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Assessment of antibody responses against gp41 in HIV-1-infected patients using soluble gp41 fusion proteins and peptides derived from M group consensus envelope

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Abstract

Human immunodeficiency virus type 1 (HIV-1) transmembrane glycoprotein gp41 is targeted by broadly-reactive neutralizing antibodies 2F5 and 4E10, making it an attractive target for vaccine development. To better assess immunogenic properties of gp41, we generated five soluble glutathione S-transferase fusion proteins encompassing C-terminal 30, 64, 100, 142, or 172 (full-length) amino acids of gp41 ectodomain from M group consensus envelope sequence. Antibody responses in HIV-1-infected patients were evaluated using these proteins and overlapping peptides. We found (i) antibody responses against different regions of gp41 varied tremendously among individual patients, (ii) patients with stronger antibody responses against membrane-proximal external region exhibit broader and more potent neutralizing activity, and (iii) several patients mounted antibodies against epitopes that are near, or overlap with, those targeted by 2F5 or 4E10. These soluble gp41 fusion proteins could be an important source of antigens for future vaccine development efforts.

Keywords: HIV-1; gp41; Neutralizing antibody; MPER

Introduction

During human immunodeficiency virus type 1 (HIV-1) infection, many antibodies are elicited against viral envelope glycoprotein. The vast majority of these antibodies are non-neutralizing, and those that neutralize are mostly isolate-specific (for reviews, see (Burton et al., 2005; Poignard et al., 2001)). Results from both active and passive immunization studies indicate that pre-existing neutralizing antibodies (Nabs) can confer protection against HIV-1 infection (Baba et al., 2000; Cho et al., 2001; Conley et al., 1996; Mascola et al., 2000; Parren et al., 1995; Shibata et al., 1999; Trkola et al., 2005). However, the precise role of humoral immunity in controlling natural HIV-1 infection is not yet clear. Better understanding of antibody responses against HIV-1 envelope glycoproteins in virus-infected patients may facilitate development of a protective vaccine against the virus.

Antisera that exhibit broadly neutralizing activity against diverse HIV-1 isolates have been observed in some long-term non-progressors (LTNP) (Braibant et al., 2006; Cecilia et al., 1999; Pilgrim et al., 1997). However, they are rare; despite over two decades of AIDS research, only a handful of broadly reactive Nabs (BR-Nabs) have been identified, including monoclonal antibodies (mAbs) b12, 2G12, 447-52D, 2F5, 4E10 and m48 (Gorny et al., 1992; Muster et al., 1993; Roben et al., 1994; Stiegler et al., 2001; Trkola et al., 1996; Zhang et al., 2006; Zwick et al., 2001). While the first three antibodies are gp120-

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specific, the latter three target gp41. Antibodies that target gp41 are of great interest from a vaccine development standpoint because they are more cross-reactive against viruses from different clades than those directed against gp120 (Binley et al., 2004; Burton et al., 2004; Opalka et al., 2004; Yuste et al., 2006; Zwick et al., 2001).

Monoclonal antibodies 2F5 and 4E10 target adjacent, but distinct, linear epitopes in a highly conserved region of gp41 near the viral membrane known as the membrane-proximal external region (MPER) (Muster et al., 1993; Stiegler et al., 2001; Zhang et al., 2006; Zwick et al., 2001), a determinant that plays a critical role in HIV-1 fusion with the cell membrane (Salzwedel et al., 1999; Suarez et al., 2000). Epitope mapping studies of 2F5 with synthetic peptides (Barbato et al., 2003; Biron et al., 2002; Joyce et al., 2002), phage displayed peptide libraries (Menendez et al., 2004; Muster et al., 1993; Zwick et al., 2001), and protease protection assays (Parker et al., 2001) have identified ELDKWA as the core antibody binding site. 4E10 binds primarily to the hexapeptide NWFNIT, which lies just four amino acids downstream of the 2F5 epitope (Brunel et al., 2006; Cardoso et al., 2005; Stiegler et al., 2001; Zwick et al., 2001). The epitope for m48 has not yet been precisely defined although it is thought to be distinct from those recognized by 2F5 or 4E10, and highly conformational, requiring proper disulfide bond formation (Zhang et al., 2006).

During the past two decades, much of HIV-1 vaccine development efforts have focused on gp120. Consequently, much less is known about the immunological properties of gp41. Efforts to evaluate immunogenicity of gp41 have been hampered by the fact that the protein, either as a whole or in part, is difficult to express in soluble forms in the absence of gp120 (Gairin et al., 1991; Luo et al., 2006; Qiao et al., 2005; Scholz et al., 2005; Weissenhorn et al., 1997a). Moreover, the large number of highly immunogenic epitopes on gp120 renders gp160 or gp140 unsuitable for assessing immunogenic properties of gp41, particularly against the MPER (Pantophlet and Burton, 2003; Wei et al., 2003). In addition, assessment of antibody responses against gp41 in virus-infected patients has been done mostly in the context of short, synthetic peptides (Calarota et al., 1996; Gnann et al., 1987; Goudsmit et al., 1990; Horal et al., 1991; Schrier et al., 1988). While peptides are suitable for identifying linear epitopes, antibody responses to non-contiguous, conformational epitopes cannot be assessed.

To date, most efforts to express soluble forms of gp41 have been limited to small fragments of the protein, including the heptad repeat (HR) regions (Root et al., 2001), the immunodominant loop between HR1 and HR2 known as cluster I (Gnann et al., 1987), a region between HR2 and the 2F5 epitope known as cluster II (Binley et al., 1996; Goudsmit et al., 1990; Xu et al., 1991), and the MPER (Luo et al., 2006; Opalka et al., 2004). Although Scholz et al. (Scholz et al., 2005) have reported generating a larger 146 amino acid gp41 fragment (residues 536–681) fused to *E. coli* chaperone SlyD and showed its immunoreactivity with HIV-1 patient sera, no other immunoprobing was performed using mAbs to confirm antigenic integrity of the protein.

In this study, we systematically generated five glutathione *S*-transferase (GST)-fusion proteins, which consist of C-terminal 30, 64, 100, 142, or 172 (full-length) amino acid residues of the gp41 ectodomain from an M group consensus envelope sequence (MCON6). They were expressed in *E. coli*, solubilized and purified. These proteins, in conjunction with consensus overlapping peptides, were used to evaluate antibody responses against gp41 in HIV-1-infected patients.

Results

Design, expression and purification of GST-gp41 fusion proteins

To assess antibody responses against conformational, non-contiguous epitopes on gp41 in HIV-1-infected patients, we constructed five gp41 fragments fused to glutathione-S-transferase (GST). GST was chosen as a fusion partner to facilitate protein refolding process. Proteins are truncated N-terminally, each about 30–40 amino acids apart, containing the C-terminal 30, 64, 100, 142, or the entire 172 a.a. of the protein (Fig. 1A). Therefore, each is designed to encompass the MPER containing the epitopes recognized by mAbs 2F5 and 4E10. The truncation sites were chosen to be either just up- or down-stream of HR domains. In addition, sites were chosen so that the N-terminal ends of the gp41 fragments would be charged amino acids (i.e. RQ for -142, ER for -100, DE for -64, and EK for -30). Proteins were tagged with six-histidine residues at the C-terminus to facilitate protein renaturation and purification. In order to enhance protein recognition by antisera from patients infected with a wide range of primary isolates from different clades, we chose to generate our gp41 fragments from an envelope from the M group consensus sequence (MCON6).

All five fusion proteins were expressed efficiently in *E. coli* BL21(DE3) upon induction with IPTG (Figs. 1B and C). As expected, all five proteins were insoluble. Anticipating difficulty in solubilizing two larger gp41 fragments, our initial efforts focused on GST-gp41-30, -64 and -100. Bacterial pellets were sonicated, and inclusion bodies were isolated and denatured in 8 M urea. Solubilized proteins were bound to Ni-NTA resin. Subsequently, proteins were renatured gradually by sequential incubation with decreasing concentrations of urea (8 M, 6 M, 4 M, 3 M, 2 M and 1 M) before a final wash with PBS and elution from the resin using imidazole. Eluted proteins were finally dialyzed in PBS. This single-step purification/renaturation procedure yields about 37, 40, and 20 mg per liter for GST-gp41-30, -64, and -100, respectively, with >85–90% purity (Fig. 2A). The identities of the purified proteins were confirmed by Western immunoblot using anti-GST antibody (Fig. 2B). A minor band of unknown identity (~23 kDa) was co-purified with GST-gp41-64 (Fig. 2A). The contaminant is likely a cleavage product of GST since it is immunoreactive to anti-GST-antibody (Fig. 2B). To verify that our fusion proteins are antigenically correct, they were subjected to immunoprecipitation analyses using BR-Nabs 2F5 and 4E10, followed by Western immunoblot with anti-GST antibody. As shown in Figs. 2C and D, all three proteins were recognized by 2F5 and 4E10, respectively. Recognition of GST-gp41-30 by 4E10, however, appeared somewhat weaker than that seen against GST-gp41-64 and -100. No binding was observed for GST

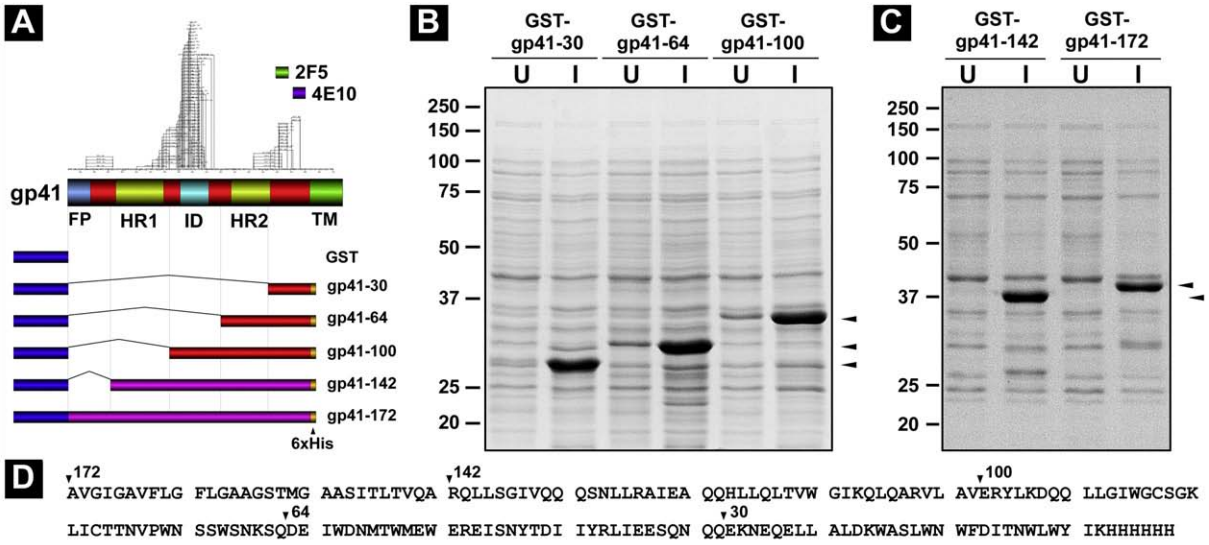

Fig. 1. Construction and expression of GST-gp41 fusion proteins. (A) A schematic diagram of gp41 and the five GST-gp41 fusion proteins generated. Key features of gp41 are indicated. Linear B cell epitopes identified to date are shown above gp41, and 2F5 and 4E10 binding sites are indicated. FP = Fusion Peptide; HR = Heptad Repeat; ID = Immunodominant region; TM = Transmembrane domain. (B and C) SDS-PAGE analyses of GST-gp41 fusion protein expression in uninduced (U) and induced (I) *E. coli* cells. Arrows indicate GST-gp41 fusion proteins. Gels were stained with Coomassie Blue. (D) Sequence of MCON6 gp41 ectodomain with 6x-His tag. The starting residues of each construct are indicated by an inverted triangle.

protein, demonstrating specific recognition of gp41 MPER by 2F5 and 4E10.

Having successfully generated soluble GST-gp41-30, -64, and -100, we pursued generating soluble GST-gp41-142 and -172. Solubilization of the two larger proteins was more difficult. Initially, we followed the same protocol used to solubilize the smaller proteins. However, this resulted in precipitation of the proteins even at 6 M urea. We hypothesized that a slower transition from 8 M to 6 M should provide more time needed for the protein to refold into the conformation that would render the protein soluble. Using a continuous, shallow gradient (see Materials and methods for details), we were able to maintain a significant portion of the protein soluble (Fig. 2E). Typical final yields for GST-gp41-142 and -172 were about 5.4 and 4.5 mg/l, respectively, which are significantly lower than for the smaller fusion proteins, but sufficient for our studies. In addition to the difficulty in solubilizing these proteins, we observed that these proteins would precipitate upon repeated freezing-and-thawing. To prevent protein precipitation, working stocks of the proteins were kept at near 0 °C (ice bath) in a cold room.

Characterization of antigenic properties of GST-gp41 fragments

ELISA was performed to evaluate the antigenic properties of purified gp41 fusion proteins more quantitatively. Proteins were probed with BR-Nabs 2F5 and 4E10, polyclonal HIV-Ig (from pooled HIV-1 patient sera), and mAb 98-6, which recognizes the coiled-coil structure of the HR1 and HR2 regions (5-helix or

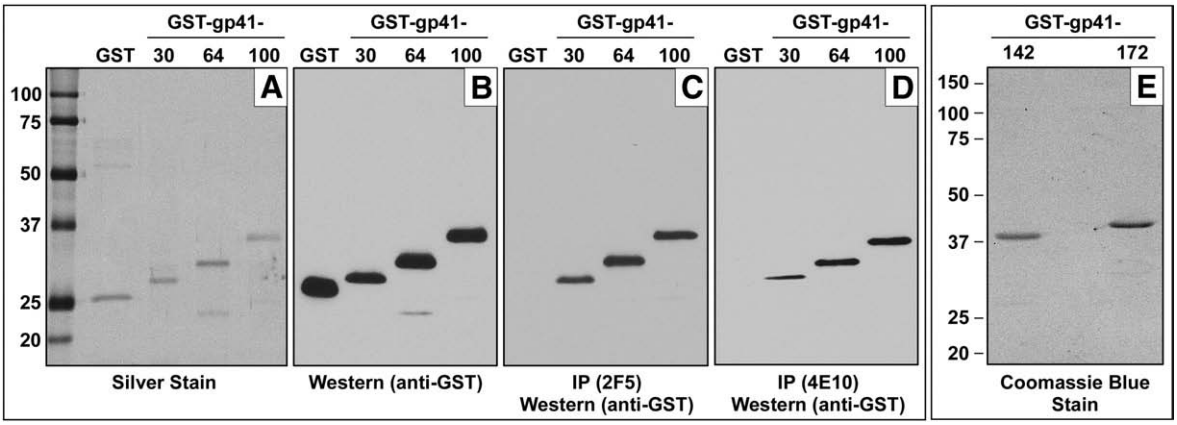

Fig. 2. Purification and immunoprecipitation analyses of GST-gp41 fusion proteins. GST and three gp41 variants (-30, -64 and -100) were initially expressed and purified. Proteins were analyzed by silver stain (A), Western blot with anti-GST antibody (B), and immunoprecipitation with 2F5 (C) or 4E10 (D) followed by Western blot with anti-GST antibody. (E) Coomassie Blue-stained SDS-PAGE analyses of purified GST-gp41-142 and -172.

6-helix bundle) (Gorny et al., 1989; Taniguchi et al., 2000). Wells were coated with equimolar amounts of the proteins to assess relative antigenicity.

2F5 was similarly reactive against all five gp41 protein fragments, indicating that the epitope recognized by the antibody is conformationally similar and equally exposed among all five proteins (Fig. 3A). In contrast, some variations in recognition of the proteins by 4E10 were observed despite the fact that all proteins contain the same epitope recognized by the antibody (Fig. 3B). Compared to GST-gp41-64, -142, and -172, which were equally reactive, GST-gp41-30 and -100 were about 20- and 5-fold less reactive, respectively. This was somewhat surprising considering that the 4E10 binding site is only 4 amino acids downstream of 2F5 epitope. In addition, 4E10 binding strength was significantly weaker against the five proteins compared with 2F5, requiring approximately 5 to 100 fold higher concentrations (Fig. 3B). This was not unexpected since 4E10 exhibits relatively weak, albeit broad, neutralizing activity (Binley et al., 2004).

98-6 reacted strongly against GST-gp41-142 and -172, indicating that these proteins exist in post-hairpin formation configuration (Fig. 3C). As predicted, the three smaller proteins were not detected by 98-6 since they do not contain both heptad repeat regions required to form the coiled-coil structure. Similar to 98-6, HIV-Ig reacted most strongly against GST-gp41-172 and -142 (Fig. 3D). Although GST-gp41-100, -64 and -30 fusion proteins were significantly less reactive with HIV-Ig compared to -172 and -142, antibodies against them could be detected using higher concentrations of the antibody and longer enzymatic reaction time (Fig. 3E). Among the three smaller proteins, HIV-Ig reacted most strongly against GST-gp41-100, which was expected since it contains the immunodominant domain of gp41 (Fig. 1A). GST-gp41-64 was also reactive, albeit less than GST-gp41-100. In contrast, GST-gp41-30 reacted very weakly, supporting the notion that the MPER is poorly immunogenic in the context of natural HIV-1 infection.

Characterization of antibody responses against gp41 in HIV-1-infected patients

Evaluating antibody responses against HIV-1 envelope glycoproteins using HIV-Ig provides only an overall picture because antibodies are prepared from a large number of virus-infected patient sera. We hypothesized that we might see some differences amongst individual patients considering significant polymorphism in the host immune system and/or variation in viral genome. The results from studies described above demonstrated that the soluble GST-fusion proteins we generated are antigenically intact and suitable for assessing immune responses against gp41 in HIV-1-infected patients.

Since all of the epitopes targeted by BR-Nabs identified to date map within the C-terminal 100 amino acids, we focused our efforts on characterizing antibody responses against GST-gp41-30, -64, and -100. Archived plasma samples from 44 HIV-1-infected patients were evaluated by ELISA using equimolar amounts of the three GST fusion proteins. Purified GST protein was used as a negative control. Results are shown in Fig. 4. Patient samples were arranged in descending order of magnitude of antibody reactivity against GST-gp41-30 (numbered CWRU-

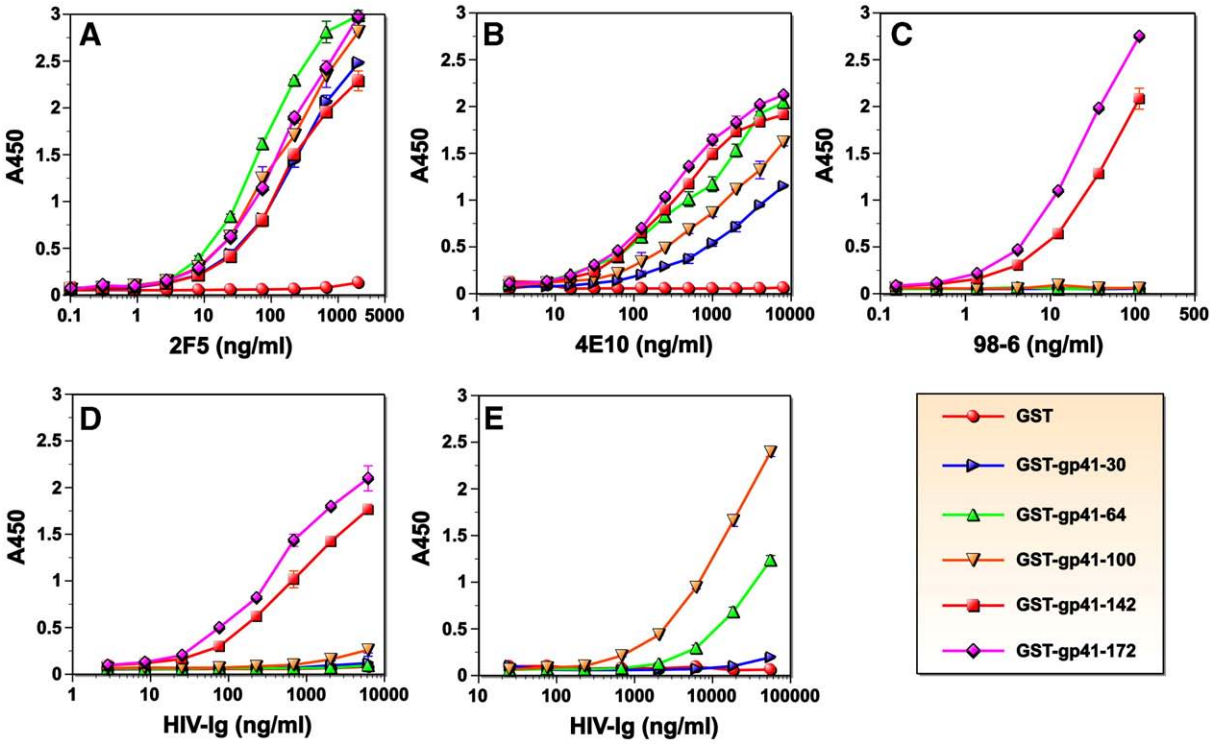

Fig. 3. Characterization of antigenic properties of GST-gp41 fusion proteins. Purified GST and GST-gp41 fusion proteins were analyzed by ELISA using mAbs 2F5 (A), 4E10 (B), 98-6 (C) and polyclonal HIV-Ig (D and E). Equimolar amounts (0.5 pmol) of GST or GST-gp41 fusion proteins were coated in each well. To detect three smaller fusion proteins by HIV-Ig, higher concentrations of the antibody were used with extended enzymatic reaction time in panel (E).

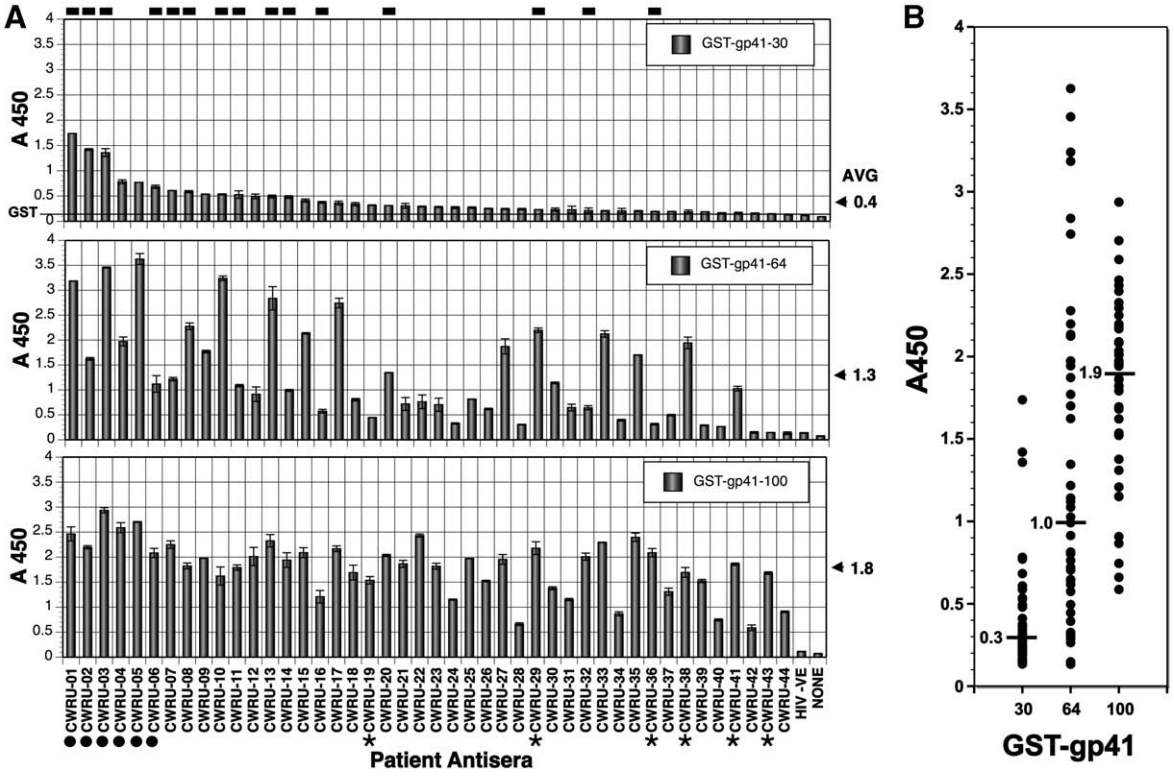

Fig. 4. Antibody responses against gp41 in individual patients. (A) ELISA was performed with plasma samples from HIV-1-infected patients at a dilution factor of 1:300. Plasma from an uninfected individual (HIV-ve) and no plasma were used as controls. Plasma samples are arranged in descending order of reactivity to GST-gp41-30. Six samples exhibiting the highest reactivity (●) and six samples with lower reactivity (*), which are further evaluated in Figs. 5 and 6, are indicated. Patients who have been infected longer than 5 years with CD4 counts >500 and never been on anti-retroviral therapy are indicated by black bars on the top. For clarity, average A450 value of GST only is shown as a line indicated as GST. Arrowheads indicate average A450 values against each fusion protein for all patients. Equimolar amounts (0.5 pmol) of GST or GST-gp41 fusion proteins were coated in each well. (B) Dot plot analyses of ELISA data showing distribution of antibody reactivity against each fusion protein. Median values are shown.

1 though -44). Of all forty-four patients, only two (CWRU-19 and -41) were on anti-retroviral therapy at the time when the plasma samples were taken.

Overall, the strongest antibody responses were detected using GST-gp41-100, followed by -64 and -30, consistent with what we observed using HIV-Ig (Fig. 3E); the mean A450 values were 1.8, 1.3 and 0.4 (Fig. 4A), and the median values were 1.9, 1.0 and 0.3 (Fig. 4B), respectively. Interestingly, we observed tremendous variation amongst individual patients in antibody reactivity against our three gp41 fragments, both in terms of the magnitude and binding pattern, as we hypothesized. Some of the patients exhibited very low antibody titers against all three gp41 fragments (e.g. CWRU-28, -34, -40 and -42), whereas others mounted strong antibody responses against all of them (e.g. CWRU-1, -3 and -5). While some patients showed good binding against only the GST-gp41-100 (e.g. CWRU-22, -25, -32, -36, -39, -43), other patients exhibited good reactivity against both GST-gp41-100 and -64, but not -30 (e.g. CWRU-29, -33, -35, -38). In general, antibody responses against GST-gp41-64 were most variable with standard deviation of 0.98, compared to 0.35 and 0.55 of GST-gp41-30 and -100, respectively (Fig. 4B). Interestingly, markedly greater antibody responses were observed against GST-gp41-64 than against -100 in seven patients (CWRU-1, -3, -5, -8, -10, -13 and -17), despite the fact that the latter protein is larger. One possible explanation is that immunogenic epitopes recognized on GST-gp41-64 in these particular patients are buried in the context of GST-gp41-100.

Perhaps the most intriguing aspect of this study was that there were many patients who mounted strong antibody responses against gp41 MPER, three of them in particular, as demonstrated by antibody reactivity to GST-gp41-30. Retrospective analyses of the patients showed that many patients who might be considered as “slow progressors” (infected longer than 5 years, CD4 T-cell count >500, and never been on anti-retroviral therapy; indicated by black bars on top of Fig. 4A) generally exhibited higher antibody reactivity against GST-gp41-30. Previous studies have shown that BR-Nabs are stronger and more frequent in LTNPs than other infected patients (Cecilia et al., 1999; Pilgrim et al., 1997). This result raised a possibility that some of these patients could have BR-Nabs targeting the MPER, some of which could exhibit 2F5- or 4E10-like properties.

Antisera from HIV-1-infected patients with stronger reactivity against gp41 MPER exhibit broader and more potent neutralizing activity

To test our hypothesis that patients who mount stronger antibody responses against gp41-MPER might have broader neutralizing activity, we compared neutralizing activity of antisera

from six patients with the highest reactivity against GST-gp41-30 (CWRU-1 to -6) and six other patients with lower reactivity (CWRU-19, -29, -36, -38, -41 and -43) indicated by dots and asterisks in Fig. 4A, respectively. The latter samples were chosen semi-randomly to make sure they had good antibody reactivity against GST-gp41-100. Neutralization assays were performed against four primary R5 or R5/X4 HIV-1 isolates (BAL, AD8, DH12 and 89.6) using a single-round pseudovirus infection assay. Viruses pseudotyped with vesicular stomatitis virus G protein (VSV-G) were used as a negative control.

Although there was no absolute correlation between antibody reactivity against GST-gp41-30 and breadth or potency of neutralizing activity on an individual patient basis, overall, the plasma from patients with higher reactivity did exhibit broader and more potent activity (Fig. 5A; $p < 0.01$). This was particularly true for patients CWRU-3, -4 and -6. It is also noteworthy that plasma samples from these patients efficiently neutralized HIV-1_{AD8}, which is one of the most difficult viruses to elicit neutralizing antibodies against (Bower et al., 2006; Cho et al., 2001; Kim et al., 2005, 2003); none of the six samples with lower GST-gp41-30 reactivity exhibited strong neutralizing activity against this virus. In contrast, plasma samples from all twelve patients neutralized HIV-$1_{89.6}$ quite effectively, suggesting that this virus might be particularly sensitive to neutralization despite being a primary isolate. Titration analyses of plasma samples from six patients with strong reactivity against GST-gp41-30 confirmed potent neutralizing activity of those from patients CWRU-3, -4 and -6 (Fig. 5B). The analyses showed that patient CWRU-5 also exhibited quite potent neutralizing activity against all viruses, albeit somewhat less effectively against HIV-1_{AD8}. None of the plasma samples neutralized viruses pseudotyped with VSV-G (data not shown), indicating specific, antibody-mediated neutralization of HIV-1.

Based on neutralization assays against four HIV-1 isolates, CWRU-4 exhibited the broadest and the most potent neutralizing activity. To determine further the extent of its breadth, neutralization assays were performed against 24 virus isolates, including sixteen from clade B, four from clade C, and four from clade A. As shown in Table 1, plasma samples from patient CWRU-4 were able to neutralize all of the viruses tested, albeit at different potency. Some isolates such as MN and SF162.LS are historically easily neutralizable "Tier 1" viruses (Mascola et al., 2005), whereas other viruses from a Standard Reference Panel of Subtype B HIV-1 Env Clones (NIH ARRRP Cat no. 11227) and Subtype A and C designed for Tier 2 and 3 studies are considered neither unusually sensitive nor resistant to neutralization. Based on our past experience (D. Montefiori), very few patient samples have this level of breadth and potency of neutralizing activity. At present, the epitope(s) targeted by these neutralizing antibodies are not known.

Identification of immunogenic linear epitopes within the C-terminal half of gp41 ectodomain

Despite the lack of absolute correlation between antibody reactivity against GST-gp41-30 and breadth or potency of neutralizing activity on an individual patient basis, the fact that four of six patients (CWRU-3, -4, -5 and -6) with the highest reactivity against the protein exhibited strong neutralizing activity suggested that something might be unique about these patients. To

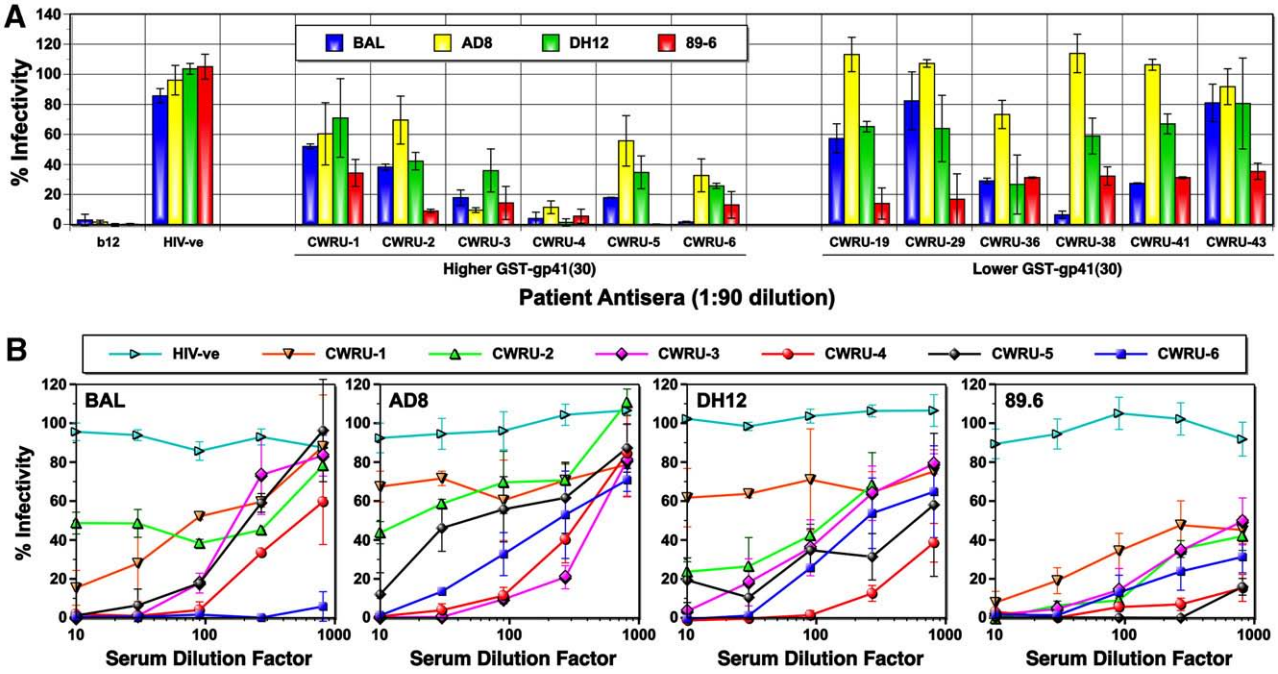

Fig. 5. Neutralizing activity of patient plasma samples. HIV-1 Env pseudotyped viruses (BAL, AD8, DH12 and 89.6) were used to assess neutralizing activity in a TZM-bl cell-based assay. Data are shown as a percentage of virus infectivity in the absence of plasma. (A) Twelve patients identified as having either high or low reactivity to GST-gp41-30 were analyzed at a single plasma dilution factor of 1:90. MAb b12 (12.5 μg/ml) and uninfected patient (HIV-ve) were used as controls. None of the plasma samples neutralized VSV-G pseudotyped virus (data not shown). (B) Titration analyses of neutralizing activity of plasma samples from six patients who exhibited strong reactivity to GST-gp41-30.

Table 1
Neutralizing activity of plasma samples from patient CWRU-4

Virus	Clade	ID50	Virus	Clade	ID50	Virus	Clade	ID50
MN	B	7103	TRO.11	B	828	Du156.12	C	511
Bal.26	B	1093	AC10.0.29	B	912	Du172.17	C	969
SF162.LS	B	43,740	RHPA4259.7	B	1,229	ZM197M.PB7	C	176
SS1196.1	B	807	THRO4156.18	B	311	CAP210.2.00.E8	C	175
6535.3	B	1091	REJO4541.67	B	1,517	Q842.d12	A	139
QH0692.42	B	129	TRJO4551.58	B	262	Q168.a2	A	216
SC422661.8	B	144	WITO4160.33	B	60	Q461.e2	A	37
PVO.4	B	457	CAAN5342.A2	B	215	Q769.d22	A	181

further characterize antibody responses in these patients, immunogenic linear epitopes were examined by ELISA using 30 overlapping peptides (15-mers, 11 a.a. overlap) spanning amino acid residues 572 through 702 (numbering based on MCON6; 569–699 on HxB2).

Out of 30 peptides, three were recognized strongly by all six patients (Fig. 6A). Two peptides (596–610: LGIWGCSGKLICTTT, and 600–614: GCSGKLICTTTVPWN) were within the immunodominant domain of cluster I (designated a.a. 579–604 on HxB2: RVLAVERYLKDQQLLGIWGCSGKLIC; (Binley et al., 1996)). Since both peptides exhibited very similar antibody reactivity profile, the core epitope likely consist of residues GCSGKLICTTT, which contains both cysteine residues previously reported to be critical for recognition (Gnann et al., 1987).

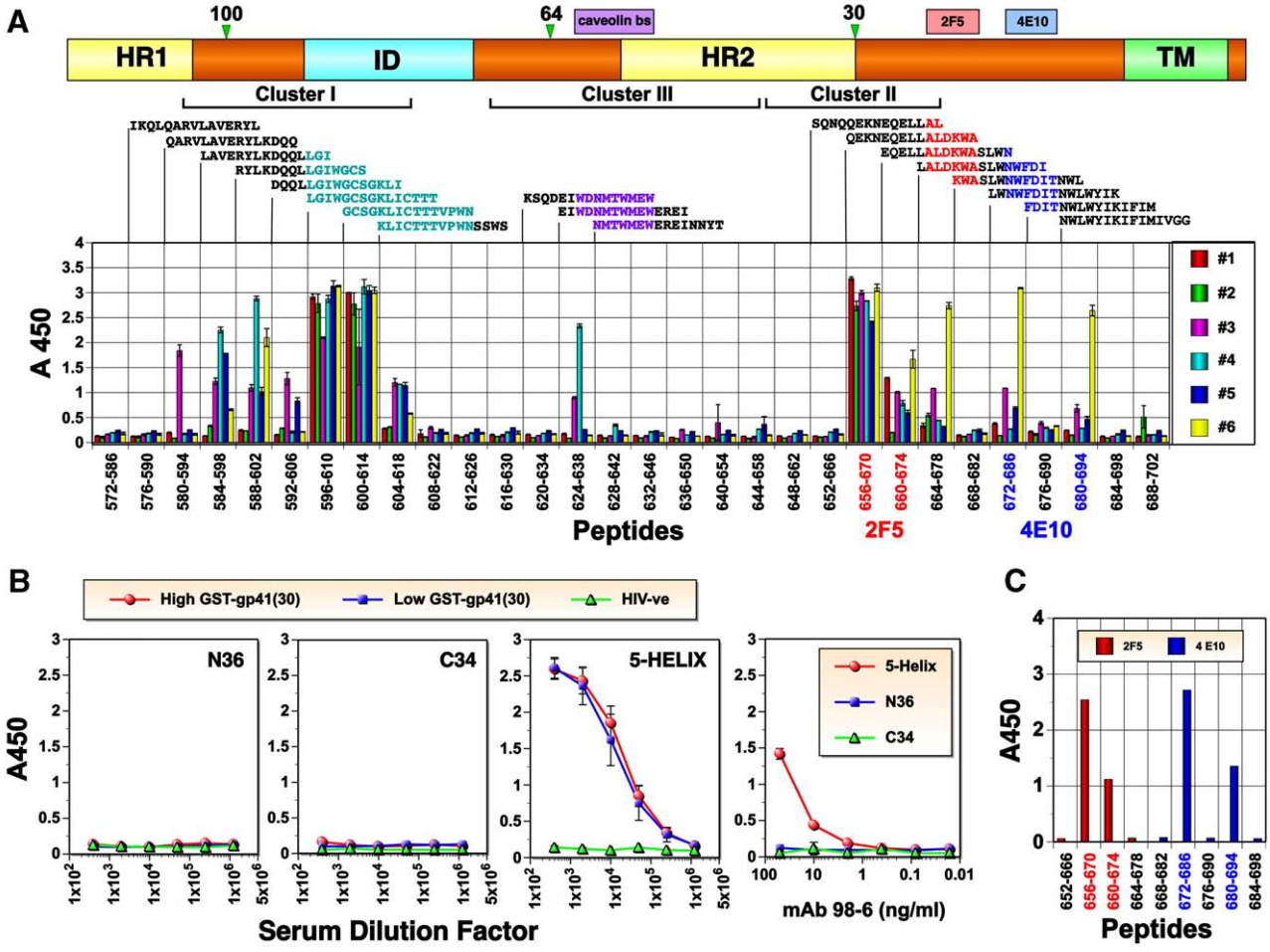

Fig. 6. Identification of immunogenic linear epitopes targeted by patients using overlapping peptide ELISA. (A) Plasma samples (1:100 dilution) from six patients that exhibited the strongest reactivity against GST-gp41-30 were further analyzed by peptide ELISA (20 pmol/well). A schematic diagram of a section of gp41 is shown on the top and the key regions are indicated, including the binding sites of caveolin-1, 2F5 and 4E10, and three immunogenic clusters as defined by Binley et al. (Binley et al., 1996). Aligned amino acid sequences of peptides that are immunoreactive are shown. Peptides are numbered based on MCON6 envelope. Those recognized by mAbs 2F5 and 4E10 are indicated in red and blue, respectively (see panel C). (B) Plasma samples were further evaluated with larger peptides encompassing HR1 (N36: SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL) and HR2 (C34: WMEWDREINN-YTSLIHSLIEESQNQQEKNEQELL), and 5-helix bundle. The plot shows average reactivity of six patients in either high or low GST-gp41-30-reactive groups. MAb 98-6 was used as a positive control. (C) Immunoreactivity of peptides to 2F5 (1 μg/ml) and 4E10 (20 μg/ml).

Four peptides upstream and one peptide downstream of the two immunodominant peptides in cluster I were also reactive, but only for certain patient plasma: 580–594 (CWRU-3), 584–598 and 588–602 (CWRU-4, -5 and -6), 592–606 (CWRU-3 and -5), and 604–618 (CWRU-3, -4 and -5). It is interesting to note that these peptides are recognized only by patient plasma that exhibit broader, more potent neutralizing activity. That is, patients CWRU-1 and -2 did not mount detectable antibodies against epitopes within these peptides. It has been shown that antibodies that target the immunodominant epitopes do not neutralize HIV-1. It remains to be determined whether any of the antibodies that bind to epitopes adjacent to these immunodominant epitopes have neutralizing activity or not.

The third highly immunoreactive peptide (656–670: QEKNEQELLALDKWA) was found in a region that overlaps cluster II (designated a.a. 644–663 on HxB2: RLIEESQNQQEKNEQELLAL; (Binley et al., 1996)). This peptide contains residues "ALDKWA", which is the core epitope for mAb 2F5 (ELDKWA) (Muster et al., 1993) and not considered to be a part of cluster II (Binley et al., 1996; Goudsmit et al., 1990). The fact that a peptide just upstream of it (652–666: SQNQQEKNEQELLAL) is recognized by none of the patient plasma indicates critical participation of and/or contribution from residues "DKWA" in forming the correct conformation of epitope(s) within the peptide. This is interesting for two reasons. Firstly, most patients (i.e. all except CWRU-2) also recognized a peptide just downstream of it (660–674: EQELLALDKWASLWN). Secondly, direct ELISA analyses with 2F5 showed that the antibody binds to both peptides 656–670 and 660–674 (Fig. 6C). Therefore, we believe some of these patients may have mounted antibodies similar to 2F5 in epitope recognition, if not neutralizing activity, during natural infection.

One of the most striking findings of this study was that antibodies from patient CWRU-6 bound strongly to peptides 672–686 (LWNWFDITNWLWYIK) and 680–694 (NWLWYIKIFIMIVGG) (Fig. 6A), the same two peptides recognized by mAb 4E10 (Fig. 6C). Although 4E10 binds primarily to residues "NWFDIT", the antibody is known to also interact with sequences downstream of it (e.g. L682 and W683; L679 and W680 for HxB2; (Brunel et al., 2006)). This strongly suggests, although does not prove, that CWRU-6 may have mounted 4E10-like antibodies. Moreover, antibodies from patient CWRU-6 bound strongly to peptide 664–678 (LALDKWASLWNWFDI), which contains epitopes recognized by both 2F5 and 4E10 in almost complete entirety. In this regard, the antibody that recognizes this peptide might have Z13-like properties, a Fab fragment that has been shown to bind to an epitope overlapping residues of both 2F5 and 4E10 epitopes (Nelson et al., 2007; Zwick et al., 2001).

Another interesting observation is that antibodies from patient CWRU-4, which exhibited the most potent and broad neutralizing activity, strongly reacted with peptide 624–638 (EIWDNMTWMEWEREI). This peptide encompasses a motif highly homologous to caveolin-1-binding domain (WNNMTWMEW). Caveolin-1 is a scaffolding protein that organizes and concentrates specific ligands within the caveolae membranes. The caveolin-1 binding site has recently been shown to play an important role during the formation of a fusion pore or endocytosis of HIV-1 (Huang et al., 2007). What draws our interest is the finding that rabbits immunized with a peptide encompassing the caveolin-1 binding domain (SLEQIWNNMTWMQWDK) mounted quite broad neutralizing activity against multiple primary HIV-1 isolates from different clades (Hovanessian et al., 2004). Therefore, one possible source of broadly neutralizing activity for patient CWRU-4 is antibodies directed against the caveolin-1 binding domain.

As shown in Fig. 4, antibody reactivity against GST-gp41-64 was significantly greater than that against GST-gp41-30 for most patients. This was the case even for the six patients with the highest reactivity against GST-gp41-30, except for CWRU-2 and -6, who showed similar reactivity. However, ELISA analyses using 15 a.a. peptides revealed almost no antibody reactivity against those in C-terminal 64 residues upstream of the MPER (i.e. a.a. 623–656; Fig. 6A). Antibody reactivity was not observed even when much longer 34 a.a. peptide was used (C34), which encompasses the entire HR2 domain (Fig. 6B, second panel). These results indicate that antibodies that specifically recognize GST-gp41-64 most likely bind to highly conformational and/or non-contiguous epitopes, which are formed only in the presence of the C-terminal 30 residues (i.e. MPER). This interpretation is consistent with a previous study, which reported that antibodies directed against cluster III (a.a. 617–646; 614–643 for HxB2) are highly conformational (Binley et al., 1996). Although none of the 12 patients (6 high- and 6 low-reactivity against GST-gp41-30) we analyzed mounted detectable antibody responses against either N36 or C34 peptides corresponding to HR1 and HR2 regions, respectively (Fig. 6B, first and second panel), all of them showed equally strong reactivity against a 5-helix bundle protein complex (Fig. 6B, third panel), a monomeric polypeptide consisting of three HR1 and two HR2 segments connected by linker sequences designed to mimic a trimeric, HR1-HR2 coiled-coil structure (Root et al., 2001). This strong response is likely a result of antibodies against non-functional, trimeric gp41 stumps on the surface of virions exposed after gp120 shedding (Moore et al., 2006). As expected, mAb 98-6 recognized 5-helix bundle, but not N36 or C34 peptides (Fig. 6B, fourth panel).

ELISA results (Fig. 6A) revealed that plasma samples from some of the patients exhibited peptide reactivity profiles that resembled those of 2F5 or 4E10 antibodies (i.e. recognition of peptides 656–670 and 660–674 by 2F5, and peptides 672–686 and 680–694 by 4E10). Considering that some of these patients might indeed have 2F5- or 4E10-like antibodies, we were curious as to the prevalence of patient antisera that exhibit similar profiles. We conducted ELISA analyses of all 44 patient samples simultaneously (except for CWRU-5 due to insufficient amount) using eight peptides that span the entire MPER. Two peptides from the cluster I immunodominant domain (596–610 and 600–614) were also evaluated, and an unrelated peptide (SARS-CoV spike glycoprotein a.a. 61–76) was used as a negative control.

Although the two immunodominant peptides were highly reactive for most individuals, about one fourth of the patients mounted surprisingly weak antibody responses against the region (A450 values less than 0.5; Fig. 7). In general, antibody reactivity against peptide 656–670 correlated with that against GST-gp41-30. However, there were several notable exceptions

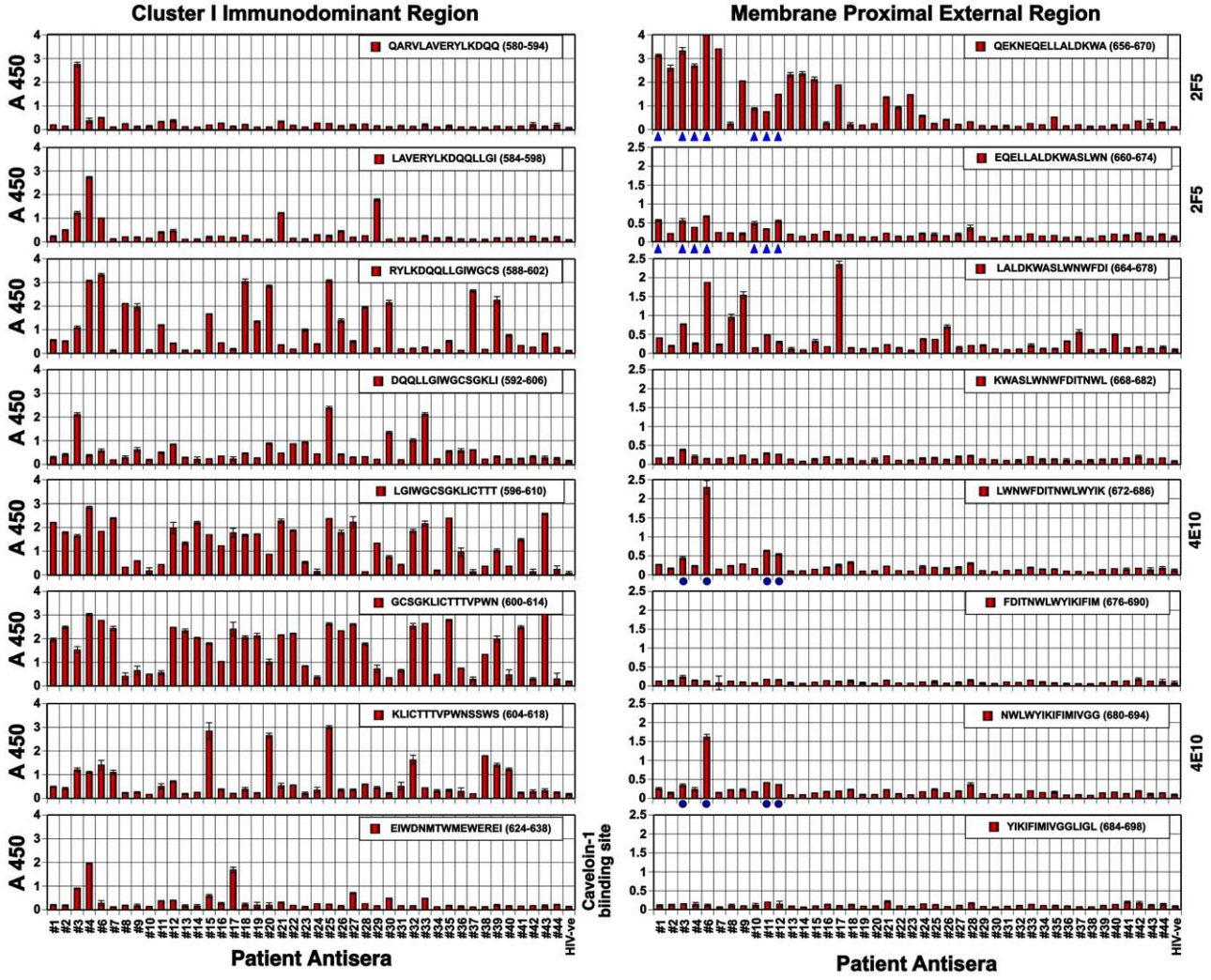

Fig. 7. Identification of immunogenic linear epitopes in gp41 by ELISA using M group consensus peptides. Immunoreactivity of plasma samples from 43 patients were analyzed against seven peptides from cluster I region (a.a. 580–618), eight peptides from the MPER (a.a. 656–698), and one peptide encompassing the caveolin-1-binding site (a.a. 624–638). A negative control peptide derived from SARS-CoV S protein showed antibody reactivity profile similar to peptides 684–698 (data not shown). CWRU-5 was not included due to insufficient amount of the sample. Peptides recognized by mAbs 2F5 and 4E10 are indicated. Patient samples that reacted significantly against both peptides recognized by 2F5 or 4E10 (arbitrarily defined as A450 value greater than twice the sum of average and standard deviation of SARS peptide background reactivity) are indicated by triangles or dots, respectively. It should be noted that *y*-axis for seven peptides from the MPER are in different scale.

for which antibody reactivity was very weak (e.g. patients CWRU-8, -16, -18, -19 and -20). Plasma samples from eight patients (indicated by triangles) reacted substantially above background levels for both peptides 656–760 and 660–674, suggesting these patients might have mounted 2F5-like antibodies. All but one of these patients had fairly strong antibody reactivity against GST-gp41-30. Plasma from several patients reacted substantially to peptide 664–678 (e.g. patients CWRU-3, -6, -8, -9, -17 and -26), suggesting that they might have Z13-like antibodies (Nelson et al., 2007; Zwick et al., 2001). Finally, plasma from four patients (CWRU-3, -6, -11 and -12) reacted against peptides 672–686 and 680–694, which are recognized by 4E10. Very weak or near background levels of antibody reactivity were observed for peptides 668–682, 676–690, and 684–698. Together, these results suggest that several patients develop antibodies against epitopes that are near, or overlap with, those targeted by 2F5 or 4E10.

Discussion

In view of the limited number of BR-Nabs that have been identified to date, better characterization of antibody responses in HIV-1-infected patients and identification of new and potent BR-Nabs is critical in designing vaccine immunogens. As a first step in our efforts toward achieving these goals, we have systematically generated and purified five soluble fusion proteins containing the C-terminal 30, 64, 100, 142 or 172 amino acids of gp41 ectodomain from an M group consensus sequence. These recombinant proteins appear to have intact antigenic structures based on immunoprobing analyses with BR-Nabs 2F5 and 4E10, 98-6, and antisera from HIV-1-infected patients. Characterization of antibody responses against gp41 had been largely limited to short peptides because the protein has been difficult to produce in soluble forms. Therefore, the set of gp41 fragments we have generated will be invaluable reagents for characterizing antibody responses against HIV-1 gp41.

Expression of eukaryotic or viral proteins in bacteria typically results in misfolding and aggregation of proteins, which accumulate in inclusion bodies. Our gp41 protein fragments were no exception despite the fact that they were fused to GST. However, we were successful in denaturing the proteins, refolding, and renaturing them into soluble forms in the absence of any detergent, although we experienced greater difficulties in solubilizing the two larger fragments and obtained substantially lower yields. Although we did not attempt to express gp41 protein fragments without GST, we are quite certain that GST played a critical role in allowing the proteins to renature into soluble forms. This conclusion is based in part from our observation that attempts to remove GST by cleaving between the two fusion partners with thrombin resulted in immediate precipitation of gp41 (data not shown). We speculate that GST could be functioning in a way analogous to gp120 by masking some of the hydrophobic patches on gp41 away from aqueous solvent to prevent non-specific aggregation.

Since GST-gp41 fusion proteins are antigenically intact, shown by their ability to bind not only BR-Nabs 4E10 and 2F5, but also conformational antibodies that bind to cluster III, they could be potential candidates for vaccine development. However, these proteins are not ideal as antigens for a vaccine as the large GST portion is likely to be immunodominant over smaller gp41 fragments. On the other hand, these proteins are highly suitable for structural studies, especially GST-gp41-64 since it is well recognized by both 2F5 and 4E10 and we can easily produce up to 40 mg of the protein from 1 L of bacterial culture. To date, the only solved crystal structures of gp41 are HR1/HR2 coiled-coil core and peptide epitopes that bind to 2F5 and 4E10 (Cardoso et al., 2005; Chan et al., 1997; Ofek et al., 2004; Tan et al., 1997; Weissenhorn et al., 1997b). Determining the structure of the entire MPER with the HR2 domain would facilitate better understanding of gp41 function and designing antigens that can elicit antibodies such as 2F5 or 4E10.

All five GST-gp41 fusion proteins were equally well recognized by 2F5, indicating that the epitope is conformationally identical and similarly exposed amongst all proteins (Fig. 3A). Although all five proteins were also recognized by 4E10, GST-gp41-100 and -30 were about 5- and 20-fold less reactive, respectively, when compared to the other three proteins (Fig. 3B). This result was somewhat unexpected taking into account that the 4E10 epitope is considered to be linear and is only four amino acids downstream of the 2F5 epitope. One likely explanation is that although the epitope is linear, it is highly conformational, requiring other regions of gp41 for proper formation of the antigenic epitope structure. In this regard, it has previously been reported that the 4E10 epitope assumes a helical conformation (Cardoso et al., 2005) and modifications that enhance helical properties increase antibody-binding affinity (Brunel et al., 2006; Cardoso et al., 2007). Based on these findings, our current hypotheses are (1) sequences within GST-gp41-64 upstream of the MPER (i.e. between C-terminal residues 64 and 30) provide constraints on the helical conformation of 4E10 epitope, thereby enhancing antibody binding compared to GST-gp41-30; (2) additional sequences between HR1 and HR2 within GST-gp41-100 are either disrupting or masking the epitope partially to reduce 4E10 binding; and (3) this inhibition is reversed when the HR2 domain forms coiled-coil structure with HR1, as demonstrated by reactivity to mAb 98-6 (Fig. 3C), in GST-gp41-142 and -172. More detailed biochemical and structural analyses are needed to test these hypotheses.

We observed tremendous variation in antibody responses to gp41 amongst different patients, not only with respect to the magnitude, but also the pattern of antibody reactivity against different gp41 fragments and peptides. One parameter that could affect the magnitude of antibody responses is plasma viral load. Greater antigenic stimulation might be expected to induce stronger antibody responses. In this regard, the duration of infection since seroconversion and possible treatment of patients with anti-retroviral therapy could potentially influence antibody levels (Binley et al., 2000; Lafeuillade et al., 1997; Morris et al., 2001). An important contribution may also stem from the overall responsiveness of the patients' immune system to antigenic stimulation; higher viral load could also mean greater deterioration of helper T cell immunity, and therefore weaker B cell response. In the present study, the patients were chosen randomly, without considerations to these parameters, because we were primarily interested in studying differential epitope targeting in individual patients and identifying those who mounted strong antibody responses against potential neutralization epitopes (*viz.* MPER).

The variation in antibody responses against different gp41 regions or peptides in different patients could be attributable to differences in patients' immune system (e.g. immunoglobulin gene repertoire) and/or HIV-1 isolates the patients are infected with (e.g. envelope protein sequence). We saw that many patients who mounted strong antibody responses against the MPER (i.e. reactivity against GST-gp41-30) exhibited broader, more potent neutralizing activity compared to those who did not. It should be emphasized, however, that we presently do not know the epitope(s) targeted by Nabs. They could be directed against the MPER, other regions of gp41, or epitopes within gp120. Furthermore, the breadth of neutralizing activity could be polyclonal with multiple Nabs targeting different epitopes. In any event, further characterization of B cell repertoire, virus isolates from these patients, and mAbs generated from the patients could provide clues as to how BR-Nabs could be elicited.

Considering that plasma samples from four of six patients that showed strong antibody reactivity against GST-gp41-30 had potent neutralizing activity, this fusion protein could be used as a tool for rapidly screening patient sera to identify those who might have BR-Nabs. In this regard, we were somewhat disappointed with the fact that patients CWRU-1 and -2, whose antisera reacted most strongly against the protein, did not exhibit potent neutralizing activity as we had hoped. One possible explanation is that there might be epitopes in the MPER that elicit non-neutralizing antibodies. It is conceivable that these antibodies could prevent binding of Nabs that target epitopes in close proximity due to steric hindrance (e.g. 2F5 or 4E10). In this case, observed neutralizing activity would be determined by relative antibody titers and affinity between neutralizing and non-neutralizing antibodies that compete for the adjacent epitopes. If this hypothesis is true, then it would have strong

implication in terms of vaccine design; not only does the antigen have to have correct neutralization epitopes, the antigen should not have competing non-neutralizing epitopes nearby.

Attempts to elicit antibodies with similar properties to 2F5 and 4E10 in animals immunized with antigens containing the gp41 MPER have not been successful. It has been proposed that this difficulty is due to autoantigen mimicry by HIV-1 based on observations that 2F5 and 4E10 cross-react with phospholipid cardiolipin (Alam et al., 2007; Haynes et al., 2005; Sanchez-Martinez et al., 2006). This view that 2F5 and 4E10 have properties of autoantibodies is quite controversial since they have been used in passive immunization studies without any complication (Ferrantelli et al., 2004; Joos et al., 2006; Trkola et al., 2005). In addition, a more recently published study reports that 2F5 fails to exhibit any cardiolipin reactivity under their set of experimental conditions (Scherer et al., 2007). Also, while 4E10 does have general affinity to lipids, this reactivity resembles that of anti-phospholipid antibodies elicited during many infections rather than that of autoimmune antiphospholipid syndrome. Thus, the inability to elicit antibodies with similar properties to 2F5 and 4E10 might not be attributable to immune tolerance mechanisms. In this study, we have identified many patients who mounted antibodies against the same peptides recognized by 2F5 and, to a lesser extent, by 4E10 (Fig. 7). Although we have yet to determine the precise epitopes and specificities of these antibodies, the results of our study suggest that patients who mount antibodies against epitopes that are near, or overlap with, those targeted by 2F5 or 4E10 may not be as rare as has previously been thought. In agreement with our findings, Gray et al. (Gray et al., 2007) have recently reported up to one third of HIV-1-infected patients mount Nabs against the MPER. Additional studies with a larger panel of patient samples and detailed biochemical analyses of purified antibodies that target the MPER could provide more definitive answers. In this regard, the fusion proteins we generated could be ideal reagents for rapid assessment of antibody responses against gp41 and for affinity purification of MPER-directed antibodies.

Materials and methods

Cloning GST-gp41 fusion constructs

To generate plasmid constructs expressing five GST-gp41 fusion proteins, regions encoding C-terminal 30, 64, 100, 142 or 172 amino acids of M group consensus gp41 ectodomain were PCR-amplified from pcDNA-MCON6gp160 (kindly provided by Dr. Beatrice Hahn, University of Alabama; (Gao et al., 2005)). PCR reactions were carried out using a common reverse primer 5′-GAATTCTTAATGGTGATGATGGTGATGCTT-GATGTACCA-CAGCCAGTTGG-3′ for all five constructs, and individual forward primers, 5′-CGCGGATCCGAGAA-GAACGAGCAGGAGC-3′ (for -30); 5′-CGCGGATCCGAC-GAGA-TCTGGGACAACATGACC-3′ (for -64); 5′-CGC-GGATCCGAGCGCTACCTGAAGGACC-AGC-3′ (for -100); 5′-CGCGGATCCCGCCAGCTGCTGTCCGGCATC G-3′ (for -142); 5′-CGCGGATCCGCCGTGGGCATCGGCGCC-3′ (for -172). Underlined and double underlined sequences indicate restriction enzyme sites *Bam*HI and *Eco*RI, respectively. Amplified DNA fragments were digested with *Bam*HI and *Eco*RI and ligated into corresponding sites on vector pGEX-2T (GE Healthcare Life Sciences). GST-gp41-64 contains inadvertent, inconsequential I649V mutation (numbering based on MCON6).

Protein expression and purification

E. coli BL21(DE3) was transformed with recombinant plasmid or pGEX-2T and cultured overnight at 37 °C in superbroth containing ampicillin (50 μg/ml). Cells were diluted 1:100 in fresh superbroth and cultured to 0.6 OD_{600} at 37 °C, at which time fusion protein expression was induced with 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) for 2 h. Cells were harvested, washed and lysed in PBS (phosphate-buffered saline) by sonication. The cell lysate was subjected to centrifugation at 10,000 rpm (HB4 rotor) for 20 min in a Sorvall Superspeed centrifuge. Pellets containing inclusion bodies were washed with PBS and solubilized in PBS containing 8 M urea. Insoluble debris was removed by centrifugation and soluble proteins were bound to Ni-NTA resin (QIAGEN) on a column. GST-gp41-30, -64 and -100 were renatured through serial incubations with 10 bed volumes of PBS containing a decreasing step gradient of urea at 8 M, 6 M, 4 M, 3 M, 2 M, 1 M and 0 M. Renaturation of GST-gp41-142 and -172 fusion proteins required much slower, continuous gradient of reduction in urea concentration, particularly below 4 M. The process typically took place over the period of 2–3 days. The column was washed with PBS containing 20 mM imidazole and proteins were eluted in PBS containing 200 mM of imidazole. The eluted proteins were dialyzed against PBS, concentrations were determined by Bradford assay, and purity was assessed by SDS-PAGE followed by silver staining.

Non-fusion GST was expressed similarly. To purify the protein, cells were lysed in PBS by sonication and the lysate was subjected to centrifugation at 5000 rpm (HB4 rotor) for 30 min. GST was purified according to the manufacturer's protocol (Novagen). Briefly, GST·Bind™ Resin was added to the supernatant and incubated at 4 °C for 60 min. GST-bound resin was pelleted by centrifugation, washed twice with PBS, loaded onto a column, and washed again with GST Binding/Wash buffer (4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.137 M NaCl, 2.7 mM KCl, pH 7.3). GST was eluted in a buffer (50 mM Tris–HCl, pH 8.0) containing 10 mM reduced glutathione. The eluted proteins were dialyzed against PBS, and concentrations were determined by a Bradford assay.

Immunoprecipitation and Western blot

HIV-1 gp41 mAbs 2F5 (Buchacher et al., 1994; Purtscher et al., 1996, 1994) and 4E10 (Stiegler et al., 2001) were obtained from Dr. Hermann Katinger through the NIH AIDS Research and Reference Reagent Program (NIH ARRRP). Immunoprecipitation (IP) was done by incubating GST or GST-gp41 fusion proteins with either 2F5 or 4E10 and protein A agarose in IP buffer (10 mM Tris, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5% Triton X-100). The mixture was agitated overnight at 4 °C. Subsequently, antigen–antibody complex bound to

protein A agarose was washed three times with IP buffer. Proteins were denatured and eluted from the resin by heating in boiling water for 3 min in 2× SDS-PAGE sample buffer. Immunoprecipitated or purified proteins were subjected to SDS-PAGE (10% acrylamide), followed by Western blot analyses using rabbit anti-GST IgG (Molecular Probes) and goat anti-rabbit IgG conjugated horseradish peroxidase (Pierce). Protein bands were visualized with SuperSignal chemiluminescent substrates (Pierce) according to the manufacturer's protocol.

ELISA

Plasma samples from 44 HIV-1-infected patients were obtained through the Case Western Reserve University Center for AIDS Research Clinical Core. Samples were heat inactivated at 56 °C for 30 min. prior to use. 6-helix and 5-helix bundle proteins (Root et al., 2001) were kindly provided by Dr. Michael Root of Thomas Jefferson University. The following reagents were obtained through the NIH ARRRP: HIV-Ig from NABI and NHLBL; HIV-1 M group consensus envelope overlapping peptides (Cat #9487), HIV-1 IIIB N36 and C34 Peptides ((Gallo et al., 2004); Cat #9822 and 9824, respectively) from DAIDS; mAb 98-6 (Gorny et al., 1989; Robinson et al., 1991; Tyler et al., 1990; Xu et al., 1991) from Dr. Susan Zolla-Pazner. SARS-CoV S protein peptide (PFYSNVTGFH-TINHTF; Cat #9605) was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository.

Purified GST-gp41 fusion proteins or overlapping peptides (0.5 or 20 pmol per well, respectively) were coated onto 96-well Nunc-Immuno Plates (Nunc; Cat #439454) using antigen coating buffer (150 mM Na_2CO_3, 350 mM $NaHCO_3$, 30 mM NaN_3, pH 9.6) at 4 °C overnight. N36, C34, 5-Helix and 6-Helix antigens were coated at 33 ng/well. Wells were blocked with PBS (pH 7.5) containing 2.5% skim milk and 25% FBS at 37 °C for 1 h, then washed four times with 0.1% Tween 20 in PBS. NP-40 was added to patient plasma samples (0.1% final) before dilution in blocking buffer. Antibodies and plasma samples were diluted as indicated, added to wells and incubated for 2 h at 37 °C in 200 µl blocking buffer. Wells were washed 4 times, and secondary antibody goat anti-human IgG conjugated to horseradish peroxidase (Pierce; Cat #31410) was incubated at 1:3000 dilution at 37 °C for 1 h. Wells were washed 4 times, and developed by adding 100 µl TMB HRP-substrate (Bio-Rad) for 5–10 min. Reactions were stopped with 50 µl of 2 N H_2SO_4. Plates were read on a microplate reader (Versamax by Molecular Devices) at 450 nm. Experiments were done in duplicates.

Neutralization assays

Single round infection assays in TZM-bl cells (Derdeyn et al., 2000; Platt et al., 1998; Wei et al., 2002) using pseudoviruses were performed as we and others have previously described (Derdeyn et al., 2000; Kim et al., 2001; Wei et al., 2002, 2003). Assays were done in two laboratories with slightly different protocols. In Cho lab, pseudoviruses were generated using pNL4-3.Luc.R-E- (Connor et al., 1995; He et al., 1995), pCMV-Tat (Jeang et al., 1987), and pLTR-gp140 constructs encoding gp140 of HIV-1 isolates BAL, 89.6, DH-12, and AD8 (Kim et al., 2001) or pHCMV-G encoding VSV-G protein (Burns et al., 1993). Briefly, 3×10^7 293T cells at 85% confluency in T225 flask were co-transfected by calcium phosphate method with 150 µg pNL4-3Luc.R-E-, 150 µg pLTR-gp140 or pHCMV-G constructs, and 25 µg pCMV-Tat. Transfected cells were incubated for 16 h before replacing medium. Cells were cultured for two more days, at which time culture medium was collected and clarified by centrifugation. Cell-free virus stocks were aliquoted and stored at −80 °C. Pseudoviruses were titered in TZM-bl cells by β-galactosidase staining as previously described (Kim et al., 2001; Wei et al., 2002). All cells were cultured at 37 °C, 5% CO_2, in DMEM supplemented with 10% FBS, penicillin/streptomycin, and glutamine.

Heat-inactivated plasma samples or mAbs diluted in serum-free DMEM at indicated concentrations were mixed with about 100–150 infectious units of pseudoviruses. The mixture was incubated at 37 °C for 1 h, and then added to TZM-bl target cells in 96-well plates in 50 µl. After 1 h adsorption, the virus inoculum was removed and 200 µl of fresh medium was added. Two days post infection, cells were lysed and virus infectivity was determined using β-Glo luminescence assay as per manufacturer's protocol (Promega). Relative luminescence units (RLU) were measured using a luminometer (Bio-Rad). Assays were performed in duplicates or in quadruplicates. Uninfected TZM-bl cells were used to determine background luminescence and mean background was subtracted from all readings. Virus infectivity was determined as a %age of no-serum controls (i.e. virus only).

In Montefiori lab, assays were also done in TZM-bl cells as described (Li et al., 2005; Montefiori, 2004). Briefly, 200 $TCID_{50}$ of virus was incubated with serial 3-fold dilutions of serum sample in triplicate in a total volume of 150 µl for 1 hr at 37 °C in 96-well flat-bottom culture plates. Freshly trypsinized cells (10,000 cells in 100 µl of growth medium containing 75 µg/ml DEAE dextran) were added to each well. One set of control wells received cells + virus (virus control) and another set received cells only (background control). After a 48-h incubation, 100 µl of cells was transferred to 96-well black solid plates (Costar) for measurements of luminescence using the Britelite Luminescence Reporter Gene Assay System (PerkinElmer Life Sciences). Assay stocks of Env-pseudotyped viruses were prepared by transfection in 293T cells and were titrated in TZM-bl cells as described (Li et al., 2005).

Statistical analyses

For the purpose of comparing high and low GST-gp41-30-binding patient groups for the breadth of neutralizing activity, summary % infectivity for individual patients was calculated as the average % infectivity observed for each individual across the four viruses. These statistically independent summary values of % infectivity were then used to compare groups using both the two group *t*-test and the non-parametric Wilcoxon Rank Sum Test. Observed % neutralization of all four viruses was significantly higher ($p<0.01$) in the high GST-gp41-30 antibody binding group for both tests.

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