Profiles of neutralizing antibody response in chronically human immunodeficiency virus type 1 clade B’-infected former plasma donors from China naïve to antiretroviral therapy

Xintao Hu,† Kunxue Hong,† Chunhong Zhao, Yang Zheng, Lijing Ma, Yuhua Ruan, Hongmei Gao, Kelli Greene, Marcella Sarzotti-Kelsoe, David C. Montefiori and Yiming Shao

Correspondence
Yiming Shao
yshao08@gmail.com

1State Key Laboratory for Infectious Disease Control and Prevention, National Center for AIDS/STD Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, PR China
2Department of Experimental Surgery, Duke University Medical Center, Durham, NC 27710, USA
3Department of Immunology, Duke University Medical Center, Durham, NC 27710, USA

Broadly neutralizing antibodies (NAbs) such as those generated in chronic human immunodeficiency virus type 1 (HIV-1) infection are considered a key component for an effective HIV-1 vaccine. Here, we measured NAb responses using a panel of 25 Env-pseudotyped viruses, including clade B, C, A, CRF07_BC and CRF01_AE strains, against plasma samples from 103 subjects in a former plasma donor cohort in central China, who were infected with HIV-1 clade B’ for at least 10 years and naïve to antiretroviral therapy at the time of sampling. We found that 64% of samples (n=66) neutralized at least half of the viruses tested and 2% (n=2) neutralized all of the viruses, while 5% (n=5) neutralized none of the viruses tested. Strikingly, 29% of plasma samples (n=30) neutralized ≥80% of the viral strains tested, indicating the presence of broadly reactive NAbs in these patients. When the magnitude (geometric mean ID₅₀ titres, GMTs) or breadth of neutralization was assessed for correlation with CD4 count or plasma viral load, the only significant positive correlations were observed between viral load and neutralization magnitude (r=0.2189, P=0.0263) and between viral load and neutralization breadth (r=0.1970, P=0.0461). A moderate difference between progressors and long-term non-progressors was observed in both the breadth (P=0.0316) and the potency (P=0.0300). A significant difference was found in the GMTs between intra-clade and inter-clade strains (P<0.001). Heat-map analysis based on k-means clustering of plasma determined a statistically stable cluster of plasma with cross-reactive and potent neutralizing reactivity. These samples could provide physical biomaterials for further virological and serological studies from which useful insights into rational HIV-1 vaccine development and therapeutic design might be derived.

INTRODUCTION

Results from many existing studies of viral vaccines, e.g. for influenza and measles, demonstrated that neutralizing antibodies (NAbs) play a pivotal role in protective immunity (Ehrlich et al., 2008; Galli et al., 2009; Nair et al., 2007; Schlereth et al., 2000). Passive transfer of broadly neutralizing Abs completely blocked infection by a chimeric simian–human immunodeficiency virus in non-human primate studies (Baba et al., 2000; Hessel et al., 2009; Mascola et al., 2000; Parren et al., 2001; Shibata et al., 1999; Veazey et al., 2003). Similarly, passive transfer of broadly neutralizing Abs also delayed human immunodeficiency virus type 1 (HIV-1) rebound after cessation of antiretroviral therapy (ART) in a clinical study (Trkola et al., 2005). These studies in animal models and humans reaffirm that broadly reactive humoral immune responses may be required to provide protective immune functions (Hoxie, 2010). Thus, an optimally effective HIV-1 vaccine will probably require the elicitation of an antibody response capable of neutralizing a broad spectrum of circulating virus strains.

Despite a multitude of effort in the design of HIV immunogens capable of inducing protective NAbs, researchers in this field have met with limited success (Mascola & Montefiori, 2010; Phogat & Wyatt, 2007). Compared with
many other viruses, HIV-1 possesses a high level of genetic variability, particularly in its envelope glycoproteins (Env), with multiple distinct clades and recombinant forms circulating in various regions of the world. This characteristic of HIV causes one of the greatest obstacles in the development of an effective HIV-1 vaccine, in particular in the elicitation of broadly neutralizing Abs. Moreover, HIV-1 has evolved multiple immune-escape mechanisms to prevent the generation of broadly neutralizing Abs, including extensive glycosylation patterns of Env, protection of conserved structures of Env by variable loops, occlusion within the oligomer and conformational masking of receptor-binding sites (Burton et al., 2004; Kwong et al., 2002; Mascola & Montefiori, 2003).

However, recent studies have suggested that a significant proportion of chronically HIV-1-infected subjects could mount broadly neutralizing Ab responses several years after infection (Binley et al., 2008; Doria-Rose et al., 2009; Li et al., 2007) and the development of cross-reactive NAbs during HIV-1 infection correlates with duration of infection and higher plasma viral RNA load (Sather et al., 2009; van Gils et al., 2010a), although the exact mechanism of such correlation remains elusive. Therefore, characterization of the cross-reactive NAb responses developed during natural HIV-1 infection, as well as identification of factors associated with their generation in geographically distinct populations infected with diverse HIV-1 strains, is relevant to the development of an effective HIV-1 vaccine.

NAb response data derived from naturally HIV-1-infected cohorts in the USA (Sather et al., 2009; Simek et al., 2009), South Africa (Gray et al., 2009), Europe (van Gils et al., 2010b), Kenya (Piantadosi et al., 2009) and India (Lakhashi et al., 2007) provide useful information for vaccine development. However, there has been no equally comprehensive study of NAb responses to HIV-1 among subjects in China. In this study, we evaluated the prevalence of naturally occurring cross-reactive neutralizing activity in a relatively large and unique cohort in central China composed of 103 individuals infected with HIV-1 clade B’ (a subset of clade B) for at least 10 years (Su et al., 2003). We also compared NAb parameters with clinical variables, and used a clustering analysis tool (based on k-means, from the Los Alamos HIV database) to define neutralizing profiles in plasma with activity against diverse strains, to determine whether subtype-specific neutralization serotypes exist in our Chinese cohort.

RESULTS

Neutralization profiles of the study population

The demographic and epidemiological data of the study cohort are shown in Table 1. The plasma samples were tested in the TZM-bl NAb assay against a panel of 25 Env-pseudotyped viruses, including eight clade B strains, four A strains, four C strains, four CRF07_BC strains and three CRF01_AE strains, as well as two tier 1 viruses (SF162.LS for clade B and MW965.26 for clade C) (see Table 2).

All 103 plasma samples tested in this study neutralized the two tier 1 strains, MW965.26 and SF162.LS, at higher titres than the other 23 viruses tested. The geometric mean ID_{50} titres (GMTs) of these samples tested against MW965.26 and SF162.LS were 4519 and 2077, respectively, which is in agreement with the generally higher neutralization sensitivity of these viruses (Seaman et al., 2010; Simek et al., 2009). These tier 1 strains are so sensitive that they may provide little or no benefit in helping to predict the overall breadth or potency, so we excluded these two strains from further assessment of the overall neutralization breadth and potency.

Using the remaining 23 strains, neutralization breadth was assessed as the fraction of strains neutralized at a detectable titre (ID_{50} >20). We found that 64 % of the samples (n=66) neutralized >50 % of the viruses tested and that 2 % (n=2) neutralized all of the viruses tested, whereas 5 % (n=5) failed to neutralize any of the viruses tested. Strikingly, 29 % of the samples (n=30) neutralized >80 % of the strains tested, indicating a high prevalence of broadly cross-reactive NAb

Table 1. Major characteristics of FPD cohorts

<table>
<thead>
<tr>
<th>Category</th>
<th>Anhui (n=81)</th>
<th>Henan (n=22)</th>
<th>Total (n=103)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years; mean ± SD)</td>
<td>44.41 ± 7.08</td>
<td>46.09 ± 5.24</td>
<td>44.77 ± 6.76</td>
</tr>
<tr>
<td>Gender [% (n)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>56.8 (46)</td>
<td>68.2 (15)</td>
<td>59.2 (61)</td>
</tr>
<tr>
<td>Female</td>
<td>43.2 (35)</td>
<td>31.8 (7)</td>
<td>40.8 (42)</td>
</tr>
<tr>
<td>Ethnic group [% (n)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Han</td>
<td>100 (81)</td>
<td>100 (22)</td>
<td>100 (103)</td>
</tr>
<tr>
<td>High-risk behaviour [% (n)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Former plasma donation</td>
<td>81 (100.0)</td>
<td>22 (100.0)</td>
<td>103 (100.0)</td>
</tr>
<tr>
<td>Intravenous drug use</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HIV-1 clade</td>
<td>B’</td>
<td>B’</td>
<td>B’</td>
</tr>
</tbody>
</table>
Table 2. Neutralization magnitude distribution between different sample groups based on neutralization breadth

GMTs are shown. ID<sub>50</sub> values <20 (lowest sample dilution tested) were assigned a value of 10. Bold type indicates clade-specific values.

<table>
<thead>
<tr>
<th>Group based on NAb breadth</th>
<th>Control</th>
<th>Tier 1A</th>
<th>Clade B</th>
<th>Clade C</th>
<th>Clade A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SVA-MLV</td>
<td>MW965.26</td>
<td>REJO4541.67</td>
<td>QH0692.42</td>
<td>SC422661.8</td>
</tr>
<tr>
<td>BCN (n=30)</td>
<td>10</td>
<td>5706</td>
<td>216.3</td>
<td>117.6</td>
<td>106.4</td>
</tr>
<tr>
<td>M-BCN (n=36)</td>
<td>10</td>
<td>5078</td>
<td>123.9</td>
<td>65.3</td>
<td>61.2</td>
</tr>
<tr>
<td>Non-BCN (n=37)</td>
<td>10</td>
<td>3279</td>
<td>33.7</td>
<td>26.3</td>
<td>17.0</td>
</tr>
<tr>
<td>Overall (n=103)</td>
<td>10</td>
<td>4519</td>
<td>91.3</td>
<td>55.9</td>
<td>45.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control</th>
<th>SVA-MLV</th>
<th>CH110.2</th>
<th>CH181.12</th>
<th>CH117.4</th>
<th>CH120.6*</th>
<th>ZM109F. PB4†</th>
<th>ZM249M. PL1</th>
<th>Du422.1</th>
<th>CAP45. 2.00.G3</th>
<th>Clade-specific</th>
<th>Q769. d22</th>
<th>Q842. d12</th>
<th>Q259. d2.17</th>
<th>Q461. e2</th>
<th>Clade-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCN (n=30)</td>
<td>10</td>
<td>94.9</td>
<td>82.2</td>
<td>77.4</td>
<td>31.8</td>
<td>66.2</td>
<td>70.8</td>
<td>71.1</td>
<td>60.7</td>
<td>49.7</td>
<td>62.4</td>
<td>117.2</td>
<td>76.3</td>
<td>35.8</td>
<td>19.3</td>
</tr>
<tr>
<td>M-BCN (n=36)</td>
<td>10</td>
<td>36.0</td>
<td>32.0</td>
<td>24.5</td>
<td>14.0</td>
<td>25.1</td>
<td>48.6</td>
<td>23.8</td>
<td>22.0</td>
<td>11.4</td>
<td>23.2</td>
<td>44.5</td>
<td>36.2</td>
<td>15.8</td>
<td>11.2</td>
</tr>
<tr>
<td>Non-BCN (n=37)</td>
<td>10</td>
<td>14.1</td>
<td>12.2</td>
<td>10.3</td>
<td>11.0</td>
<td>11.8</td>
<td>18.0</td>
<td>11.6</td>
<td>11.3</td>
<td>10.3</td>
<td>12.5</td>
<td>15.9</td>
<td>10.4</td>
<td>10.4</td>
<td>10.0</td>
</tr>
<tr>
<td>Overall (n=103)</td>
<td>10</td>
<td>34.1</td>
<td>29.7</td>
<td>25.1</td>
<td>16.3</td>
<td>25.4</td>
<td>38.0</td>
<td>25.3</td>
<td>23.3</td>
<td>16.8</td>
<td>24.8</td>
<td>40.8</td>
<td>28.7</td>
<td>17.3</td>
<td>12.6</td>
</tr>
</tbody>
</table>

* Tier 3 virus.
† Tier 1B virus.
activities among this unique study population (Fig. 1). Based on the neutralization breadth among the overall study population, the sample was divided into three groups. Subjects with >80% neutralization breadth were defined as the broadly cross-reactive neutralization group (BCN, \(n=30\)). Subjects with <50% neutralization breadth were defined as the non-broadly cross-reactive neutralization group (Non-BCN, \(n=37\)). Other individuals with moderate neutralization breadth were defined as the intermediate broadly cross-reactive neutralization group (M-BCN, \(n=36\)).

For each group of subjects, a GMT was computed for each strain tested, so that neutralization potency could be compared more easily with plasma neutralization breadth (Table 2). Additionally, clade-specific GMTs were calculated for each group. Of the virus strains tested, clade B viruses exhibited the highest NAb sensitivities, with clade-specific GMTs of 109.2 (BCN), 52.3 (M-BCN), 11.4 (Non-BCN) and 43.6 (overall study population), which are consistent with the clade’s history of infection (Su et al., 2003). In contrast, subtype A viruses exhibited the lowest NAb sensitivities, with clade-specific GMTs of 49.8 (BCN), 23.1 (M-BCN), 11.4 (Non-BCN) and 22.5 (overall study population). The clade-specific GMTs of five clades tested were in the following order: clade B>CRF07_BC>clade C>CRF01_AE>clade A in all study groups except for the Non-BCN group. Among all individual strains, REJO4541.67 (clade B, tier 2 strain) exhibited the highest GMT value in all study groups, whereas Q461.e2 (clade A, tier 2 strain) exhibited the lowest GMT in all study groups (Table 2).

We noticed that the samples in the BCN group, which exhibited broader neutralizing responses, tended to have higher neutralization titres than other groups that exhibited intermediate or low level breadth. Based on this observation, we analysed the association of neutralization breadth and potency using a Spearman rank order correlation analysis; our results demonstrated that there was a significant positive correlation between neutralization breadth and potency across all 103 samples (\(r=0.9718, P<0.0001\)), as well as within the BCN (\(r=0.7805, P<0.0001\)), M-BCN (\(r=0.7644, P<0.0001\)) and Non-BCN (\(r=0.9613, P<0.0001\)) groups. These data indicated that the breadth and potency of neutralization developed in parallel.

Association between neutralization breadth/ magnitude and viral load

To investigate the association between neutralization breadth and potency and clinical parameters, we performed a Spearman rank order correlation analysis between the observed breadth or magnitude and plasma viral load or CD4 counts. A positive correlation between neutralization breadth and viral load was observed (Fig. 2a, left: \(r=0.1970, P=0.0461\)) in the study population. There was also a positive correlation between neutralization breadth and viral load (Fig. 2b, left: \(r=0.2189, P=0.0263\)). When the analysis was restricted to the 30 samples with broadly cross-reactive NAb activities (BCN group), this correlation still existed and became more significant (Fig. 2a, right: \(r=0.4236, P=0.0197\) for breadth/viral load; Fig. 2b, right: \(r=0.6581, P<0.0001\) for magnitude/viral load). No significant correlation between neutralization breadth/potency and CD4 count was observed, although there was a comparable negative correlation trend (Fig. 2c, d).

Progressors generate stronger NAb responses than long-term non-progressors (LTNPs)

To determine whether there is a difference in neutralization breadth and potency between progressors, AIDS patients and LTNPs, we compared the neutralization breadth and potency among the three study groups using Mann–Whitney U statistical analysis. We observed that there was a modest difference between progressors and LTNPs in neutralization breadth (Fig. 3a, \(P=0.0316\)) and potency (Fig. 3b, \(P=0.0300\)). This observation concurs with data from other cohorts located in different geographical areas, including the USA (Doria-Rose et al., 2010; Sather et al., 2009), South Africa (Gray et al., 2009), Europe (van Gils et al., 2010b) and Kenya (Piantadosi et al., 2009), indicating that progressors, who exhibit higher plasma viral loads, generate stronger NAb responses than LTNPs. This observation is probably due to persistent antigenic stimulation of B-cells (Doria-Rose et al., 2009; Mahalanabis et al., 2009).
Fig. 2. Correlation between neutralization breadth/magnitude and plasma viral loads/CD4 counts. (a, b) For the total population of 103 samples (left) and for 30 samples with broadly cross-reactive NAb activities (right), the neutralization breadth (a) and magnitude (b) of NAb response are compared with individuals' viral loads, respectively. The numbers on the x-axis represent the values of the log_{10}(viral load). The values on the y-axis indicate the breadth (a) and GMTs (b). (c, d) For the total population of 103 samples (left) and for 30 samples with broadly cross-reactive NAb activities (right), the neutralization breadth (c) and magnitude (d) of NAb response are compared with individuals' CD4 counts. The numbers on the x-axis represent the CD4 count (cells mm^{-3}). The values on the y-axis indicate the breadth (c) and GMTs (d). P-values (two-sided) and r values are based on Spearman's rank test.
Preferential intra-clade neutralization of clade B in comparison with inter-clade /CRF07_BC/clade C/clade A/CRF01_AE

We compared the GMTs for the sample set (all clade B') against a set of clade-matched viruses (clade B) and other non-clade-matched viruses (CRF07_BC, clade C, clade A, and CRF01_AE viruses, and all non-clade-matched viruses as a whole). We found that GMTs between intra-clade-matched (B) and non-clade-matched specific virus sets, including CRF07_BC, clade C, clade A, CRF01_AE and inter-clade (all non-clade matched viruses as whole), showed significant differences (Fig. 4a, P<0.0001 for all pairs). Even

Fig. 3. Neutralization breadth and magnitude comparison between different sample populations. Neutralization breadth (a) and magnitude (b) are compared between progressors (n=82), AIDS patients (n=6) and LTNPs (n=15). The x-axis lists different groups including 82 progressors, six AIDS patients and 15 LTNPs. The y-axis indicates the breadth (a) and GMTs (b), respectively. P-values (two-sided) are based on a Mann–Whitney U test. The error bars show the median with the interquartile range. Significant differences between the three groups are indicated: *P<0.05 (Mann–Whitney U test).

Fig. 4. Comparison of GMTs between clade-matched (B) and CRF07_BC/clade C/clade A/CRF01_AE/inter-clade virus strain sets. The difference of GMTs between clade-matched (B) and the CRF07_BC, clade C, clade A, CRF01_AE, inter-clade (excluding all the clade B viruses)-specific viruses set is illustrated. P-values (two-sided) are based on the Mann–Whitney U test. The x-axis indicates the clade-specific virus groups. The y-axis values represent GMTs. Significant differences between the six groups are indicated: *P<0.05; **P<0.01; ***P<0.001 (Mann–Whitney U test).
in the 30 samples with broadly cross-reactive NAb activities (BCN group), a significant difference in the GMTs between the clade-matched (B) virus set and other virus sets, including CRF07_BC, clade C, clade A, CRF01_AE and inter-clade, was still observed (Fig. 4b, P<0.05 for all pairs).

**Heat-map analysis based on k-means clustering of neutralization data**

In order to discern patterns of NAb responses in our unique cohort and to define underlying similarities in neutralization pattern, we utilized a new web-based heat-map tool to cluster the 103 samples and 25 strains on the basis of the natural log ID₅₀ values. After k-means clustering (k=3) and synchronous treatment of two statistical indices ('Bootstrap' and 'Noise'), three robust subgroups were identified for both plasma and virus strains, as illustrated in Fig. 5.

The panel of 25 diverse viral strains that were used to study breadth could be grouped into three clusters (Fig. 5). Strain cluster 1 (S1) consisted of two tier 1 Env clones, SF162.LS and MW965.26, which were the most neutralization-sensitive viruses in the panel. Strains of different subtypes were included in the other two clusters. Cluster S2 was predominantly composed of six subtype B envelopes. One tier 1B, subtype C envelope strain (ZM109F.PB4) and one relatively neutralization-sensitive subtype A strain (Q769.d22) were also categorized into cluster S2. One exception is AC10.0.29, which is a clade B virus but was neutralized more easily by clade C plasma than clade B plasma, so this virus was located outside cluster S2 (Li et al., 2005). PVO.4 (clade B, tier 3 strain) was located between clusters S2 and S3. The most resistant cluster is S3, which included eight strains of different clades and did not include clade B. Two of the four clade C strains and two of the four CRF07_BC strains were in cluster S3; these strains are known to be sensitive to clade C plasma, but more resistant to clade B plasma (Gray et al., 2009; Li et al., 2006; Seaman et al., 2010).

A considerable proportion of the plasma samples from LTNPs and a few samples from non-LTNPs were found to form plasma cluster P1, which is characterized by a lack of neutralization breadth and low or no neutralization activity (Fig. 5). Plasma cluster P3 was composed of 11 plasmas with the greatest potency and breadth of neutralization, all of which had been included in the BCN group based on breadth. Among the plasma with intermediate reactivity, one robust cluster was identified; plasma in this cluster (P2) did not neutralize the most resistant strains (GMT of 18.54 for P2 plasma versus S3 strains), but did neutralize strains in the other two strain clusters with higher titres (GMTs of 4813.73 and 66.23 for P2 plasma versus S1 and S2, respectively). Using this strategy, plasma clusters can be identified by overall breadth or potency and the viral strain types that they neutralized. When clinical parameters were included in this analysis, only viral load was associated significantly with a specific cluster; median viral load was higher in patients with plasma in cluster P3 than in patients with plasma in P1 and P2, which contained a significant proportion of the LTNPs.

**DISCUSSION**

To our knowledge, our study is the first to profile the NAb responses comprehensively in a relatively large and unique former plasma donor (FPD) cohort of subjects chronically infected with HIV-1 clade B’ for at least 10 years in central China. We demonstrated that a high prevalence of NAbS was detected, whereby 64% of the samples neutralized >50% of the viruses tested, and that broadly neutralizing Abs were found in 29% of the study subjects (Fig. 1), similar to observations in other recent studies (Doria-Rose et al., 2009; Sather et al., 2009; Simek et al., 2009).

Previous studies show that the development of broadly neutralizing Abs may benefit from durative antigen stimulation. In our cohort, a modest positive correlation was observed between viral load and neutralization breadth/magnitude (Fig. 2a, b), similar to findings in cohorts in the USA (Sather et al., 2009), South Africa (Gray et al., 2009), Europe (van Gils et al., 2010b) and Kenya (Piantadosi et al., 2009). A study by Sather et al. (2009) observed an association between neutralization breadth and the duration of infection in their study cohort, indicating that the development of broadly neutralizing Abs needs long-term antigenic stimulation. Scheid et al. (2009) found that Env-specific IgG genes are highly mutated in subjects with broadly neutralizing Abs compared with other IgG genes, suggesting multiple rounds of selection and hypermutation in response to persistence or turnover of viral antigen. A study by Wu et al. (2011) also suggested that high-affinity maturation of broadly neutralizing Abs may be driven by extensive viral antigen exposure. In contrast, data from several recent studies of HIV-1-infected individuals who were followed for up to 7 years suggested that, if broadly NAb responses had not developed by 4 years, they did not develop later (Gray et al., 2011; Mikell et al., 2011). The reason for this phenomenon remains unknown. One possible explanation is that dysregulation of the immune system over time may result in a reduced ability to mount new antibody responses after 2–3 years infection, independent of disease progression (van Gils et al., 2010a).

The prevalence of broadly neutralizing Abs observed in our FPD cohort of subjects who were chronically infected for at least 10 years may add support to the notion that broadly neutralizing Abs may be driven by extensive viral antigen exposure. Further longitudinal follow-up studies are needed to compare the dynamic profiles of NAb responses at various time points post-infection and to determine whether no further increase in neutralization breadth occurs after a certain time period following initial infection (Gray et al., 2011; Mikell et al., 2011).

We observed that the subjects’ plasma generally neutralized clade-matched pseudoviruses at higher titres than non-clade matched strains. Our clade B’ plasma neutralized clade B strains better than CRF01_AE strains, which corresponded with a previous study (Mascola et al., 1996). It should be noted that this preferential neutralization does not suggest bona fide clade-specific serotypes, as many of the subjects were also found to have a high coverage of potent neutralizing
reactivity to pseudoviruses from other clades. For example, sample DRVI01 demonstrated GMT values of 375.59, 337.50 and 439.80 respectively against the CRF07_BC, clade C and clade A virus strain sets, which is higher than the GMT value of 307.62 against the clade B virus strain set. Similarly, sample F438 also showed a higher GMT of 249.13 against the CRF07_BC strain set compared with a GMT of 232.59 against the clade B strain set. Sample F433 even exhibited a reversed trend, with GMTs of 229.86, 133.81 and 135.64 respectively against the CRF07_BC, clade C and CRF01_AE strain sets, which are higher than the GMT of 92.5 against the clade B virus strain set. In total, seven samples (F433, F260, F466, F462, F461, F432 and F445) exhibited higher GMTs against inter-subtype strains than against intra-subtype strains. Thus, regarding vaccine development, a polyvalent vaccine strategy may be a promising direction (Lu et al., 2010).

The new heat-map tool based on \( k \)-means clustering identified three significantly robust clusters of plasma with higher, moderate and lower neutralizing reactivity, providing guidance for the further delineation of neutralization specificities in different clusters of samples. Specifically, the subjects identified as top potently and broadly cross-reactive neutralizers (DRVI01, DRVI02 and DRVI03) in this study deserve further investigation. This includes the isolation and identification of potentially novel mAbs with broad and potent neutralizing activity from these subjects, and the identification of potential genetic and amino acid signatures associated with the broadly neutralizing Ab responses from the pathogen.

Further studies are needed to define the fine specificities responsible for the broadly neutralizing activity observed in our FPD cohort samples and also the host factors responsible for generating and maintaining such a NAb response. Recent studies led by Li et al. (2007), Binley et al. (2008), Sather et al. (2009) and Tomaras et al. (2011) found that a significant proportion of the cross-reactive neutralization activity and epitope specificity in sera from many elite neutralizers could not be mapped to currently known neutralization epitopes, despite the fact that CD4-binding site (CD4bs) antibodies are found in many individuals with broadly neutralizing activity. Identification of the specificities responsible for the broad and potent neutralization activity in these elite neutralizers may provide new insight and guidance for rational HIV-1 vaccine design. Preliminary identification of the specificities present in our FPD cohort plasma, using validated CD4bs epitope-specific pairing probes (RSC3/ΔRSC3) (Wu et al., 2010), indicated that the top three neutralizers (DRVI01, DRVI02 and DRVI03) possess CD4bs-binding activities (unpublished data). The fact that elite neutralizers could make broad and potent NAb, even in the presence of B-cell dysfunction caused by HIV (Moir & Fauci, 2009), calls for a deeper understanding of B-cell biology during the course of natural HIV-1 infection and will facilitate the development of an effective AIDS vaccine.

**METHODS**

**Study population.** Our study population consisted of 103 subjects with chronic infection who donated plasma during 1992 and 1995 (Xu et al., 2006; Zhang et al., 2008). All participants were naïve to ART at the time of sampling during the period June 2007–May 2009. The study subjects consisted of 61 males and 42 females, with a mean age of 44.77 ± 6.76 years. The median values for CD4 counts and plasma viral loads at the time of sampling were 455 cells mm\(^{-3}\) (50–997 cells mm\(^{-3}\)) and 25 200 copies ml\(^{-1}\) (<50 100 000 copies ml\(^{-1}\)), respectively. Fifteen patients were LTNPs, as described previously (Zhang et al., 2008), who typically maintain a viral load (VL) of <50 RNA copies ml\(^{-1}\) and a stable CD4 count in the absence of ART; this group had a median CD4 count of 561 cells mm\(^{-3}\). The other 88 patients include six AIDS patients whose CD4 counts are <200 cells mm\(^{-3}\) and 82 progressors without apparent CD4 T-cell decline. The 82 progressors had a median viral load of 35 550 RNA copies ml\(^{-1}\) and a median CD4 count of 454 cells mm\(^{-3}\). For the six AIDS patients, the median viral load was 212 500 RNA copies ml\(^{-1}\) and the median CD4 count was 162 cells mm\(^{-3}\).

In this cohort, subjects became infected during unregulated commercial plasma donation whereby whole blood was collected from multiple donors, with unknown HIV-1 infection status, and pooled prior to processing. Following this practice, the pooled crythrocytes were reinfused into the donors. In some instances, the equipment was also unknowingly contaminated with blood from HIV-positive donors. This practice was discontinued at the end of 1995 (Kaufman & Jing, 2002). The outbreak of HIV-1 infection occurred within a narrowed period of time and the patients were infected with very similar virus strains of clade B’, which was confirmed by previous studies (Su et al., 2003; Wang et al., 2007). This study was reviewed and approved by the Institutional Review Boards of the National Center for AIDS/STD Control and Prevention, the Chinese Center for Disease Control and Prevention, and all subjects provided written informed consent prior to blood and data collection.

**Cells.** TZM-bl (also called JC53-BL) cells were obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP, catalogue no. 8129) as contributed by John Kappes and Xiaoyun Wu. This is a genetically engineered HeLa cell clone that expresses CD4, CXCR4 and CCR5 and contains Tat-responsive reporter genes for...
firefly luciferase and *Escherichia coli* β-galactosidase under the regulatory control of the HIV-1 long-terminal repeat (Platt et al., 1998; Wei et al., 2002). 293T/17 cells were obtained from the ATCC (catalogue no. 11268). Both cell lines were maintained in Dulbecco's 1998; Wei cultures were incubated at 37 °C in a humidified 5 % CO2, 95 % air environment. Cell monolayers were split 1:10 at confluence by treatment with 0.25 % trypsin, 1 mM EDTA (Invitrogen).

**Virus isolates.** Molecularly cloned gp160 genes for HIV-1 Env pseudovirus production were obtained as follows. Env clones SF162.LS (subtype B) and MW965.26 (subtype C) were obtained from the NIH ARRRP. Clones representing the standard panel of subtype B HIV-1 reference strains (QA0692.42, SC422661.8, PVO.4, AC10.0.29, RHPA4259.7, RHEO4541.67, TROD4551.58 and CAAN3534.2.A2) and the standard panel of subtype C HIV-1 reference strains (Du422.1, CAP45.2.00.G3, ZM249M.P1l and ZM1099.FB4) were also obtained from the NIH ARRRP. The four Kenyan acute/early subtype A Env clones (Q461.e2, Q769.d22, Q259.d2.17 and Q842.d12) were obtained from the NIH ARRRP as contributed by Julie Overbaugh (Long 2002). Three recently transmitted CRF01_AE Env clones (BM2249, BM2316 and BM2498) from China (CH181.12, CH117.4, CH110.2, CH120.6) are from intravenous drug use transmissions and were co-developed by Dr David C. Montefiori’s laboratory and Dr Yiming Shao’s laboratory. Three recently transmitted CRF01_AE Env clones (BM2249, BM2316 and BM2498) from a MSM cohort (men who have sex with men) were also developed by our laboratory (unpublished data).

**HIV-1 Env-pseudotyped virus preparation and titration.** Stocks of a single-round infection of HIV-1 Env pseudoviruses were produced by co-transfecting 293T/17 cells (4 × 106 cells per T75 flask) with 4 μg of an HIV-1 *rev*/*env* expression plasmid and 8 μg of an *env*-deficient HIV-1 backbone plasmid (pSG3Env) using the transfection reagent Fugene 6 (Roche). Supernatants were harvested 24 h following transfection and clarified by 0.45 μm filtration. Single-use aliquots (1.0 ml) were stored at −80 °C. The recommended virus dilution to use in the TZM-bl NAb assay was calculated to ensure a standardized virus dose in the assays (Li et al., 2005; Todd et al., 2012).

**NAb assay in TZM-bl cells.** Plasma samples were heat-inactivated at 56 °C for 1 h followed by a 1 min centrifugation at 14 000 r.p.m. (18-place spin rotor F-45-18-11; Eppendorf) prior to use in the NAb assay. NABs were measured as a function of reduction in Tat-induced luciferase reporter gene expression after a single round of infection in TZM-bl cells with Env-pseudotyped viruses as described previously (Li et al., 2005; Montefiori, 2005). This assay is a modified version of the assay as described previously (Li et al., 2005; Wei et al., 2002). Briefly, 50 μl of Env-pseudotyped virus that was previously titrated for optimal infectivity was incubated with serial threefold dilutions of plasma sample, assayed in duplicate, in a total volume of 150 μl for 1 h at 37 °C in 96-well flat-bottom culture plates. Freshly trypsinized TZM-bl cells were then added (1 × 104 per well in a 100 μl volume) in 10 % DMEM growth medium containing DEAE–dextran (Sigma) at a final concentration of 10 μg ml−1. One set of control wells received cells plus pseudovirus (virus control) and another set received cells only (background control). Following 48 h incubation, 150 μl culture medium was removed from each well and 100 μl luciferase reporter gene assay system reagent was added (Bright-Glo; Promega). After a short incubation (minimum of 2 min), 150 μl lysate from each well was transferred to 96-well black solid plates (PerkinElmer Life Sciences) for measurement of luminescence in a luminometer. The 50 % inhibitory dose (ID50) was defined as the reciprocal of the plasma reagent dilution that caused a 50 % reduction in relative luminescence units (RLU) compared with virus control wells after subtraction of background RLU. All NAB assays were conducted using the validated TZM-bl NAb assay, following standard operating procedures, in accordance with Good Clinical Laboratory Practice compliance (Ozaki et al., 2012).

**Viral load testing.** Plasma viral load was measured using the Amplicor ultrasensitive assay (Hoffman La Roche) according to the manufacturer’s instructions; this assay has a lower detection limit of 50 copies HIV-1 RNA ml−1.

**CD4 count testing.** CD4+ cell counts from EDTA-anticoagulated whole blood were assessed by using TruCounts (HTC-CD3Ab, PE-CD4Ab, PerCP-CD45Ab, APC-CD8Ab) and FACSCalibur (both from BD).

**Heat-map analysis based on k-means (k=3) clustering.** A heat-map is a graphical way of displaying a table of numbers by using colours to represent the numerical values. To display the neutralization data underlying the clustering pattern visually, we utilized the heat-map tool based on k-means clustering (k=3), as publicly available on the Los Alamos HIV database (http://www.hiv.lanl.gov/content/sequence/HEATMAP_KMEANS/heatmap_kmeans.html), to generate a heat-map (Fig. 5). This web-based heat-map tool used is a modified version of ‘heatmap.2’ of the gplots package of the statistical environment R, whose principles were listed by Tibshirani et al. (2001). Bootstrap, a common procedure, was used to evaluate the stability of the components in a certain subgroup by resampling with replacement 10 000 times. Similarly, noise data representing the repeated iterations data were used to evaluate the impact of assay-to-assay variability. The resulting degree of consensus is shown in the row or column labelled ‘Bootstrap’ in Fig. 5. Stability of categories for these data is shown in the row and column labelled ‘Noise’ in Fig. 5. We defined three subgroups (k=3) as the maximum number such that three robust clusters, representing higher, moderate and lower levels for both plasma and strains, were formed at a consistency of at least 90 % by both indices of stability. These methods were also described previously (Doria-Rose et al., 2010; Seaman et al., 2010).

**Statistical analyses.** Statistical analysis and basic graphical delineation were performed using GraphPad Prism 5 (GraphPad Software Inc.) and Microsoft Excel 2007 (Microsoft Corp.) based on a Spearman rank correlation, a Mann–Whitney test or a Fisher’s exact test, where appropriate; P<0.05 was considered significant. Viral load values below the limit of detection (50 RNA copies ml−1) were assigned a value of 49 for statistical analysis purposes.

**ACKNOWLEDGEMENTS**

We would especially like to thank the study participants for their generosity. This study was supported by the Bill and Melinda Gates Foundation’s Collaboration for AIDS Vaccine Discovery’s (CAVD) Comprehensive Antibody–Vaccine Immune Monitoring Consortium (38619), the National Nature Science Foundation of China (81172809), the National Major Project for Infectious Disease Control and Prevention (2008ZX10001-010, 2012ZX10001-008), the SKLID key project (2011 SKLID 103) and an International Cooperation Grant from the Ministry of Science and Technology of China (2009DFB30420). We are grateful to the Vaccine Research Center of the National Institute of Allergy and Infectious Diseases at National Institutes of Health for providing the RSC3/ARSC3 probes directed to CD4bs on gp120. We also thank the CAVD HIV Specimen Cryopreservation (HSC) for their contribution of HIV-1 Env-pseudotyped viruses for this study. We are grateful to Dr Xueling Wu for her critical review of the manuscript.
REFERENCES


