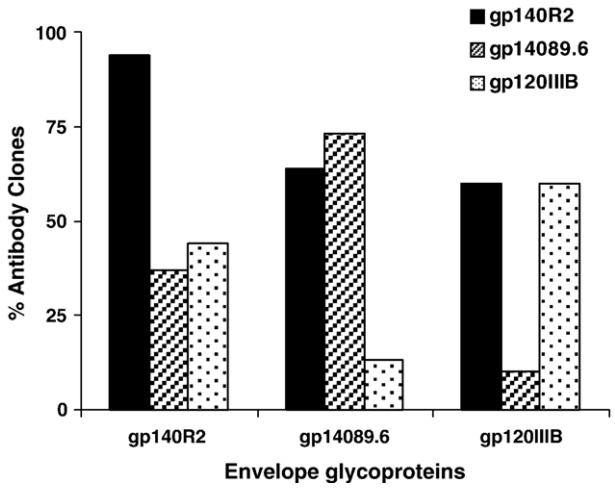

Fig. 1. Monoclonal phage ELISA reactivity of antibody clones. Screening antigens ($gp140_{R2}$, $gp140_{89.6}$, $gp140_{IIIB}$) were captured by the polyclonal sheep anti-gp120 antibody D7324 (5 μg/ml) coated on 96-well plates. Phage supernatants of 30 clones from each panning experiment were added, bound phage was detected by anti-M13-HRP antibody and optical density was measured at 450 nm. The number of antibody clones that bind to the screening antigen is shown as percentage of the total number of clones (30).

With the second strategy (CAP), we selected antibodies that bind to gp41 by using a mixture of tagged $gp140_{R2}$ as a selecting antigen and $gp120_{R2}$ as a competitor. After the third and fourth round of panning, 96 antibody clones were screened by phage ELISA against $gp140_{R2}$ and $gp120_{R2}$. One clone, m46, with the highest binding to $gp140_{R2}$ and no significant binding to $gp120_{R2}$ was selected and sequenced. Its sequence was unique with a 12-residue long complementarity determining region 3 (CDR3) of the heavy chain (VIIRGSHYKDDY). Interestingly, m46 was not selected by using other Envs (89.6, CM243) as antigens.

These observations indicate that $gp140_{R2}$ is very antigenic and contains exposed conserved structures that serve as epitopes for bcnAbs.

Binding of m22, m24 and m46 to gp120 and gp140 from different isolates

To further characterize the selected antibodies (m22, m24 and m46) they were expressed as Fabs, purified and their binding to gp120 and gp140 was measured under various conditions. M22 and m24 bound to gp140 from 89.6, and gp120 from JR-FL and IIIB with high (nM) affinity and also bound to several gp140s and gp120s from primary isolates that were not used in the panning and screening procedures suggesting that their epitope is exposed in gp120s of isolates from different clades as measured by an ELISA assay (data not shown). The kinetics of interaction of m22 and m24 with different Envs was measured by using an optical biosensor system (Biacore) based

Table 2
Sequence similarity of antibody clones with mAbs m14 and m18

Panning antigen (number of sequenced clones)	m14	m18
$gp140_{R2}$ (14)	2	9
$gp140_{R2}$ (12)	3	5
$gp140_{89.6}$ (10)	–	1
$gp120_{IIIB}$ (12)	1	3

The numbers of clones with sequence similarity to m14 and m18 are shown. The total numbers of sequenced clones from each panning procedure using $gp140_{R2}$, $gp120_{IIIB}$ or $gp140_{89.6}$ as antigens are in parentheses. The results of two independent pannings with R2 are shown.

on surface plasmon resonance. The association and dissociation rate constants for m22 and m24 binding to $gp140_{R2}$ and $gp140_{89.6}$ (tethered) were in a range typical for binding of antibodies with nM affinity (Table 3). The equilibrium dissociation constants, derived from the kinetic parameters, were comparable to the binding affinity observed for m18 and m14.

To approximately localize the m22 and m24 epitopes we evaluated the competitive binding activity of Fab m22 and Fab m24 in presence of sCD4 and other anti-gp120 antibodies. M22 and m24 competed significantly with sCD4 and IgG b12, and two other CD4bs antibodies (m14 and m18) but only weakly with IgG 17b and another CD4i antibody Fab X5 (Fig. 2). These results further suggest that m22 and m24 strongly bind to both gp120 and gp140, and behave as CD4bs antibodies, as observed earlier with m14 and m18.

To further characterize Fab m46 it was purified and its binding to gp140s and gp120s from R2 and 89.6 was measured by ELISA (Fig. 3). We observed high level of binding to the gp140s but no significant reactivity for the corresponding gp120s. To determine whether m46 binds to conformational epitopes, we prepared denatured $gp140_{89.6\ (tethered)}$, by adding a reducing reagent. Unlike binding to native gp140, binding of m46 to denatured gp140 was abolished suggesting that m46 recognizes a conformational epitope on gp41 (Figs. 4A and B). The binding of the other tested gp41 antibodies, IgG 2F5 and Fab Z13 was not affected by the reducing condition.

To further characterize the binding of m46 to gp41, competition experiments were performed with different anti-gp41 antibodies (Fig. 5); the anti-gp120 antibodies IgG b12, IgG 2G12, and Fab X5 were used as controls for gp41 specificity. We observed competition with two recently identified gp41 antibodies, Fab m43 and Fab m45 (Zhang et al., unpublished) and lack of competition with two other gp41 antibodies, IgG 2F5 and IgG 4E10. In contrast to 2F5 and Z13, m46 strongly competed with the mouse cluster IV antibody T3 (Fig. 6A) but did not compete with the mouse cluster V antibody D3 (Fig. 6B). These results indicate that in contrast to the well-characterized HIV-1 neutralizing anti-gp41 antibodies 2F5, 4E10 and Z13, which can bind linear epitopes, m46 binds to a conformational epitope on gp41 that is likely localized in a region different than the epitopes of 2F5, 4E10 and Z13.

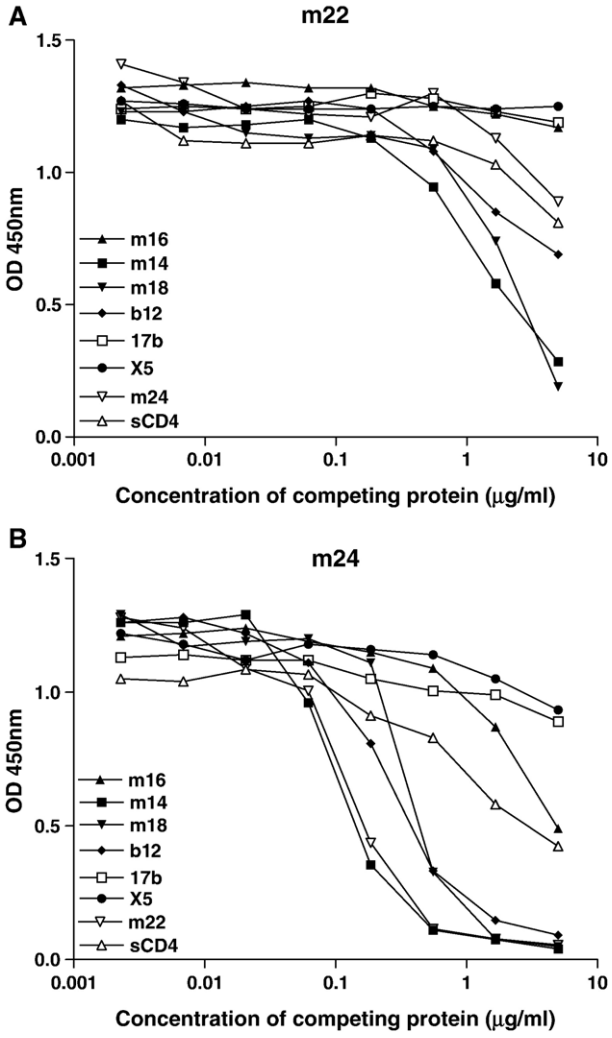

Fig. 2. Competition of m22 and m24 with other CD4bs and CD4i antibodies. Gp140R2 was captured by the polyclonal sheep anti-gp120 antibody D7324 (5 μg/ml) coated in 96-well plates. Three-fold serially diluted sCD4, Fabs (m14, m16, m18, X5, m22, m24), and IgGs (b12, 17b) followed by addition of biotinylated m22 (A) or m24 (B), at a constant concentration (which leads to 70% of maximum binding) simultaneously to the wells. Bound antibodies were detected by streptavidin–HRP and measured as optical densities at 450 nm.

Table 3
Binding kinetics of m22 and m24 to $gp140_{R2}$ and $gp140_{89.6}$ measured by Biacore

HIV-1 isolate	ka (10^5 M^{-1} s^{-1})		kd (10^{-4} s^{-1})		K_D (nM)	
	m22	m24	m22	m24	m22	m24
$gp140_{R2}$ (clade B)	2.1	1.2	1.6	1.5	0.8	1.3
$gp140_{89.6\ (tethered)}$ (clade B)	0.6	0.4	1.6	2.2	2.9	5.2

The experiments were performed as described in the Materials and methods section — m22 and m24 were injected (30 μl/min) at different concentrations (50, 25, 12.5, 6.25 and 3.125 nM) over a CM5 sensor chip, containing captured $gp140_{R2}$ and $gp140_{89.6\ (tethered)}$. The association, ka, and dissociation, kd, rate constants were calculated by fitting both association and dissociation phases with a 1:1 Langmuir global model using BIAevaluation 4.1 software, and the equilibrium dissociation constant, K_D, was determined from their ratio.

To further characterize the epitope of m46, we measured by ELISA m46 binding to thirty-four peptides derived from different regions of gp41, including six peptides from the membrane-proximal external region (MPER) where 2F5, 4E10 and Z13 bind. We did not observe any significant binding of m46 to the tested peptides including DP178 (T20), C34, N36 and the peptide 2031 (Fig. 7 and data not shown). These results were further confirmed by competition ELISA with T20 (Fig. 8), where T20 competed with IgG 2F5 but did not compete with IgG m46 and Fab Z13. These observations further indicate that m46 recognizes a conformational epitope that is different from the epitopes of other known anti-gp41 antibodies such as 2F5, Z13 and 4E10.

In an attempt to localize the m46 epitope we measured its binding to five soluble trimeric polypeptides derived from gp41:

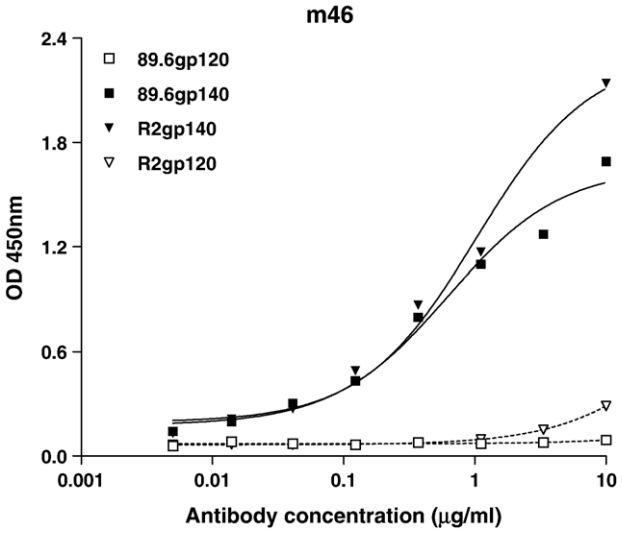

Fig. 3. Binding of m46 to gp140/120 from primary isolates. Gp140/gp120s were coated on 96-well plates, washed and m46 was added at the indicated concentrations. Bound antibodies were detected by anti-human IgG F(ab′)$_2$-HRP and measured as optical densities. The background, determined as the amount of Fab bound to BSA, was subtracted. Standard variation of triplicate wells was on average around 5%.

N_{CCG}-gp41, $N35_{CCG}$-N13, $N34_{CCG}$, the minimal thermostable ectodomain core of gp41 – the 6-helix bundle (6-HB) (Louis et al., 2005) and a single chain 5-helix bundle (5-HB), which folds into a structure similar to the trimers-of hairpins, but with an unoccupied binding site for a C peptide (Root et al., 2001). Fab m46 bound weakly only to the 5-HB (Fig. 9 and data not shown) indicating that its conformational epitope includes structures from both (N and C) heptad repeats.

Neutralizing activity of m46 against primary HIV-1 isolates from different clades

The neutralizing activity of Fab m46 to primary isolates from different clades was tested by using assays based on cell lines infected by pseudovirus and on PBMCs infected by replication-competent virus. For a panel of clade C isolates, which are not neutralized by 2F5, m46 was much more potent in the PBMC-based assay than in the cell line/pseudovirus-based assay (Table 4). To account for possible differences between pseudovirus and virus derived from PBMCs, m46 was also tested in the same cell line (TZM-bl) but with a PBMC-derived virus. The results indicate that the use of PBMCs as target cells but not the nature of the virus containing the same Env is a major determinant of the high neutralizing activity of m46 (Table 4). PBMCs have lower CCR5 concentration in comparison to cell lines used in neutralization assays (Choudhry et al., 2006). Experiments with HeLa cell lines expressing high or low levels of CCR5 were performed to evaluate the effect of the CCR5 concentration on the neutralizing activity of m46 and other gp41 antibodies. For the cell line with low CCR5 cell surface concentration we observed a significant increase in the neutralizing activity of both Fab and IgG m46, and of two other gp41 antibodies, 2F5 and 4E10, against different primary isolates of HIV-1 (Table 5).

In a separate set of experiments we compared the neutralizing activity of IgG m46 with the potent inhibitor of HIV-1 entry, the gp41-derived peptide T20 which is the only entry inhibitor currently in clinical use (Table 6). On a molar basis the inhibitory activity of m46 was on average approximately comparable to that of T20–for two isolates (from clades A and C) the activity of the two inhibitors was comparable, for two other isolates (from clades B and E) the IC_{50} for T20 was several fold lower than that for IgG m46 and for one isolate (from clade D) IgG m46 was much better inhibitor than T20. The inhibitory activities of IgG m46 and T20 were also compared in a PBMC based assay for an isolate from clade B (92BR020) (Fig. 10). Not only the IC_{50}s for IgG m46 and T20 were comparable (173 ± 26 nM and 154 ± 17 nM, respectively) but also the functional dependences of the virus concentration on the inhibitor concentration were similar. These results suggest that m46 is a cross-reactive HIV-1-neutralizing antibody which exhibits neutralizing potency for

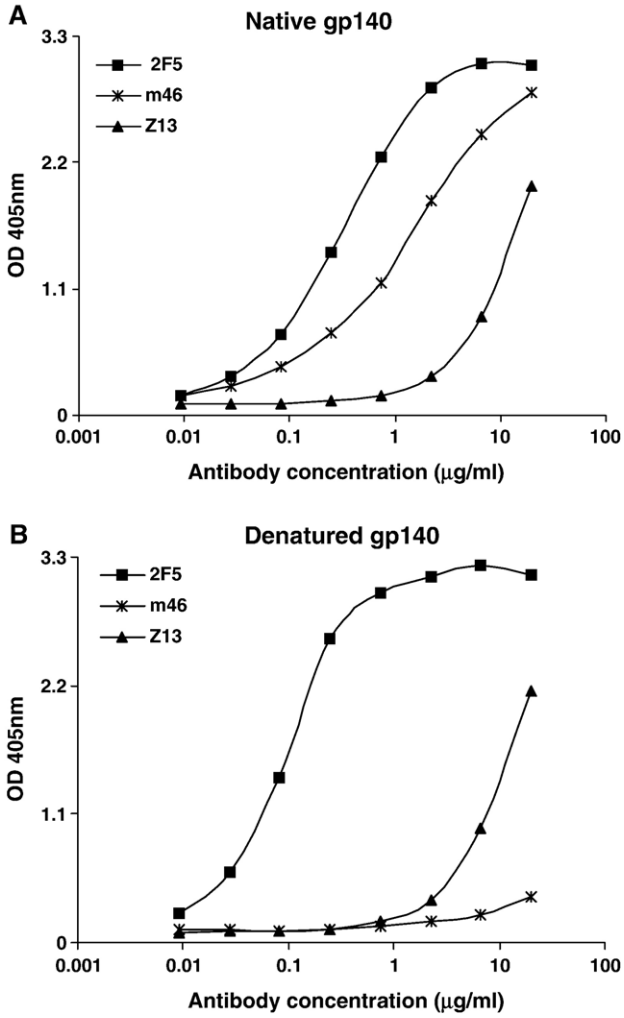

Fig. 4. Binding of m46 to native (A) and denatured tethered $gp140_{89.6}$ (B). Tethered $gp140_{89.6}$ or denatured $gp140_{89.6}$ at 1 μg/ml (prepared as described in Materials and methods) was coated on 96-well plates. The plates were blocked using 3% BSA in PBS, and 3-fold serially diluted anti-gp41 antibodies with starting concentration of 10 μg/ml were added to the wells. Bound antibodies were detected using HRP-conjugated anti-human IgG, F(ab′)2 and ABTS as substrate. Optical density at 405 nm was measured.

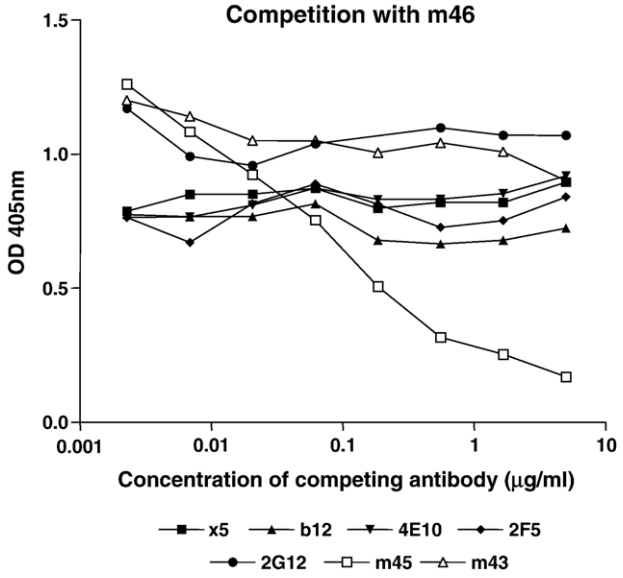

Fig. 5. Competition of m46 with anti-gp41, CD4bs and CD4i antibodies. Gp140R2 was captured by the polyclonal sheep anti-gp120 antibody D7324 (5 μg/ml) coated on 96-well plates. Serially diluted sCD4, different Fabs (X5, m43, m45) and IgGs (b12, 2F5, 4E10, 2G12) were added, along with biotinylated m46 at a constant concentration that led to 70% of maximum binding, simultaneously to the wells. Bound antibodies were detected by streptavidin–HRP and measured as optical densities at 405 nm.

the tested isolates approximately comparable to that of T20 on a molar basis with a mechanism dependent on the CCR5 concentration.

Discussion

The major findings of this study are the demonstration of high level of exposed conserved epitopes on the R2 Env and the identification of novel bcnAbs. The finding that the use of an Env (R2) isolated from a patient with high level of bcnAbs as an antigen for panning of an immune human antibody library resulted in the efficient selection of such bcnAbs further supports the proposition that donors with broadly cross-reactive HIV-1 neutralizing sera are likely to be infected with viruses encoding Envs with unusual immunogenic properties (Cham et al., 2006). Interestingly, the immune antibody library was derived not from the R2 donor but from a pool of three other individuals with high levels of bcnAbs. It is tempting to speculate that it could be the R2 Env that is capable of eliciting bcnAbs and not the specific properties of the immune system of the R2 donor although one might argue that all individuals with high levels of bcnAbs have such specific properties of their immune systems leading to enhanced elicitation of bcnAbs. One can further speculate that R2-like viruses or other viruses with Envs exposing conserved epitopes caused the elicitation of bcnAbs in the three donors from which the immune library was constructed. Whatever the mechanism is, the use of the R2 Env helped to identify novel bcnAbs; this efficient selection of bcnAbs indicates its potential as selecting antigen for screening of antibody libraries and as vaccine immunogen, and provides novel tools for elucidating mechanisms and developing of inhibitors and vaccines. Indeed, recently the use of R2 as an immunogen mixed with an adjuvant elicited antibodies in rabbits that neutralized all 47 tested primary HIV-1 isolates (G. Quinnan et al., AIDS Vaccine Meeting, Amsterdam, abstract # P09A-76).

Of the large number of mAbs and Fabs against Envs until recently only several human mAbs have been identified that exhibit broad and potent HIV-1 neutralizing activity (D'Souza et al., 1997; Binley et al., 2004). The identification of new broadly cross-reactive HIV-1 neutralizing mAbs suggests that such antibodies could play even more important role in vivo than anticipated although we do not have direct evidence that the antibodies we have identified exist in vivo.

We observed that the R2 Env used as an antigen enables selection of antibody clones from an immune library, which cross-react with Envs from the other isolates used in this study ($gp140_{89.6}$ and $gp120_{IIIB}$). However, in phage ELISA antibody clones selected from panning against $gp140_{89.6}$ had high cross-

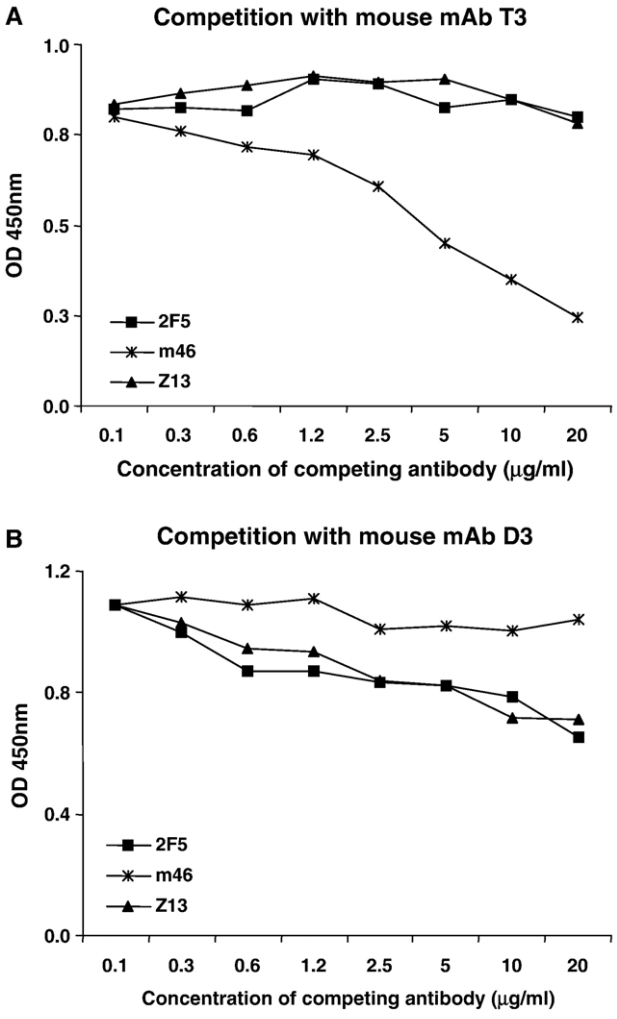

Fig. 6. Competition of m46 with anti-gp41 antibodies. 1 mg/ml $gp140_{89.6}$ was coated on 96-well plates. Two-fold serially diluted IgG m46, IgG 2F5 and Fab Z13 were added to the wells and biotinylated mouse monoclonal antibody (mAb) T3 and D3 at a constant concentration corresponding to 70% maximum binding was simultaneously added to the wells. Bound biotinylated T3 (A) and D3 (B) were detected using streptavidin–HRP at 450 nm.

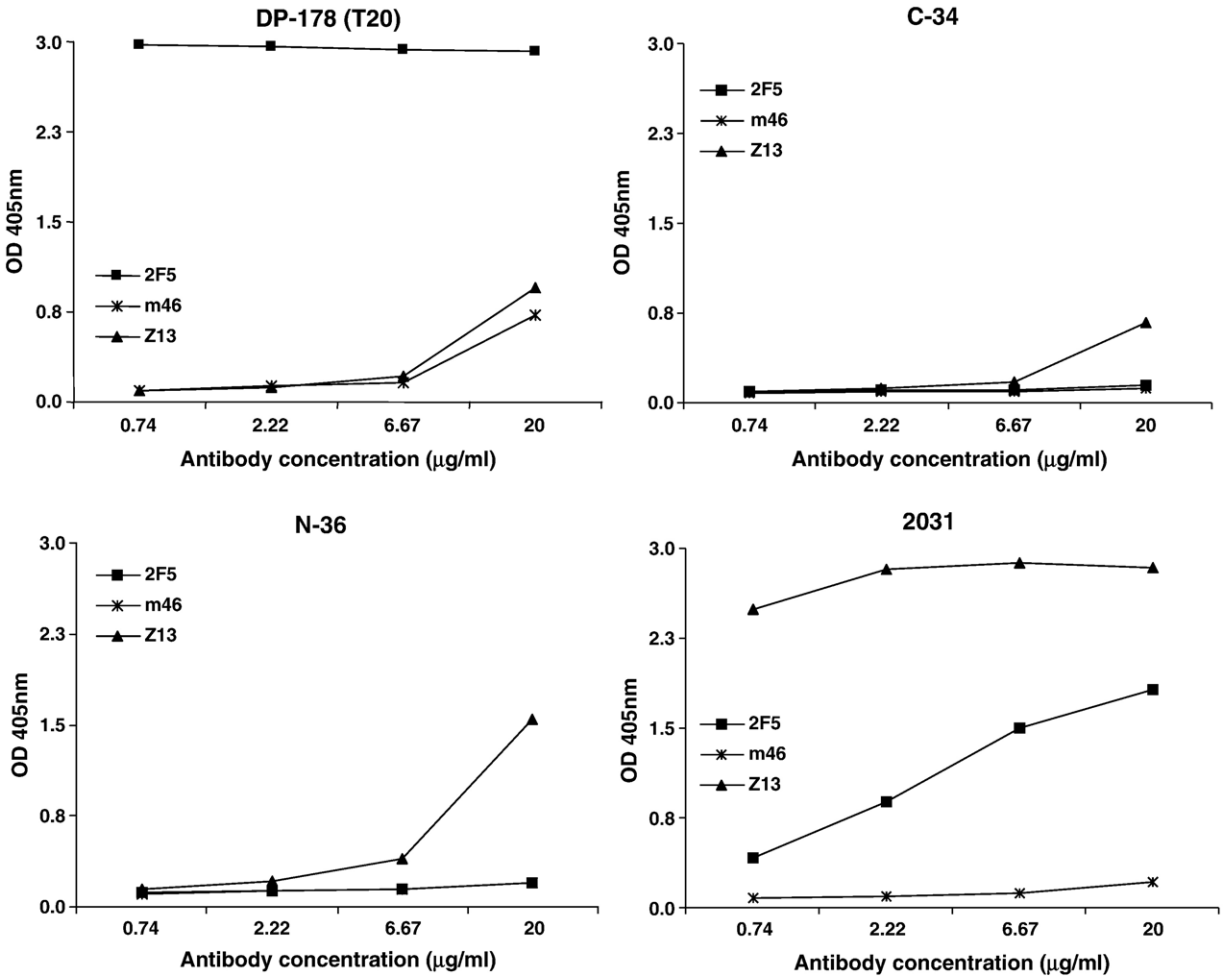

Fig. 7. ELISA reactivity of m46 with selected peptides derived from gp41. Different peptides derived from gp41 were coated at 4 μg/ml concentration on a 96-well plate. Three-fold serially diluted Fab m46, Fab Z13 and IgG 2F5 were added to the blocked plates. Bound antibodies were detected by addition of HRP-conjugated anti-human IgG, F(ab′)2 and the absorbance measured by using ABTS substrate.

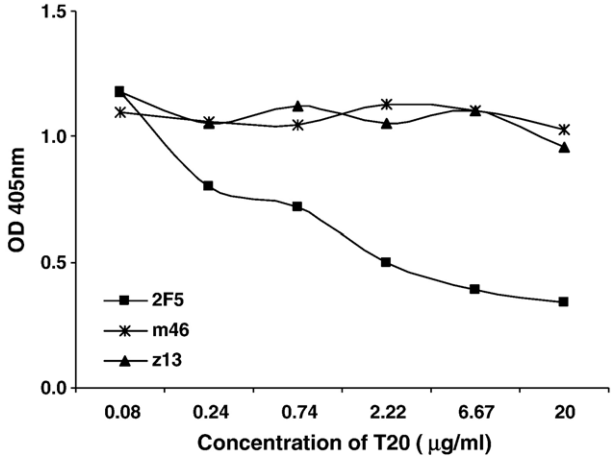

Fig. 8. Competition of m46 with T20. 1 mg/ml $gp140_{89.6}$ was coated on 96-well plates. Two-fold serially diluted T20 was added to the wells and IgG m46, Fab Z13 or IgG 2F5 was added to the wells at a constant concentration corresponding to 70% maximum binding. Bound antibodies were detected using HRP-conjugated anti-human IgG, F(ab′)2 and measuring absorbance at 405 nm.

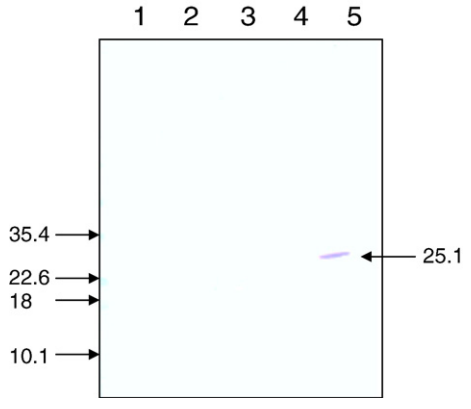

Fig. 9. Western blot analysis of Fab 46 with soluble gp41 constructs containing heptad repeats. SDS-PAGE of non-reduced N_{CCG}-gp41 (lane 1), $N35_{CCG}$-N13 (lane 2), $N34_{CCG}$ (lane 3), 6-HB (lane 4) and 5-HB (lane 5) was electrophoresed (500 ng/lane) on 20% homogeneous PhastGel and immunoblotted with Fab m46, and detected by goat anti-human Fab antibody–alkaline phosphatase conjugate using Fast 5-bromo-4-cloro-3-indolyl phosphate/nitro blue tetrazolium solution. Arrows indicate the molecular mass markers.

Table 4
Neutralization of HIV-1 primary isolates by the gp41-specific antibody IgG1 m46

Virus	PBMC virus/ TZM-bl assay	293T Pseudovirus/ TZM-bl assay	PBMC virus/ PBMC assay
Du123.6	>35	>35	1.5
Du151.2	>35	>35	25
Du156.12	>35	>35	>35
Du172.17	>35	>35	4.5
Du422.1	>35	>35	25

Neutralization assays were performed either using pseudovirus produced in 293T cells or viruses produced in PBMCs and TZM-bl cells or PBMCs as targets. Shown are IC_{50}s in μg/ml.

reactivity to $gp140_{R2}$ and $gp140_{89.6}$ but not significant binding to $gp120_{IIIB}$; similarly antibodies obtained by panning against $gp120_{IIIB}$ had high cross-reactivity to $gp140_{R2}$ but on average low level of binding to $gp140_{89.6}$. These results suggest that the R2 Env glycoprotein shares common epitopes with the other Env glycoproteins ($gp140_{89.6}$ and $gp120_{IIIB}$) used in panning but the other Env glycoproteins do not exhibit some of these common epitopes. Thus the selected antibodies, m22 and m24, had neutralization activity similar to that of the previously characterized antibodies m18 (Zhang et al., 2003) and m14 (Zhang et al., 2004). Panning against the other two tested Envs (89.6 and III B) by using standard panning procedures did not lead to selection of clones that are broadly cross-reactive or exhibit potent neutralizing activity (data not shown). Interestingly, although the R2 Env can mediate CD4-independent entry, we were not able to select CD4i antibodies from the panning against the R2 Env; this could be partially due to the screening based on gp140s and gp120s in the absence of CD4 and experiments are in progress to evaluate the possibility for identification of CD4i antibodies by using R2 Env as an antigen.

Table 5
Neutralization of HIV-1 primary isolates from different clades by m46, 2F5 and 4E10 measured by a pseudovirus assay using cell lines with different CCR5 surface concentrations

HIV-1 clade	Antibody	High CCR5	Low CCR5
92UG037.8 (clade A)	Fab m46	>50 (40%)	18
	IgG m46	>50 (35%)	25
	IgG 2F5	0.3	0.2
	IgG 4E10	1	0.2
Bal (clade B)	Fab m46	45	5
	IgG m46	30	8
	IgG 2F5	6	1
	IgG 4E10	12	0.1
GXC-44 (clade C)	Fab m46	>50 (44%)	15
	IgG m46	>50 (39 %)	25
	IgG 2F5	>25 (20 %)	>25 (30%)
	IgG 4E10	15	8
Z2Z6 (clade D)	Fab m46	20	10
	IgG m46	10	3
	IgG 2F5	18	9
	IgG 4E10	22	10
CM243 (clade E)	Fab m46	28	5
	IgG m46	25	6
	IgG 2F5	3.2	1
	IgG 4E10	1.5	0.6

Antibodies at various concentrations were mixed with viruses pseudotyped with the Env from different clades of HIV-1 for 30 min at 37 °C and mixed with cells expressing high or low CCR5 concentrations in triplicate wells. The mean luminescence readings were determined 3 days later and IC_{50} (μg/ml) was calculated at the antibody concentration at which 50% neutralization was observed. The mean of three measured IC_{50}s for each antibody/virus/cell combination are presented (in μg/ml); the standard deviation was on average 5% and did not exceed 10%, the numbers in parentheses are the percentages neutralization measured at those concentrations used (50 or 25 μg/ml).

Table 6
Comparison of neutralization of HIV-1 primary isolates from different clades by IgG m46 and T20 measured by a pseudovirus-based assay using cell lines with different CCR5 surface concentrations

HIV-1 clade	Antibody	High CCR5	Low CCR5
92UG037.8 (clade A)	IgG m46	>334 (41%)	214
	T20	445	111
Bal (clade B)	IgG m46	247	80
	T20	89	8.9
GXC-44 (clade C)	IgG m46	>334 (32%)	147
	T20	668	89
Z2Z6 (clade D)	IgG m46	80	30
	T20	734	111
CM243 (clade E)	IgG m46	214	67
	T20	45	8.9

Various concentrations of IgG m46 and T20 were mixed with viruses pseudotyped with the Env from different clades of HIV-1 for 30 min at 37 °C and mixed with cells expressing high or low CCR5 concentrations in triplicate wells. The IC_{50} (in nM) was calculated as described in Table 5.

We also selected one gp41-specific antibody (m46) by using the competitive antigen panning methodology (Zhang et al., 2006). This antibody binds to a conformational epitope (in contrast to 2F5 and Z13) and exhibits broad and relatively potent neutralizing activity. Although currently the exact localization of its epitope is not known because of lack of

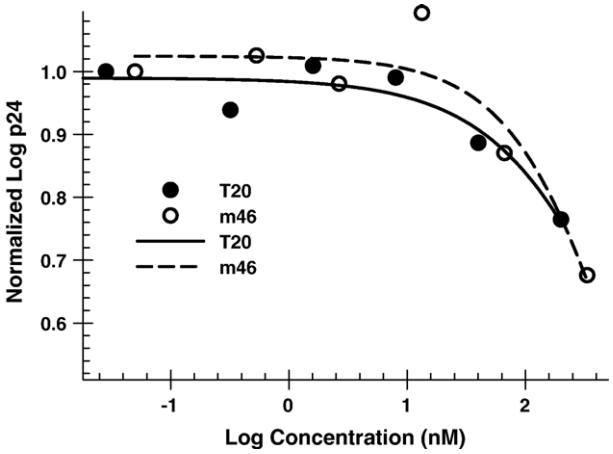

Fig. 10. Inhibitory activity of IgG m46 and T20 against an isolate from clade B (92BR020) measured by a PBMC assay. The PBMCs were activated with PHA-P for 3 days, infected with HIV-1 in presence of the IgG m46 (open circles and dashed line) or T20 (solid circles and solid line) for 24 h, washed twice with PBS and left to grow in RPMI-1640 supplemented with IL-2. Lysis buffer was added to the cell suspension 3 days later and p24 was detected by standard capture ELISA. To account for variations in viral replication in the absence of inhibitors, the Log of p24 was normalized to Log p24 in the absence of inhibitors. Experimental data were fitted by using the sigmoidal 3-parameter function of SigmaPlot software (Sysstat Software, Inc., San Jose, CA). IC_{50}s were calculated from the fitted curves.

crystal structure of its complex with gp140 we demonstrated that it does not overlap with the epitopes of 2F5, 4E10 and Z13. In addition, we found that m46 does not bind to any gp41 peptide tested including N-36, C-34 and T20 (Fig. 7), and it does not bind to soluble constructs from gp41 containing the N-terminal heptad repeat and the 6-helix bundle (6-HB) (Fig. 9). However, it did bind, although weakly, to a construct containing the 5-HB indicating that its conformational epitope contains structures from both the N- and C-terminal heptad repeats (Fig. 9). Interestingly, T20, which also binds to the 5-HB, exhibited similar neutralizing activity on molar basis for several isolates from different clades (Table 6) and for one isolate tested in a PBMC-based assay the neutralizing activity as function of the inhibitor concentration was the same (Fig. 10). It is tempting to speculate that some of the components of the neutralizing mechanisms of m46 and T20 are similar and include binding to fusion intermediates. We also observed that similarly to other gp41-specific antibodies (2F5, 4E10, Z13) and some CD4-induced antibodies, the m46 neutralizing activity increased with a decrease in the cell surface concentration of CCR5. The concentration of CCR5 on the cell surface has been shown previously to affect the neutralizing activity of HIV-1-specific antibodies likely by changing the kinetics of entry (Choudhry et al., 2006). Further characterization of m46 and its epitope, and elucidation of its mechanism of inhibition could help in the development of novel vaccine immunogens and inhibitors. Overall, these results suggest that the R2 Env exposes conserved epitopes that could be used as templates for design of vaccine immunogens and as targets for inhibitors, and that the new antibodies, especially m46, offer new tools for development of inhibitors, vaccines and research reagents.

Materials and methods

Cells, viruses, plasmids, soluble CD4 (sCD4), gp120, gp140, and antibodies

HEK 293T cells were purchased from American Type Culture Collection (Manassas, VA). HeLa cell lines, expressing different levels of CCR5, were a gift from D. Kabat (Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, Oregon). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Plasmids used for expression of various Envs were obtained through the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, from B. Hahn (University of Alabama at Birmingham), and the remaining clones are described previously (Vujcic and Quinnan, 1995; Quinnan et al., 1999; Zhang et al., 2002). The R2 (clade B) Env was from a patient whose sera demonstrated broad cross-neutralizing activity previously cloned and has been described elsewhere (Earl et al., 1994, 2001).

Two-domain soluble CD4 (sCD4) was obtained from the ARRRP. Thirty-four peptides derived from gp41, including DP178 (T-20), C34, N36, 2031 were also obtained from the ARRRP. $Gp120_{89.6}$ and $gp140_{89.6}$ were produced from recombinant vaccinia viruses, provided by R. Doms (University of Pennsylvania, PA). $Gp120_{IIIB}$, and a battery of gp140s including R2 were also prepared from recombinant vaccinia viruses and have been extensively detailed elsewhere (Quinnan et al., 2005). Briefly, recombinant gp140 glycoprotein was produced by infecting BS-C-1 cells with an appropriate vaccinia virus encoding the indicated HIV-1 gp140 cassette. The gp140 coding regions have the proteolytic cleavage site between gp120 and gp41 ectodomain mutated to enhance the yield of oligomeric glycoprotein. Oligomeric gp140/120 was purified from serum-free culture supernatants using a combination of Lentil lectin Sepharose 4B affinity and size exclusion chromatography. HIV-1 gp140 preparations have been shown to consist of approximately 40% trimer and 60% dimer (Earl et al., 1997).

The human mAbs X5, Z13, m9, m14, m16 and m18, and the mouse mAbs T3 (cluster IV) and D3 (cluster V) (Dreyer et al., 1999) were produced in our laboratories; b12 was a gift from D. Burton (The Scripps Research Institute, La Jolla, CA), and 17b was from J. Robinson (Tulane University Medical Center, New Orleans, LA). The human monoclonal antibodies 2F5 and 4E10 were a gift from H. Katinger (Institute of Applied Microbiology, University for Agricultural Sciences, Vienna, Austria). The following antibodies were purchased: polyclonal sheep anti-gp120 antibody D7324 (Cliniqa, Fallbrook, CA), HRP-conjugated monoclonal mouse anti-M13 antibody (Pharmacia, Uppsala, Sweden) and HRP-conjugated polyclonal anti-human IgG F(ab′)2 antibodies (Jackson ImmunoResearch, Westgrove, PA).

Antigen panning of phage library and analysis of selected phage clones

The phage library was constructed using pComb3H phagemid vector and 30 cm^3 of bone marrow obtained from three long-term nonprogressors (A, H and K) (Montefiori et al., 1996) whose sera exhibited the broadest (Zhang et al., 2003) and most potent HIV-1 neutralization (provided by T. Evans, University of California, Davis, CA). Three immunotubes (Nunc, Denmark) were coated with 10 μg/ml of $gp140_{R2}$, $gp120_{IIIB}$ and $gp140_{89.6}$ each at 4 °C overnight and blocked with 3% BSA for 2 h at 37 °C. Phage (5×10^{12} cfu/ml) were adsorbed for 2 h at 37 °C and immunotubes were washed 20 times with 1 ml of PBS containing 0.1% Tween-20 and another 20 times with 1 ml of PBS. Bound phage were eluted by incubation at room temperature (RT) for 10 min with 1 ml of 100 mM triethylamine followed by neutralization with 0.5 ml of 1 M, pH 7.5 Tris–HCl. Eluted phage were rescued by infection of *Escherichia coli* TG1 cells and phage library was prepared for the next round of panning. For each round of panning similar procedure was used; 30 individual clones after the 3rd and 4th round of panning were screened by phage ELISA for binding to Envs of primary isolates of HIV-1. Competitive antigen panning (CAP) using $gp140/120_{R2}$ was done by mixing labeled gp140 with 5-fold excess of gp120 (Barbas et al., 2001; Zhang et al., 2006). Individual clones from each panned library were

screened for binding to gp140/120$_{IIIB}$, gp140/120$_{89.6}$, and gp140/120$_{R2}$ by phage ELISA.

Phage ELISA

ELISA was performed by using 96-well Nunc-ImmunoTM MaxisorpTM surface plates (Nalge Nunc International, Denmark) which were coated overnight at 4 °C with 100 μl of Envs (1 μg/ml in sodium bicarbonate buffer, pH 8.3) blocked in 100 μl of 4% non-fat dry milk in PBS for 1 h at 37 °C. After 4 washes with 0.05% Tween-20/PBS washing buffer, wells were incubated with 100 μl of phage supernatant for 2 h at 37 °C. Bound phage were detected by using horse radish peroxidase (HRP) labeled anti-M13 monoclonal antibody (Amersham Biosciences, Uppsala, Sweden) with incubation for 1 h at 37 °C and revealed by adding ready-to-use TBS substrate (Sigma, St. Louis, USA). Color development was performed at RT for 15 min, quenched by 2 N HCl and monitored at 450 nm.

Preparation of soluble Fabs and binding assays

Phagemid DNA from the selected clones was prepared and digested with *Nhe*I and *Spe*I to remove gene III fragment, self-religated and transformed in *E. coli* XL-1 Blue. Soluble Fabs were produced and purified using protein G columns as described (Zhang et al., 2004). ELISA D7324 capture assays with soluble Fabs (m22, m24 and m46) and recombinant HIV-1 gp120s or gp140s from different isolates were performed by using 96-well Nunc-ImmunoTM MaxisorpTM surface plates (Nalgen Nunc International, Denmark) as described (Zhang et al., 2006). Direct ELISA was carried out by coating gp120/140s on the plates at a concentration of 1 μg/ml. In cases using denatured gp140s, purified gp140s were diluted in 1% sodium dodecyl sulfate and 50 mM dithiothreitol to 10 μg/ml and boiled for 5 min, and then diluted 1:10 in PBS and coated on 96-well plates. Competition ELISA of mAbs (m22, m24 and m46) with other anti-gp120/gp41 antibodies and sCD4 were carried out using D7324 capture assay as following: 1 μg/ml gp140$_{R2}$ was captured by the polyclonal sheep anti-gp120 antibody D7324 (5 μg/ml) coated on 96-well plates. Following the addition of 3-fold serially diluted Fabs (m14, m16, m18, X5), IgGs (b12, 17b) and sCD4, equal volume of biotinylated Fabs (m22, m24 and m46) at a concentration, which led to 70% maximum binding, was simultaneously added to each well. Bound biotinylated Fabs were detected by streptavidin–HRP (Pierce, Rockford, IL) and optical densities were measured. In another set of experiments, 1 mg/ml gp140$_{89.6}$ was coated on 96-well plates. Two-fold serially diluted m46, 2F5 and Z13 were added to the wells and biotinylated mouse monoclonal antibody T3 and D3 at a constant concentration corresponding to 70% maximum binding was simultaneously added to the wells. Bound biotinylated T3 and D3 were detected using HRP conjugated to streptavidin and ABTS substrate and measured the absorbance at 405 nm.

The kinetics of m22 and m24 binding was measured by Biacore 1000 optical instrument (Biacore, Pharmacia, Piscataway, NJ). An anti-gp41 human monoclonal antibody Fab (m43) developed recently in our laboratory (Zhang et al., 2006) was immobilized on a (CM5) sensor chip using carbodiimide coupling chemistry. Gp140 (200 nM) was injected at a flow rate of 10 μl/min and varying concentrations of antibodies (Fabs) were then injected at a flow rate of 30 μl/min using PBS buffer (pH 7.4) with 0.05% Tween-20. Control surface was prepared similarly and the experiments were performed by injecting solutions containing different concentrations of antibodies using running buffer in lieu of gp140s. All the sensograms were corrected by subtracting the low signal from the control reference surface. The association and dissociation phase data were fitted simultaneously to a 1:1 Langumir global model by using the nonlinear data analysis program BIAevaluation 4.1.

Binding of Fab m46 to soluble trimeric coiled-coil constructs from gp41 was performed by Western blot analysis as previously described (Louis et al., 2005).

HIV-1 Env clones and pseudovirus preparation

Viruses pseudotyped with Envs from HIV-1 primary isolates representing HIV-1 group M, clades A–F (Zhang et al., 2003), and laboratory adapted HIV-1 isolates and JRCSF were used in this study. Cloning of HIV-1 envelope genes and preparation of pseudoviruses have been previously described (Zhang et al., 2003). Briefly, pseudotyped viruses were prepared by cotransfection of 70% to 80% confluent HEK 293T cells with pNL4-3.luc.E-R- and HIV-1 Env plasmid using the calcium phosphate/HEPES buffer, according to manufacturer's instruction (Promega, Madison, WI). Eighteen hours after the transfection, medium was replaced with medium supplemented with 0.1 mM sodium butyrate (Sigma, St. Louis, USA). Cells were allowed to grow for an additional 24 h. The supernatant was harvested, centrifuged at 16,000 rpm for 5 min at 4 °C, filtered through a 0.45-μm pore filter (Millipore, Bedford, MA), prior to use in neutralization assays.

HIV-1 neutralization assays

Single-round infectious molecular clones, produced by envelope complementation as described above, were used. The degree of virus neutralization by antibody was achieved by measuring luciferase activity as described previously (Platt et al., 1998). Briefly, neutralization assays were carried out in triplicate by preincubation of 25 μl of 2-fold serial dilution of mAbs with 25 μl of pseudovirus suspension for 1 h at 4 °C. Virus–antibody mixtures were then combined with 150 μl suspensions of 1–2 × 10^4 HOS CD4$^+$ CCR5$^+$/CXCR4$^+$, in 96 wells of tissue culture plates (Costar, Corning, NY). Similar experiments were done with HeLa cells expressing different concentrations of CCR5, high CCR5 (clone JC.53) or low CCR5 (clone JC.10) (Choudhry et al., 2006). Plates were incubated at 37 °C in 5% CO_2 for 3 days and then washed with PBS and lysed for 30 min with 15 μl of 1 × Luciferase Assay System cell lysis buffer (Promega, Madison, WI), and luminescence readings for triplicate wells were determined by lumiCount microplate luminometer (Turner Designs). Neutralization titers were determined based on relative luminescence

units (RLU) and neutralization endpoint was the last concentration of mAbs at which mean results from the test samples were less than 50% of non-neutralized control mean. IC_{50} of neutralization was assigned for the antibody concentration at which 50% neutralization was observed. Neutralization assays for each Env were generally repeated in at least two independent experiments

Testing of m46 with a panel of clade C isolates (CCR5 phenotype), was also performed by a pseudovirus assay (designated here as TZM-bl assay), which is similar to the assay described above, but TZM-bl cells were used as targets for infection (Li et al., 2005). The clade C, Du gp160 clones used in TZM-bl assay were derived from acute/early sexually acquired infections in South Africa (Bures et al., 2000). The same panel of clade C and other primary isolates were also tested by using an assay based on inhibition of HIV-1 infection in PBMCs by measuring p24 (Bures et al., 2002).

Acknowledgments

We wish to thank Gabriela Stiegler and Hermann Katinger for providing 2F5 and 4E10, and Michael Zwick and Dennis Burton for providing DNA encoding Z13. This research was supported by the NIH Intramural AIDS Targeted Antiviral Program (IATAP) and the Gates Foundation to D.S.D., the Intramural Research Program of the NIH, National Cancer Institute, and Center for Cancer Research, with Federal funds from the NIH, National Cancer Institute, under Contract No. NO1-CO-12400, NIH grants AI48280 to G.V.Q. and C.C.B., and AI37438 and AI64070 to G.V.Q. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

Barbas, C.F., Burton, D.R., Scott, J.K., Silverman, G.J., 2001. Phage Display: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Binley, J.M., Wrin, T., Korber, B., Zwick, M.B., Wang, M., Chappey, C., Stiegler, G., Kunert, R., Zolla-Pazner, S., Katinger, H., Petropoulos, C.J., Burton, D.R., 2004. Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. J. Virol. 78, 13232–13252.

Bures, R., Gaitan, A., Zhu, T., Graziosi, C., McGrath, K.M., Tartaglia, J., Caudrelier, P., El, H.R., Klein, M., Lazzarin, A., Stablein, D.M., Deers, M., Corey, L., Greenberg, M.L., Schwartz, D.H., Montefiori, D.C., 2000. Immunization with recombinant canarypox vectors expressing membrane-anchored glycoprotein 120 followed by glycoprotein 160 boosting fails to generate antibodies that neutralize R5 primary isolates of human immunodeficiency virus type 1. AIDS Res. Hum. Retroviruses 16, 2019–2035.

Bures, R., Morris, L., Williamson, C., Ramjee, G., Deers, M., Fiscus, S.A., bdool-Karim, S., Montefiori, D.C., 2002. Regional clustering of shared neutralization determinants on primary isolates of clade C human immunodeficiency virus type 1 from South Africa. J. Virol. 76, 2233–2244.

Burton, D.R., 2002. Antibodies, viruses and vaccines. Nat. Rev., Immunol. 2, 706–713.

Burton, D.R., Montefiori, D.C., 1997. The antibody response in HIV-1 infection. AIDS 11 (Suppl. A), S87–S98.

Burton, D.R., Pyati, J., Koduri, R., Sharp, S.J., Thornton, G.B., Parren, P.W., Sawyer, L.S., Hendry, R.M., Dunlop, N., Nara, P.L., et al., 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science 266, 1024–1027.

Cham, F., Zhang, P.F., Heyndrickx, L., Bouma, P., Zhong, P., Katinger, H., Robinson, J., van der, G.G., Quinnan Jr., G.V., 2006. Neutralization and infectivity characteristics of envelope glycoproteins from human immunodeficiency virus type 1 infected donors whose sera exhibit broadly cross-reactive neutralizing activity. Virology 347, 36–51.

Choudhry, V., Zhang, M.Y., Harris, I., Sidorov, I.A., Vu, B.K., Dimitrov, D.S., 2006. Increased efficacy of HIV-1 neutralization by antibodies at low CCR5 surface concentration. Biochem. Biophys. Res. Commun. 348, 1107–1115.

Dong, M., Zhang, P.F., Grieder, F., Lee, J., Krishnamurthy, G., VanCott, T., Broder, C., Polonis, V.R., Yu, X.F., Shao, Y., Faix, D., Valente, P., Quinnan Jr., G.V., 2003. Induction of primary virus-cross-reactive human immunodeficiency virus type 1-neutralizing antibodies in small animals by using an alphavirus-derived in vivo expression system. J. Virol. 77, 3119–3130.

Dreyer, K., Kallas, E.G., Planelles, V., Montefiori, D., McDermott, M.P., Hasan, M.S., Evans, T.G., 1999. Primary isolate neutralization by HIV type 1-infected patient sera in the era of highly active antiretroviral therapy. AIDS Res. Hum. Retroviruses 15, 1563–1571.

D'Souza, M.P., Livnat, D., Bradac, J.A., Bridges, S.H., AIDS Clinical Trials Group Antibody Selection Working Group, 1997. Evaluation of monoclonal antibodies to human immunodeficiency virus type 1 primary isolates by neutralization assays: performance criteria for selecting candidate antibodies for clinical trials. J. Infect. Dis. 175, 1056–1062.

Earl, P.L., Broder, C.C., Long, D., Lee, S.A., Peterson, J., Chakrabarti, S., Doms, R.W., Moss, B., 1994. Native oligomeric human immunodeficiency virus type 1 envelope glycoprotein elicits diverse monoclonal antibody reactivities. J. Virol. 68, 3015–3026.

Earl, P.L., Broder, C.C., Doms, R.W., Moss, B., 1997. Epitope map of human immunodeficiency virus type 1 gp41 derived from 47 monoclonal antibodies produced by immunization with oligomeric envelope protein. J. Virol. 71, 2674–2684.

Earl, P.L., Sugiura, W., Montefiori, D.C., Broder, C.C., Lee, S.A., Wild, C., Lifson, J., Moss, B., 2001. Immunogenicity and protective efficacy of oligomeric human immunodeficiency virus type 1 gp140. J. Virol. 75, 645–653.

Ferrantelli, F., Ruprecht, R.M., 2002. Neutralizing antibodies against HIV—Back in the major leagues? Curr. Opin. Immunol. 14, 495–502.

Garber, D.A., Silvestri, G., Feinberg, M.B., 2004. Prospects for an AIDS vaccine: three big questions, no easy answers. Lancet, Infect. Dis. 4, 397–413.

Gorny, M.K., Conley, A.J., Karwowska, S., Buchbinder, A., Xu, J.Y., Emini, E.A., Koenig, S., Zolla-Pazner, S., 1992. Neutralization of diverse human immunodeficiency virus type 1 variants by an anti-V3 human monoclonal antibody. J. Virol. 66, 7538–7542.

Johnson, W.E., Desrosiers, R.C., 2002. Viral persistance: HIV's strategies of immune system evasion. Annu. Rev. Med. 53, 499–518.

Li, M., Gao, F., Mascola, J.R., Stamatatos, L., Polonis, V.R., Koutsoukos, M., Voss, G., Goepfert, P., Gilbert, P., Greene, K.M., Bilska, M., Kothe, D.L., Salazar-Gonzalez, J.F., Wei, X., Decker, J.M., Hahn, B.H., Montefiori, D.C., 2005. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol. 79, 10108–10125.

Louis, J.M., Bewley, C.A., Gustchina, E., Aniana, A., Clore, G.M., 2005. Characterization and HIV-1 fusion inhibitory properties of monoclonal Fabs obtained from a human non-immune phage library selected against diverse epitopes of the ectodomain of HIV-1 gp41. J. Mol. Biol. 353, 945–951.

Montefiori, D.C., Pantaleo, G., Fink, L.M., Zhou, J.T., Zhou, J.Y., Bilska, M., Miralles, G.D., Fauci, A.S., 1996. Neutralizing and infection-enhancing antibody responses to human immunodeficiency virus type 1 in long-term nonprogressors. J. Infect. Dis. 173, 60–67.

Moulard, M., Phogat, S.K., Shu, Y., Labrijn, A.F., Xiao, X.D., Binley, J.M., Zhang, M.Y., Sidorov, I.A., Broder, C.C., Robinson, J., Parren, P.W.H.I., Burton, D.R., Dimitrov, D.S., 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, 6913–6918.

Muster, T., Steindl, F., Purtscher, M., Trkola, A., Klima, A., Himmler, G., Ruker, F., Katinger, H., 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J. Virol. 67, 6642–6647.

Platt, E.J., Wehrly, K., Kuhmann, S.E., Chesebro, B., Kabat, D., 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. J. Virol. 72, 2855–2864.

Poignard, P., Saphire, E.O., Parren, P.W., Burton, D.R., 2001. Gp120: biologic aspects of structural features. Annu. Rev. Immunol. 19, 253–274.

Quinnan, G.V., Zhang, P.F., Fu, D.W., Dong, M., Alter, H.J., 1999. Expression and characterization of HIV type 1 envelope protein associated with a broadly reactive neutralizing antibody response. AIDS Res. Hum. Retroviruses 15, 561–570.

Quinnan Jr., G.V., Yu, X.F., Lewis, M.G., Zhang, P.F., Sutter, G., Silvera, P., Dong, M., Choudhary, A., Sarkis, P.T., Bouma, P., Zhang, Z., Montefiori, D.C., VanCott, T.C., Broder, C.C., 2005. Protection of rhesus monkeys against infection with minimally pathogenic simian-human immunodeficiency virus: correlations with neutralizing antibodies and cytotoxic T cells. J. Virol. 79, 3358–3369.

Richman, D.D., Wrin, T., Little, S.J., Petropoulos, C.J., 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc. Natl. Acad. Sci. U.S.A. 100, 4144–4149.

Roben, P., Moore, J.P., Thali, M., Sodroski, J., Barbas III, C.F., Burton, D.R., 1994. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. J. Virol. 68, 4821–4828.

Root, M.J., Kay, M.S., Kim, P.S., 2001. Protein design of an HIV-1 entry inhibitor. Science 291, 884–888.

Sanders, R.W., Venturi, M., Schiffner, L., Kalyanaraman, R., Katinger, H., Lloyd, K.O., Kwong, P.D., Moore, J.P., 2002. The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. J. Virol. 76, 7293–7305.

Scanlan, C.N., Pantophlet, R., Wormald, M.R., Ollmann, S.E., Stanfield, R., Wilson, I.A., Katinger, H., Dwek, R.A., Rudd, P.M., Burton, D.R., 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, 7306–7321.

Stiegler, G., Kunert, R., Purtscher, M., Wolbank, S., Voglauer, R., Steindl, F., Katinger, H., 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, 1757–1765.

Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J.P., Katinger, H., 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol. 70, 1100–1108.

Vujcic, L.K., Quinnan Jr., G.V., 1995. Preparation and characterization of human HIV type 1 neutralizing reference sera. AIDS Res. Hum. Retroviruses 11, 783–787.

Wei, X., Decker, J.M., Wang, S., Hui, H., Kappes, J.C., Wu, X., Salazar-Gonzalez, J.F., Salazar, M.G., Kilby, J.M., Saag, M.S., Komarova, N.L., Nowak, M.A., Hahn, B.H., Kwong, P.D., Shaw, G.M., 2003. Antibody neutralization and escape by HIV-1. Nature 422, 307–312.

Zhang, P.F., Bouma, P., Park, E.J., Margolick, J.B., Robinson, J.E., Zolla-Pazner, S., Flora, M.N., Quinnan Jr., G.V., 2002. A variable region 3 (V3) mutation determines a global neutralization phenotype and CD4-independent infectivity of a human immunodeficiency virus type 1 envelope associated with a broadly cross-reactive, primary virus-neutralizing antibody response. J. Virol. 76, 644–655.

Zhang, M.Y., Shu, Y., Phogat, S., Xiao, X., Cham, F., Bouma, P., Choudhary, A., Feng, Y.R., Sanz, I., Rybak, S., Broder, C.C., Quinnan, G.V., Evans, T., Dimitrov, D.S., 2003. Broadly cross-reactive HIV neutralizing human monoclonal antibody Fab selected by sequential antigen panning of a phage display library. J. Immunol. Methods 283, 17–25.

Zhang, M.Y., Xiao, X., Sidorov, I.A., Choudhry, V., Cham, F., Zhang, P.F., Bouma, P., Zwick, M., Choudhary, A., Montefiori, D.C., Broder, C.C., Burton, D.R., Quinnan Jr., G.V., Dimitrov, D.S., 2004. Identification and characterization of a new cross-reactive human immunodeficiency virus type 1-neutralizing human monoclonal antibody. J. Virol. 78, 9233–9242.

Zhang, M.Y., Choudhry, V., Sidorov, I.A., Tenev, V., Vu, B.K., Choudhary, A., Lu, H., Stiegler, G.M., Katinger, H.W., Jiang, S., Broder, C.C., Dimitrov, D.S., 2006. Selection of a novel gp41-specific HIV-1 neutralizing human antibody by competitive antigen panning. J. Immunol. Methods 317, 21–30.

Zolla-Pazner, S., 2004. Identifying epitopes of HIV-1 that induce protective antibodies. Nat. Rev., Immunol. 4, 199–210.

Zwick, M.B., Labrijn, A.F., Wang, M., Spenlehauer, C., Saphire, E.O., Binley, J.M., Moore, J.P., Stiegler, G., Katinger, H., Burton, D.R., Parren, P.W., 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J. Virol. 75, 10892–10905.