Broadly Neutralizing HIV Antibodies Define a Glycan-Dependent Epitope on the Prefusion Conformation of gp41 on Cleaved Envelope Trimmers

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SUMMARY

Broadly neutralizing HIV antibodies are much sought after (a) to guide vaccine design, both as templates and as indicators of the authenticity of vaccine candidates, (b) to assist in structural studies, and (c) to serve as potential therapeutics. However, the number of targets on the viral envelope spike for such antibodies has been limited. Here, we describe a set of human monoclonal antibodies that define what is, to the best of our knowledge, a previously undefined target on HIV Env. The antibodies recognize a glycan-dependent epitope on the prefusion conformation of gp41 and unambiguously distinguish cleaved from uncleaved Env trimers, an important property given increasing evidence that cleavage is required for vaccine candidates that seek to mimic the functional HIV envelope spike. The availability of this set of antibodies expands the number of vaccine targets on HIV and provides reagents to characterize the native envelope spike.

INTRODUCTION

Broadly neutralizing antibodies (bnAbs) to highly antigenically variable viruses such as HIV, influenza virus, and HCV have attracted much attention in recent years because they provide new opportunities to counter a particularly troublesome category of pathogens (Burton et al., 2012; Kwong and Mascola, 2012; Klein et al., 2013). Researchers are using such antibodies to guide vaccine design for these pathogens by defining conserved epitopes that can then be presented to the immune system under more favorable conditions than typically occur in natural infection or in most conventional vaccination approaches. For example, grafting conserved epitopes into molecular scaffolds as candidate immunogens is one approach under intense study (Jardine et al., 2013; Kulp and Schief, 2013). BnAbs have also been critical in native envelope-protein structural studies, notably for HIV (Julien et al., 2013a; Lyumkis et al., 2013), HCV (Kong et al., 2013a), Ebola virus (Lee et al., 2008), and RSV (McLellan et al., 2013). Finally, there is increasing evidence of the potential of bnAbs as therapeutics (Buchacher et al., 1992; Klein et al., 2012b; Barouch et al., 2013; Shingai et al., 2013). However, the number of targets identified by bnAbs is limited, and the discovery of more targets would be of considerable value for vaccine design, structural studies, and therapeutic applications.

For HIV, bnAbs that recognize a gp41 region close to the virus membrane (the membrane proximal external region, or MPER) (Muster et al., 1993; Zwick et al., 2001; Huang et al., 2012), the CD4 binding site (CD4bs) of gp120 (Barbas et al., 1992; Burton et al., 1994; Zhou et al., 2010; Scheid et al., 2011; Wu et al., 2011), a gp120 V2 loop region including the glycan at N160 (Walker et al., 2009, 2011), and a gp120 region centered on the glycan at N332 (Buchacher et al., 1994; Trkola et al., 1996; Walker et al., 2013a, 2013b), Ebola virus (Lee et al., 2008), and RSV (McLellan et al., 2013). Finally, there is increasing evidence of the potential of bnAbs as therapeutics (Buchacher et al., 1992; Klein et al., 2012b; Barouch et al., 2013; Shingai et al., 2013). However, the number of targets identified by bnAbs is limited, and the discovery of more targets would be of considerable value for vaccine design, structural studies, and therapeutic applications.
et al., 2011) have been identified. Two other potential bnAb sites, the first possibly involving the V3 loop and the coreceptor site of gp120 (Klein et al., 2012a) and the second possibly involving the CD4bs of gp120 and the N-trimer structure on gp41 (Zhang et al., 2012), have been described but only partially characterized. We have previously isolated a number of potent broadly neutralizing monoclonal antibodies to the V2 and N332 regions from HIV-infected donors (Walker et al., 2009, 2011). These antibodies were isolated by direct functional screening rather than by antigen selection (Scheid et al., 2009). In this method, IgG+ memory B cells from the donor were plated at approximately a single B cell per well, and after activation for 7–8 days, supernates were screened for their ability to neutralize indicator viruses. Antibody-variable-region genes were rescued from positive wells by PCR, and IgG molecules were expressed for further studies. Here, we applied this approach to an HIV-infected donor whose outstanding potency and breadth of serum led to classification of the donor as an “elite neutralizer” (Simek et al., 2009; Walker et al., 2010). We describe a set of antibodies that specifically recognize cleaved HIV Env trimer via a glycan-dependent epitope expressed on the prefusion form of gp41. The antibodies bind a previously undefined region on Env and thus provide a target that could be useful in the development of immunogens for HIV vaccine design. Furthermore, the antibodies can help to distinguish between native and nonnative conformations of Env immunogens, facilitating the selection of appropriate immunogens.

RESULTS

Isolation of Antibodies PGT151–158, which Show Broad Neutralization of HIV

By direct functional screening of supernatants derived from activated memory B cells from an elite neutralizer infected with a clade C virus, we identified antibodies that showed notable neutralizing activity against a small set of viruses, JR-CSF (clade B), 93IN905 (clade C), and IAVI C22 (clade C), which we chose because they were potently neutralized by the donor’s serum. Eight related antibody sequences, designated PGT151–158 (the PGT151 family; Table S1), were retrieved from positive wells, expressed, and found to have cross-clade neutralization activity against a small panel of five indicator viruses in a TZM-bl pseudovirus-neutralization assay (Simek et al., 2009; Walker et al., 2010). The heavy-chain-complementarity-determining region 3s (HCDR3s) of the PGT151 family was found to be 28 amino acids in length, an unusual feature shared by several HIV-1 bnAbs targeting glycan-dependent epitopes and the MPER on gp41 (Mascola and Haynes, 2013). As shown in Figure 1, most of the antibodies neutralized four of the five viruses in the indicator panel, but none neutralized the clade B isolate 92BR020. A notable feature of the neutralization curves for some of the antibody-isolate combinations was that, although IC50 values indicated potent activity, 100% neutralization was not achieved. In a number of cases, neutralization saturation occurred in the range of 50%–80%, although there were some cases with lower saturation, particularly against the clade AE isolate 92TH021.

Two of the antibodies with the most complete neutralization against the indicator panel of viruses, PGT151 and PGT152, were then evaluated for neutralization against a much larger panel of 117 cross-clade virus isolates in a TZM-bl pseudovirus neutralization assay. PGT151 neutralized approximately 66% of viruses with a median IC50 of 8 ng/ml, and PGT152 neutralized 64% of viruses with a median IC50 of 12 ng/ml (Table 1). These bnAbs have IC50 values that are about 1 log lower than the V2 prototype bnAb PG9 (Table S2) and that are comparable to the IC50 values that have been determined for the N332-glycan-targeting bnAbs PGT121 and PGT128 on the same panel (Walker et al., 2011).

Figure 1. PGT151-158 Neutralization of an Indicator Panel of Five Pseudoviruses

Serial dilutions of antibody were preincubated with pseudovirus for 1 hr and then added to TZM-bl cells. Three days after infection luciferase values were measured, and percent neutralization was calculated. Data are from one representative experiment of at least two replicate experiments and are presented as the mean of two replicate wells ± SEM.
The composition of this panel, derived from the 117 members with that of the donor serum on a smaller 25 virus panel somewhat underestimated in a pseudovirus-TZM-bl assay relative to monomeric gp120 from several viruses from multiple clades, although they are able to potently neutralize the corresponding viruses (Figure 2A and Figure S2A). PGT151 and PGT152 also did not bind 293F-cell–produced gp41 trimers (Figure S2B), which are most likely in a 6 helix bundle conformation. Surprisingly, PGT153 did show some binding to the gp41 trimeric protein, and of note, this antibody has the least sequence identity to PGT151 and other family members (Table S1). The results suggested that the antibodies might be largely specific for native Env trimer. In order to investigate this possibility, we measured the binding of PGT151 and PGT152 to native cleaved and uncleaved variant JR-FL trimers on the surface of 293T cells by flow cytometry. We chose this particular isolate because it has previously been shown that fully cleaved JR-FL Env is efficiently displayed on the surface of transfected 293T cells, whereas for most other isolates (Pancera and Wyatt, 2005), relatively high proportions of uncleaved trimers are expressed. As shown in Figure 2B, PGT151 and PGT152 bind exclusively to cleaved variant JR-FL trimers and show no binding to the corresponding uncleaved trimers on the surface of transfected cells. Cleavage was recently shown to be important for formation of native-like structure and presentation of quaternary epitopes (Ringe et al., 2013). Together, these data strongly argue that PGT151 and PGT152 are trimer specific and, furthermore, are able to distinguish cleaved from uncleaved trimers.

PGT151 and PGT152 were shown not to bind to a number of recombinant, uncleaved gp140 preparations (Figure S2B), which, although they contain three gp120 subunits attached to trimeric gp41 ectodomains, adopt conformations that do not resemble native Env trimers (Julien et al., 2013a; Lyumkis et al., 2013; Ringe et al., 2013). However, PGT151 and PGT152 do bind well to the stabilized recombinant trimeric molecule BG505 SOSIP.664 gp140 trimer (Figure 2C), which is a close antigenic and structural mimic of the native Env trimer (Julien et al., 2013a; Lyumkis et al., 2013; Ringe et al., 2013; Sanders et al., 2013). PGT151 also distinguishes cleaved from uncleaved BG505 SOSIP.664 gp140 trimers (Figure 2C).

No antibody demonstrating such an unambiguous cleaved trimer reactivity profile has been described previously, although

### Table 1. PGT151 and PGT152 Potency and Breadth of Neutralization against a 117 Pseudovirus Panel

<table>
<thead>
<tr>
<th>mAb</th>
<th>PGT151</th>
<th>PGT152</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (μg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC80 (μg/ml)</td>
<td></td>
<td></td>
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<tr>
<td>Breadth (%)</td>
<td></td>
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</tr>
</tbody>
</table>

*See also Table S2.*

Median IC50 or IC80 (μg/ml)

<table>
<thead>
<tr>
<th>mAb</th>
<th>IC50</th>
<th>IC80</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGT151</td>
<td>0.008</td>
<td>0.024</td>
</tr>
<tr>
<td>PGT152</td>
<td>0.012</td>
<td>0.034</td>
</tr>
</tbody>
</table>

Breadth (%) 66 44 64 47
trimer (Walker et al., 2009; Sanders et al., 2013) and cleavage (Li et al., 2012) preferences have been reported.

PGT151 and PGT152 Bind to Complex Tri- and Tetra-Antennary Glycans

Given that the less than 100% neutralization observed in a number of assays is reminiscent of the previously described glycan dependency of binding for some anti-HIV antibodies, we investigated the ability of PGT151 and PGT152 to neutralize a number of glycan-modified viruses. The glycosidase inhibitor swainsonine prevents the formation of complex glycans, and pseudovirus made in the mutant GNT1-deficient HEK293S cell line (GNT1/−/−) also does not contain complex glycans. As shown in Figure 3A, PGT151 or PGT152 neutralization of JR-CSF virus grown in the presence of swainsonine or in 293S cells was greatly reduced in comparison to neutralization of wild-type virus. Moreover, although PGT151 reacted well with BG505 SOSIP.664 gp140 trimers expressed in 293T cells, it did not react with the same trimers derived from 293S cells (data not shown). These results suggest that PGT151 and PGT152 bind to complex glycans on Env.

To further investigate, we screened PGT151-158 for glycan specificity by using several glycan microarrays. PGT151-158 bound tetra-antennary-complex-type N-glycans with terminal galactose with and without terminal sialic acid residues (Figure 3B, Figure 3D, and Tables S4 and S5). Although terminal sialic acid linked to the 6 position of galactose blocks binding of the antibodies, sialic acid linked to the 3 position appears to be accommodated. We note that in Figure 3B, the signal for tetra-antennary N-glycan binding was saturated. In addition, most of the PGT151 family antibodies also bound triantennary-complex-type N-glycans with terminal galactose (Figures 3B and 3C), although with lower avidity than the binding to tetra-antennary glycans. Blattner et al. further confirmed these results by showing that PGT151 and PGT152 bound to triantennary complex glycans by using isothermal titration calorimetry (ITC) (Blattner et al., 2014, this issue). None of the antibodies were observed to interact with high-mannose glycans of the type demonstrated to interact with the antibodies 2G12 and PGT128 or the biantennary-complex glycan shown to interact with PGT121 (Figure 3C and Tables S4 and S5).

Overall, we concluded that the binding of the PGT151 family is glycan dependent but does not involve either of the high-mannose-containing regions that have previously been found to be important for broad neutralization. These regions are the high-mannose gp120 patch around N332, recognized by antibodies such as 2G12, PGT121, and PGT128, and the V2 loop region around N160, recognized by antibodies such as PG9 and PG16 (Doorey and Burton, 2010; McLellan et al., 2011; Walker et al., 2011; Julien et al., 2013b; Kong et al., 2013b). Of note, PG16 has recently been shown to interact on glycan microarrays with a terminally sialylated tetrantennary glycan. However, this glycan has a different sialic acid linkage than that of the tetra-antennary glycan that interacts with PGT151–PGT158 (Shivatare et al., 2013). Taken together, these results indicate that members of the PGT151 family of antibodies bind a glycan-dependent Env epitope that has not been previously characterized.

PGT151 Does Not Compete with Any Known bnAbs for Binding to Env Spikes

We next epitope mapped PGT151, which is the somatic variant showing the most complete neutralization, by analyzing known bnAbs for competition for binding to cleaved Env spikes (Figure S3A). When PGT151 was prebound to JR-FL ΔCT Env trimers expressed on the surface of 293T cells, it did not compete
with bnAbs targeting the V2 loop N160-glycan-dependent epitope recognized by bnAb PGT145 or the region around N332 recognized by bnAbs PGT121, PGT128, PGT135, 2G12, and PGT130. This result was consistent with the glycan array data above. Additionally, when PGT151 was prebound to JR-FL E168K trimers, PGV04 (Figure S3A) and CD4-IgG (Figure S3B) were still able to bind the trimers, suggesting that its epitope did not significantly overlap with the CD4 binding site. PGT151 did show partial competition with CD4 prebound to cell-surface-expressed trimers (Figure S3C), which most likely is due to conformational changes that occur on the trimer after CD4 binding (Liu et al., 2008) and lead to partial disruption of the PGT151 epitope.

It has previously been shown that MPER-targeting bnAbs 2F5, 4E10, and 10E8 do not bind well to cleaved JR-FL trimers on the surface of 293T cells (Chakrabarti et al., 2011; Huang et al., 2012). In contrast, 2F5 and 4E10 have been shown to bind well to uncleaved JR-FL trimers (Chakrabarti et al., 2011). Because PGT151 binds only to cleaved trimers, we were unable to perform satisfactory competition experiments with MPER-targeting bnAbs and PGT151. These results are further evidence that PGT151 binds to a previously unidentified epitope on Env.

PGT151 Binds to a Glycan-Dependent Epitope on gp41

Next, a large number of viruses containing alanine substitutions in gp120 and gp41 were evaluated in the context of PGT151 neutralization of JR-CSF. As shown in Table S6A and Table 2, only substitutions in gp41 had a significant effect on neutralization by PGT151. The substitutions that had the greatest effect on IC50 and the level of maximum neutralization of JR-CSF were N611D, N637K, E647A, and E647G. The former two substitutions are expected to lead to the loss of the glycans at N611 and N637, occupied most likely by complex glycans (Go et al., 2008, 2009). The latter two substitutions are expected to lead to the loss of the glycans at N611 and N637, occupied most likely by complex glycans (Go et al., 2008, 2009). Next, we investigated the ability of PGT151 to neutralize a panel of variants that had been generated in the process of glycosidase inhibition, which prevents the trimming of mannose residues from the Manα6 arm of the GlcNAcManα6GlcNAc2 structure, or in 293S cells (GNT1/C0/C0 cells), a cell line that is deficient in N-acetylglucosaminyltransferase I and is unable to add a GlcNAc residue to the Manα6GlcNAc2 structure to permit processing to complex glycans. Neutralization of JR-SCF pseudovirus by 2G12 is not affected by either (1) or (2) because it binds exclusively to high-mannose glycans on the glycan shield of Env. Data are presented as the mean ± SEM. (B) PGT151–PGT158 bind tetra-antennary complex carbohydrates and that PGT151 additionally binds a triantennary glycan, on the Wong glycan array. Data are presented as the mean ± SEM. (C) Most of the PGT151 family MAbs do bind triantennary complex-type glycans on the CFG microarray. (D) PGT151 and PGT152 bind a tetrantennary complex-type glycan on the neoglycolipid microarray. The symbols for common monosaccharides are as follows: purple diamonds represent sialic acid, yellow circles represent galactose, red triangles represent fucose, blue squares represent N-acetyl glucosamine, and green circles represent mannose. See also Tables S4 and S5. Data for the CFG microarray are presented as the mean ± SEM. Small differences in array results might reflect differences in glycan coating densities.
LAI strain of HIV-1 (Table S6B). Again, the gp41 residues N611 and N637 were found to be important. In addition, an A501T substitution in gp120 showed a significant if moderate effect (Table 2 and Table S6B). Finally, in the context of the isolate JR-FL, the most marked effects occurred for substitutions leading to the loss of glycans at N611 and N637. At 98.7% and 92.8% conservation, residues N611 and N637, respectively, are extremely highly conserved across the 30,324 viral sequences found in the Los Alamos database. E647 is 90.2% conserved across the viral sequences.

Given the evidence that some HIV glycan-dependent antibodies can interact with differing sets of glycans in an isolate-dependent context (McLellan et al., 2011) (D. Sok, personal communication), we tested combinations of double substitutions of N611A, N637A, and E647A in JR-CSF (clade B), BG505 (clade A), JR-FL (clade B), 92RW020 (clade A), 94UG103 (clade A), and IAVI C22 (clade C) in comparison with single substitutions in terms of their effects on PGT151 neutralization (Figure 4A). For all isolates, N611A consistently had the greatest effect on IC50 and the maximum level of neutralization. The single N637A and E647A substitutions had little to no effect on the IC50 for most isolates and varying effects on the maximum level of neutralization by PGT151. However, the N611A + N637A and N611A + E647A double substitutions completely abrogated neutralization of all isolates by PGT151. Use of PGV04 as a control showed that the substitutions did not affect the IC50 or maximum inhibition of this CD4bs bnAb (Figure 4B).

To determine whether the glycans at N611 and N637 were the only combination of gp41 glycans that PGT151 uses for binding, we made double substitutions of all four (N611, N616, N625, and N637) gp41 glycan combinations in JR-CSF (Figure S4A) and JR-FL (Figure S4C) and the four equivalent (N611, N618, N625, and N637) gp41 glycan combinations in BG505 (Figure S4B). No other combination of glycan substitution other than N611A + N637A completely eliminated neutralization by PGT151. All other double-substitution combinations that removed two glycans in gp41 (N625 and either N616 or N618) in combination with either N611 or N637 had no additional effects on neutralization beyond those elicited by the single N611 or N637 substitution alone. The double substitution N616A + N625A had no effect at all on neutralization by PGT151. PGV04 was used as a control for these substitutions for BG505 (Figure S4C), and PGV04 and 10E8 were used as controls for the substitutions in JR-FL (Figure S4C). In addition, double substitutions leading to the removal of the four gp41 glycans in JR-CSF were made in combination with N88A, which would lead to the removal of a glycan that would not be expected to be close to the PGT151 epitope (Julien et al., 2013a; Lyumkis et al., 2013). These double substitutions had no effect on PGT151 neutralization, in the same way that the double substitutions at N616 (or N618) and N625 had no effect on PGT151 neutralization.

Table 2. The Effect of Single-Amino-Acid Substitutions on PGT151 Neutralization for Three HIV Isolates

<table>
<thead>
<tr>
<th>Variant virus</th>
<th>Fold IC50 increase relative to wild-type (PGT151)</th>
<th>% Maximum neutralization</th>
<th>Fold IC50 increase relative to wild-type (PGT151)</th>
<th>% Maximum neutralization</th>
<th>Fold IC50 increase relative to wild-type (PGT151)</th>
<th>% Maximum neutralization</th>
</tr>
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<tbody>
<tr>
<td>JR-CSF</td>
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<tr>
<td>A501T</td>
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<td>44</td>
<td>6</td>
<td>75</td>
<td>&gt;350</td>
<td>31</td>
</tr>
<tr>
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<td>14</td>
<td>81</td>
<td>&gt;200</td>
<td>8</td>
</tr>
<tr>
<td>N611D</td>
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<td></td>
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<tr>
<td>S613A</td>
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<td>41</td>
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</tr>
<tr>
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<td>70</td>
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<tr>
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<td>20</td>
<td>&gt;115</td>
<td>37</td>
<td>&gt;200</td>
<td>33</td>
</tr>
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<td>T639A</td>
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<td>52</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>E647A</td>
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<td>1</td>
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<tr>
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<td>2</td>
<td>95</td>
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<td>94</td>
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<tr>
<td>WT</td>
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<td>93</td>
<td>1</td>
<td>100</td>
<td>1</td>
<td>99</td>
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</tbody>
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*Neutralization activity is reported as the n-fold increase in IC50 value relative to wild-type and was calculated with the equation (IC50 mutant)/(IC50 wild-type). Percent maximum neutralization is the plateau reached on the neutralization curve by the mAb. Boxes are color coded as follows: blue, substitutions that had a negligible effect on IC50 or maximum neutralization; yellow, 4- to 9-fold increase in IC50; orange, 10- to 99-fold increase; red, >100-fold increase in IC50; lavender, maximum neutralization ≥ 50%; brown, maximum neutralization < 50%; gray, virus not significantly infectious; and ND, not done. Data are presented as the mean of two independent experiments. See also Table S6.
Blattner et al. fitted the high-resolution EM structure of BG505 SOSIP.664 into the reconstruction of the JR-FL:PGT151 Fab complex and predicted that the PGT151 LC interacts with N276 from one gp120 protomer and with N262 and N448 from a second gp120 protomer (Blattner et al., 2014, this issue). The removal of the glycan at N276 or N448 in BG505 or JR-CSF backgrounds had no effect on PGT151 neutralization. Furthermore, the removal of the combination of glycans at N276 + N611 or N276 + N637 had no further effects on PGT151 neutralization as compared to the effects of virus lacking only the glycan at N611 or N637, respectively. In contrast, the removal of the glycan at N448 in combination with N611 or N637 increased PGT151 maximum neutralization compared to the neutralization plateaus of virus lacking the single glycans at N611 or N637. This suggests that the glycan at N448 affects PGT151 neutralization only in the context of the loss of a glycan at N611 and/or N637 or of the presence of certain glycoforms at these positions. Our data paint a complex picture of Env recognition by PGT151. Many interactions appear to be isolate dependent, and phenotypes might not manifest when single mutations are introduced (e.g., N234 and N637 in BG505; Figure S4B). Thus, PGT151 might use redundant or compensatory interactions.

**Figure 4. PGT151 Neutralization of Viruses Containing N611A, N637A, and E647A Single and Double Alanine Substitutions**

Serial dilutions of antibody (A) PGT151 and (B) PGV04, used as a control, were preincubated with pseudovirus for 1 hr and then added to TZM-bl cells. Three days after infection, luciferase values were measured and percent neutralization was calculated. See also Figure S4. Data are from a representative experiment of at least two replicate experiments and are presented as the mean values of two replicate wells ± SEM.
isolates (Walker et al., 2009, 2011; Wu et al., 2010; Scheid et al., 2011). Greater potency and/or breadth of neutralization against diverse new bnAbs are directed to epitopes defined earlier but have evolved largely to avoid the obstruction of the N276 glycan rather than to use it for a positive binding interaction. Thus, functional and structural studies can be complementary in providing understanding of the complex interaction of antibodies with epitopes involving glycans.

**PGT151 and PGT152 Mediate Antibody-Dependent, Cell-Mediated Cytotoxicity and Are Not Polyreactive**

Neutralizing antibodies of the appropriate isotype recognizing native Env spikes might be expected to mediate killing of HIV-infected cells by antibody-dependent, cell-mediated cytoxicity (ADCC). PGT151 and PGT152 were investigated in an ADCC assay against target cells infected with NL4-3, YU2, or JR-CSF viruses and were shown to mediate this effector activity (Figure 5). SIVmac239 was used as a negative control.

Finally, we found no evidence for polyreactivity of either PGT151 or PGT152 when we screened against a panel of antigens (Figure S5A), and PGT151 did not bind to HEP-2 cells in an antinuclear antibody (ANA-HEP-2) indirect immunofluorescence assay, indicating that it does not bind to nuclear or cytoplasmic self-antigens (Figure S5B). These results indicate that PGT151 and PGT152 are not polyreactive and that they have the capacity to mediate ADCC in addition to neutralizing free virus, which could be significant for the in vivo anti-viral activity of the antibodies, whether vaccine induced or administered passively.

**DISCUSSION**

The past few years have seen an explosion in the generation of bnAbs to HIV (Burton et al., 2012; Kwong and Mascola, 2012; Klein et al., 2013; Mascola and Haynes, 2013). Many of the new bnAbs are directed to epitopes defined earlier but have greater potency and/or breadth of neutralization against diverse isolates (Walker et al., 2009, 2011; Wu et al., 2010; Scheid et al., 2011; Huang et al., 2012). However, at least two gp120 epitope regions involving protein and glycan recognition have been described (Walker et al., 2009, 2011). One of these regions is a V2 epitope dependent on a glycan at N160, whereas the other is a cluster of epitopes involving conserved parts of the V loops and centered on N332. Here, we describe a set of antibodies recognizing a previously undefined glycan-dependent epitope on gp41. The two prototype antibodies from this set, PGT151 and PGT152, are very potent and neutralize about two-thirds of a large panel of viral isolates with a median IC$_{50}$ approximately 10 ng/ml, although they show incomplete neutralization with a significant fraction of viruses. The recognized epitope is centered on the highly conserved glycan site at N611, and there is involvement of another highly conserved glycan site at N637 and the less conserved residue E647. The relative contributions of these individual residues appear to be isolate dependent. Substitution of N611 consistently had an adverse effect on neutralization by PGT151 and, in combination with other substitutions at N637 or E647, invariably led to complete abrogation of antibody neutralization. Nevertheless, viruses resistant to neutralization by PGT151 were identified in the large panel that was tested; this panel contained both N611 and N637 glycan sites. This is in line with previous studies on glycan-dependent gp120 antibodies, for which crucial glycans have been identified but for which changes in non-glycan-related amino acid residues lead to resistance even in the presence of these crucial glycans (Walker et al., 2009, 2011; McLellan et al., 2011; Doria-Rose et al., 2012). Also of note, viruses lacking both the N611 and N637 glycan sites tend to show lower infectivity than wild-type viruses, suggesting that the absence of these sites might be associated with a lower fitness in vivo and that this lower fitness could be associated, at least in part, with their high levels of conservation.

For JR-CSF, the N611D substitution completely abolished PGT151 neutralization, whereas removal of the glycan at N611 by N611A or S613A substitution had somewhat lesser effects on IC$_{50}$ and percent maximum neutralization. Similarly, the N637K substitution abolished PGT151 neutralization, whereas N637A or T639A substitution only affected the percent maximum neutralization. The stronger effect of N611D and N637K as substitutions at N637 or E647, invariably led to complete abrogation of antibody neutralization. Nevertheless, viruses resistant to neutralization by PGT151 were identified in the large panel that was tested; this panel contained both N611 and N637 glycan sites. This is in line with previous studies on glycan-dependent gp120 antibodies, for which crucial glycans have been identified but for which changes in non-glycan-related amino acid residues lead to resistance even in the presence of these crucial glycans (Walker et al., 2009, 2011; McLellan et al., 2011; Doria-Rose et al., 2012). Also of note, viruses lacking both the N611 and N637 glycan sites tend to show lower infectivity than wild-type viruses, suggesting that the absence of these sites might be associated with a lower fitness in vivo and that this lower fitness could be associated, at least in part, with their high levels of conservation.

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Furthermore, at first glance our data might seem contradictory to that of Blattner et al. in that the negative-stain EM data fitted into the high-resolution EM structure indicate that the glycans at N448 and N276 contact PGT151. However, we have previously shown that removal of the N276 glycan, which forms contacts with VRC01 as determined by crystal structure, actually increases VRC01 neutralization potency in functional assays (Falkowska et al., 2012). It appears that the VRC01 antibody has evolved largely to avoid the obstruction of the N276 glycan rather than to use it for a positive binding interaction. Thus, functional and structural studies can be complementary in providing understanding of the complex interaction of antibodies with epitopes involving glycans.

**Figure 5. ADCC by PGT151 and PGT152**

(A) PGT151 and (B) PGT152 were titrated for ADCC activity against target cells infected with HIV-1 NL4-3, YU2, JR-CSF, and SIVmac239, and an NK cell line (KHYG-1) expressing CD16 was used as the effector cell. The killing of virus-infected cells by ADCC is indicated by a loss of relative light units (RLUs). SIVmac239 is used as a negative control, and HIVIG is used as a positive control. Data are presented as mean values of three replicates ± SEM.
of single substitutions at N611 and N637 might reflect the presence of the MPER of gp41 and the proximity of the viral membrane in neutralization studies but not those using the recombinant SOSIP protein. Similarly, some differences were observed between neutralization studies and SOSIP binding studies in terms of the effects of a number of gp120 substitutions on PGT151 binding. Thus, a number of substitutions led to decreased binding of PGT151 to BG505 SOSIP (Blattner et al., 2014) but did not impact neutralization. The residues that lowered binding but did not impact neutralization might have had a general effect on the trimer structure of BG505 SOSIP.664 but not on trimer structure and/or accessibility of PGT151 to its epitope in the context of virus neutralization. Alternatively, there might be a strain-specific effect given that we made substitutions in JR-CSF and LAI rather than BG505.

The glycans involved in interaction with PGT151 and PGT152 are probably complex in light of the fact that virus produced with high-mannose glycans only is not neutralized; this idea is also consistent with glycans in datasets that show optimal binding to tri- and tetra-antennary N-linked glycans. The ability of PGT151 to recognize slightly different epitopes on different isolates may at first seem surprising but has in fact been noted for both V2- and N332-dependent HIV bnAbs (McLellan et al., 2011) (D. Sok, personal communication). It appears that bnAbs recognizing neighboring glycans can employ varying modes of recognition to the extent that alternate glycans can be used on different isolates in some cases. Detailed molecular understanding of how promiscuous binding is achieved for the PGT151 family of antibodies must await high-resolution structures of these antibodies in complex with Env.

Similar to the V2-glycan-dependent bnAbs such as PG9 and PG16, the PGT151 family of antibodies shows incomplete neutralization behavior in the TZM-bl and PBMC assays, i.e., members of this family show neutralization curves that plateau at less than 100% for a fraction of viral isolates. For PG9, incomplete neutralization has been ascribed, at least in part, to glycan heterogeneity (Doorens and Burton, 2010). This seems to also be the case for the PGT151 antibodies: we have shown that gp120 glycans that are in close proximity to the PGT151 epitope can affect neutralization by PGT151. Of note, the extent of incomplete neutralization appears to be generally less in an assay involving replication-competent, PBMC-grown viruses than in assays that use the corresponding pseudo-viruses grown in 293T cells or replication-competent, PBMC-grown viruses infecting TZM-bl cells. The impact of incomplete neutralization on the protective ability of antibodies in vivo has not yet been clearly established.

One of the most striking features of PGT151 and PGT152 is that they are specific for cleaved Env trimer and do not recognize uncleaved Env trimer. This behavior is seen in the context of trimer expressed on the surface of infected cells and in the context of a recombinant trimer that mimics virion-expressed trimer. The gp41 components of the uncleaved soluble gp140 proteins have previously been shown to adopt a 6 helix bundle conformation, whereas the gp41 components in the context of the cleaved SOSIP trimers retain the prefusion form (Guttman et al., 2013; Julien et al., 2013a; Lyumkis et al., 2013; Ringe et al., 2013). Hence, PGT151 and PGT152 report on the conformation of gp41 and trimeric Env and should be valuable in distinguishing different forms of Env as found in a number of potential immunogens, e.g., VLPs. The antibodies also appear to stabilize the cleaved Env trimer (Blattner et al., 2014, this issue), and this property might be crucial in structural studies on functional spikes isolated from infected cell surfaces.

Finally, the PGT151 family of antibodies defines what is, to the best of our knowledge, a previously unidentified, broadly neutralizing target on the HIV envelope spike; after molecular characterization, this target could be incorporated into vaccine designs. The prevalence of the PGT151 specificity in donors with broadly neutralizing sera is most likely limited in light of earlier observations on the predominance of neutralizing specificities (Walker et al., 2010). However, more precise understanding of prevalence will require the use of the distinguishing features presented in this study to characterize sera from a number of cohorts of infected individuals.

**EXPERIMENTAL PROCEDURES**

**Isolation of Monoclonal Antibodies**

The method for isolating human monoclonal antibodies from memory B cells in circulation has previously been described (Walker et al., 2009).

**Pseudovirus Generation**

The generation of pseudoviruses incorporating Env from different HIV-1 strains and/or containing single alanine substitutions is fully described elsewhere (Pantophlet et al., 2003). Swainsonine was added at the time of transfection for the glycosidase inhibitor experiments and was used at 20 μM.

**Neutralization Assay**

The neutralization activity of antibodies against pseudovirus was measured with a TZM-bl assay as previously described (Li et al., 2005). Neutralization of PBMC-grown, replication-competent virus was evaluated with the TZM-bl assay as described above or with human PBMCs. For neutralization via PBMCs, antibody and virus were preincubated for 1 hr at 37°C before being added to the stimulated PBMCs in a 96-well plate. The cells were incubated for 24 hr, washed three times, and incubated for 4–7 days. p24 was read on day 4 and day 7 after infection.

**ELISA**

Ninety-six-well plates were coated overnight at 4°C with anti-gp120 antibody D7324 (International Enzymes) at 5 μg/ml in PBS. Plates were washed four times with PBS (0.05% Tween), either gp120 protein or lysed virus (931N905 or 94UG103) was added, and preparations were incubated for 2 hr at 37°C. The remainder of the experiment was conducted as previously described (Falkowska et al., 2012). The gp140 and gp41 proteins were directly coated onto the ELISA plates. The polyreactivity ELISA was performed as previously described (Walker et al., 2009).

**ELISA with BG505 SOSIP.664 Trimer**

The procedure for the Ni-NTA ELISA has been previously described (Bontjer et al., 2010). In brief, 0.1 μg/ml of His-tagged BG505 SOSIP gp140 was added in PBS to Ni-NTA His Sorb 96-well plates (QIAGEN) for 1 hr at 37°C. Wells were washed four times with PBS (0.05% Tween), either gp120 protein or lysed virus (931N905 or 94UG103) was added, and preparations were incubated for 2 hr at 37°C. The remainder of the experiment was conducted as described previously (Falkowska et al., 2012). The gp140 and gp41 proteins were directly coated onto the ELISA plates. The polyreactivity ELISA was performed as previously described (Walker et al., 2009).
BG505 SOSIP are not shown but are comparable to those for His-tagged BG505 SOSIP.

Flow Cytometry
Serial dilutions (1:5) of PGT151, PGT152, PG9, b12, and b6 starting at 20 μg/ml were added to JR-FL E168KΔCT wild-type or uncleaved (REKR replaced with SEK) transfected 293T cells and incubated for 1 hr at 37°C on a plate rocker. The plate was washed twice in FACs buffer (PBS, 10% FBS, 0.01% sodium azide) and stained with a 1:200 dilution of R-phycocerythrin (PE)-conjugated AffiniPure F(ab')2 fragment goat anti-human IgG, F(ab')2 (Jackson ImmunoResearch). Binding was analyzed with flow cytometry as previously described (Walker et al., 2009).

The Consortium for Functional Glycomics Glycan Microarray Analysis
PGT151 and PGT152 were screened on a printed glycan microarray, version 5.0, from the Consortium for Functional Glycomics (CFG) as described previously (Blixt et al., 2004). Antibodies were used at 30 μg/ml and were precomplexed with 15 μg/ml secondary antibody (goat anti-human-Fc-rPE, Jackson Immunoresearch) before addition to the slide. Complete glycan array data sets for all antibodies can be found at http://www.functionalglycomes.org through the public database of CFG under resource request number 2944.

Neoglycolipid Microarray Analysis
Analysis with neoglycolipid arrays (Feizi and Chai, 2004) was carried out as described previously (Pejchal et al., 2011). In brief, PGT151 and PGT121 were analyzed at 50 μg/ml, and biotinylated anti-human IgG (Vector) was then added at 5 μg/ml. The results for PGT128 and 2G12 were taken from earlier experiments performed with different versions of the microarrays, as described (Pejchal et al., 2011). The analyses were performed at 20°C. The full array data with 38 oligosaccharide probes are in Table S4.

Wong Glycan Microarray Analysis
Amine-functional glycans were printed in replicates of three onto NHS-activated glass slides at a 100 mM concentration as previously described (Liang et al., 2011; Shivatavare et al., 2013). Printed slides were incubated for 1 hr at 4°C. The sample was added to a glycan array and incubated at 1 hr at 4°C. The slides were washed sequentially in PBS with 0.05% Tween-20, in PBS, and then in water. Arrays were scanned on a ProScanArray HT (PerkinElmer) confocal slide scanner. Image analysis was carried out with Genepix Pro 6.0 analysis software ( Molecular Devices Corporation). Error bars represent the average percentage error for all data points reported. The oligosaccharide probes and array data are shown in Table S5.

ADCC
ADCC activity was measured as previously described (Alpert et al., 2012). CEM.NKR-ccR–sLTR-Luc cells (1 x 10⁶ cells), which contain a Tat-inducible luciferase reporter gene, were infected with HIV-1uA-L/L (200 ng p24), HIV-1uNL (1 μg p24), or SIVmac239 (500 ng p27) by spinoculation in the presence of polybrene (40 μg/ml) (EMD Millipore). Four days after infection, CEM.NKR-ccR–sLTR-Luc cells were washed three times and incubated in 96-well plates with an NK cell line (KHYG-1) expressing CD16 at a 10:1 E:T ratio in the presence of serial dilutions of monoclonal antibodies or purified immunoglobulin from HIV-1–infected donors (HIVIG; AIDS Reagent Program, Division of AIDS, NIAID, NIH: Catalog #9597, HIV-IG from NABI and NHLBI). After an 8 hr incubation, cells were mixed with luciferase substrate (BriteLite Plus; Perkin Elmer), and luciferase activity in relative light units (RLUs) was measured with a Victor X plate reader (Perkin Elmer). ADCC activity was calculated from the mean RLU for triplicate wells at each antibody concentration relative to the means for background and maximal RLUs from replicate wells containing uninfected and infected cells, respectively, incubated with NK cells but without antibody.

Statistics
Statistical analyses were done with Prism 6.0 for Mac (GraphPad).

ACCESS NUMBERS
The Genbank accession numbers for the PGT151-158 sequences reported in this paper are BankIt171714 PG151HC KJ700283, BankIt171714 PG152HC KJ700289, BankIt171714 PG153HC KJ700284, BankIt171714 PG154HC KJ700285, BankIt171714 PG155HC KJ700286, BankIt171714 PG156HC KJ700287, BankIt171714 PG157HC KJ700288, BankIt171714 PG158HC KJ700289, BankIt171714 PG151LC KJ700290, BankIt171714 PG152LC KJ700291, BankIt171714 PG153LC KJ700292, BankIt171714 PG154LC KJ700293, BankIt171714 PG155LC KJ700294, BankIt171714 PG156LC KJ700295, BankIt171714 PG157LC KJ700296, and BankIt171714 PG158LC KJ700297.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures, six tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.04.009.

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