A Derivative of the D5 Monoclonal Antibody That Targets the gp41 N-Heptad Repeat of HIV-1 with Broad Tier-2-Neutralizing Activity

Adonis A. Rubio,a,b Maria V. Filsinger Interrante,a,c,d Benjamin N. Bell,a,e Clayton L. Brown,a,f Theodora U. J. Bruun,a,f Celia C. LaBranche,g,h David C. Montefiori,h,i Peter S. Kim,h,i

ABSTRACT HIV-1 infection is initiated by the viral glycoprotein Env, which, after interaction with cellular coreceptors, adopts a transient conformation known as the prehairpin intermediate (PHI). The N-heptad repeat (NHR) is a highly conserved region of gp41 exposed in the PHI; it is the target of the FDA-approved drug enfuvirtide and of neutralizing monoclonal antibodies (mAbs). However, to date, these mAbs have only been weakly effective against tier-1 HIV-1 strains, which are most sensitive to neutralizing antibodies. Here, we engineered and tested 11 IgG variants of D5, an anti-NHR mAb, by recombining previously described mutations in four of D5’s six antibody complementarity-determining regions. One variant, D5_AR, demonstrated 6-fold enhancement in the 50% inhibitory dose (ID50) against lentivirus pseudotyped with HXB2 Env. D5_AR exhibited weak cross-clade neutralizing activity against a diverse set of tier-2 HIV-1 viruses, which are less sensitive to neutralizing antibodies than tier-1 viruses and are the target of current antibody-based vaccine efforts. In addition, the neutralization potency of D5_AR IgG was greatly enhanced in target cells expressing FcγRI, with ID50 values of <0.1 μg/ml; this immunoglobulin receptor is expressed on macrophages and dendritic cells, which are implicated in the early stages of HIV-1 infection of mucosal surfaces. D5 and D5_AR have equivalent neutralization potency in IgG, Fab, and single-chain variable-fragment (scFv) formats, indicating that neutralization is not impacted by steric hindrance. Taken together, these results provide support for vaccine strategies that target the PHI by eliciting antibodies against the gp41 NHR and support investigation of anti-NHR mAbs in nonhuman primate passive immunization studies.

IMPORTANCE Despite advances in antiretroviral therapy, HIV remains a global epidemic and has claimed more than 32 million lives. Accordingly, developing an effective HIV vaccine remains an urgent public health need. The gp41 N-heptad repeat (NHR) of the HIV-1 prehairpin intermediate (PHI) is highly conserved (>90%) and is inhibited by the FDA-approved drug enfuvirtide, making it an attractive vaccine target. However, to date, anti-NHR antibodies have not been potent. Here, we engineered D5_AR, a more potent variant of the anti-NHR antibody D5, and established its ability to inhibit HIV-1 strains that are more difficult to neutralize and are more representative of circulating strains (tier-2 strains). The neutralizing activity of D5_AR
was greatly potentiated in cells expressing FcγRI; FcγRI is expressed on cells that are implicated at the earliest stages of sexual HIV-1 transmission. Taken together, these results bolster efforts to target the gp41 NHR and the PHI for vaccine development.

**KEYWORDS** HIV-1, antibodies, prehairpin intermediate, virus

More than 35 million people currently live with HIV/AIDS globally (https://www.who.int/news-room/fact-sheets/detail/hiv-aids); despite the promise of antiretroviral therapy, a preventative HIV-1 vaccine remains an urgent global health need. However, after more than three decades of intense research, a prophylactic HIV/AIDS vaccine remains elusive. The RV144 trial raised expectations in 2009 (1), but the lack of efficacy reported in 2020 for the HVTN702 trial in South Africa, which was based on the prime-boost regimen utilized in RV144, was another major setback for the field (2).

Many current HIV-1 vaccine-directed research efforts are motivated by the finding that rare broadly neutralizing antibodies (bNAbs) can be isolated from patients chronically infected with HIV-1 (3–22). The main epitopes of these bNAbs include the CD4 binding site (CD4bs), the membrane proximal external region (MPER), the V1/V2 region, and the V3 glycan (23–26). Importantly, bNAbs are generally elicited as a result of complex coevolutionary pathways with viral variants and thus tend to be characterized by extensive somatic hypermutation (SHM) of the antibody genes. Indeed, germline precursors of bNAbs typically do not bind to most Env proteins (7, 27, 28). As a result, germline-lineage approaches (5, 10, 12, 15, 16, 21, 22) have emerged wherein an engineered immunogen is used to stimulate a germline precursor of a bNAb, followed by multistage multicomponent immunization aimed to direct affinity maturation toward the desired bNAb. While some progress has been made in eliciting bNAb-like responses in nonhuman primates (NHPs) (29–31), the technical and biological difficulties in eliciting rare bNAbs represent critical barriers to developing an antibody-based HIV vaccine against the native prefusion conformation of Env.

In contrast to these bNAb epitopes on the native conformation of Env, one highly conserved region of HIV-1 gp41 is exposed in a transient form of Env that is critical for viral infection, the prehairpin intermediate (PHI). Upon binding cellular receptors, Env undergoes substantial conformational changes to form the PHI, in which the N-heptad repeat (NHR) and C-heptad repeat (CHR) regions are exposed before the cellular and viral membranes come together for membrane fusion (32–34). Due to its high sequence conservation (≈93%) (https://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html) and critical role in viral entry (32), the NHR is a promising target for blocking HIV-1 infection. Several inhibitors of HIV-1 infection that target the NHR and prevent viral membrane fusion have been identified (35–48). Cyclic D-peptide inhibitors that target the NHR have been used to prevent and treat simian-human immunodeficiency virus (SHIV) infection in rhesus macaques (35, 49). Most notably, the FDA-approved HIV-1 fusion inhibitor enfuvirtide (50–52) validates the NHR as a therapeutic target in humans.

Of relevance for vaccine applications, several monoclonal antibodies (mAbs) that target the NHR and inhibit HIV-1 infection have been characterized (53–56). The first of these mAbs was D5 (53), which was isolated from a human B cell-derived phage display library using two synthetic mimetics of the gp41 PHI: 5-helix (36) and IZN36 (58). X-ray crystallographic studies reveal that D5 binds a conserved hydrophobic pocket of the NHR (59). In contrast to most HIV-1 bNAbs (9, 60–66), D5 and other neutralizing mAbs that target the NHR (53–56, 67) contain far fewer amino acid changes resulting from SHM (Fig. 1). Thus, eliciting anti-PHI antibodies via vaccination is not expected to require extensive germline-targeting strategies. Indeed, various NHR-based vaccine candidates have been shown to elicit HIV-1-neutralizing antisera in animals (56, 68–70). These antisera, however, demonstrate only weak neutralization potency. Similarly, while exhibiting broad neutralizing activity, the potencies of mAbs targeting the NHR, including D5, are weak and mostly limited to tier-1 HIV-1 isolates (53–56). Taken
together, these findings have led to understandable skepticism about the PHI as a vaccine target.

Here, we engineered more-potent versions of D5 by combining multiple mutations in the complementarity-determining region (CDR) loops of the antibody that were individually shown to increase the neutralization activity of D5 by Montgomery et al. (67). The most enhanced recombined variant, D5_AR, shows neutralization efficacy in several diverse tier-2 HIV-1 viruses. Thus, D5_AR presents proof of concept that an anti-PHI mAb, with low levels of SHM, can neutralize tier-2 HIV-1 viruses. In addition, as recently reported for D5 (71) and earlier for MPER mAbs (72, 73), the neutralization potency of D5_AR was enhanced ~1,000-fold in target cells expressing the high-affinity immunoglobulin receptor FcγRI compared to those without. These results bolster attempts to target the PHI as an alternative and orthogonal approach toward an HIV-1 vaccine that is fundamentally different from the prevalent germline-targeting strategies to elicit bNAbs.

RESULTS

D5_AR, a recombined CDR mutant of D5, has enhanced neutralization potency against HIV-1 in vitro. X-ray crystallography revealed that complementarity-determining region (CDR) loops in the antibody variable regions of both the heavy and light chains (VH and VL, respectively) contribute to the binding of D5 to the NHR (59). Informed by this insight, Montgomery et al. (67) sought to increase the neutralization potency of D5 by randomizing residues in five of the six CDRs (VH CDR1, 2, and 3 and VL CDR1 and 2). Four D5 IgG variants, each with only one CDR mutated, had slightly increased neutralization potency (67).

To assess the impact on neutralization after the introduction of multiple mutated CDRs, we recombinantly expressed and purified wild-type D5 IgG and 15 D5 IgG variants (Fig. 2). The variants were designated D5_HXXX_LX (Fig. 2C), with each X replaced by either 0 (representing the wild-type sequence) or 1 (representing the mutated CDR sequence) in the heavy (CDR1, 2, and 3) and light (CDR1 and 2) CDR loops. Using single-round infectivity assays in TZM-bl cells, each D5 IgG variant was screened for neutralization potency against lentivirus pseudotyped with HIV-1 HXB2 Env (74–78). Neutralization potency was reported as the 50% inhibitory dose (ID50). We confirmed that the four single-CDR mutants previously described by Montgomery et al. (67) (D5_H100_L0, D5_H010_L0, D5_H001_L0, and D5_H000_L1) displayed enhanced neutralization versus the parent D5.
Next, we screened 11 additional D5 variants with lentivirus pseudotyped with HIV-1 HXB2 Env for neutralization potency using a single-round infectivity assay. Several recombinant D5 variants had little effect or even diminished the neutralization potency compared to that of D5 (Table 1). Nevertheless, we identified six D5 variants that modestly enhanced (~2.0-fold) the neutralization potency of D5 (Table 1). Among these, D5_H011_L0, in which both CDR2 and CDR3 of the heavy chain are mutated, demonstrated the greatest enhancement (4-fold) in ID50 (Table 1). We renamed this enhanced D5 variant D5_AR.

To further characterize D5_AR, we assessed the difference in binding for D5_AR Fab compared to D5 Fab against CCIZN17, a variation of a previously described mimetic of the NHR trimer (58, 79), using biolayer interferometry (see Materials and Methods). The Fab for D5_AR has ≈38-fold higher affinity for CCIZN17 than for D5 (1.6 nM for D5_AR compared to 61 nM for D5), providing a potential explanation for the enhanced neutralization activity of D5_AR (Fig. 3).

Size exclusion chromatography purification impacts D5’s neutralization potency. Our initial neutralization screen (Table 1) utilized antibody purified via protein A affinity chromatography (see Materials and Methods). To obtain a more in-depth neutralization profile for D5_AR compared to that of D5, which included testing the constructs’ neutralization potency against various viruses and in different antibody formats, we added a size exclusion chromatography (SEC) step following protein A affinity chromatography. Notably, we found that the manner of purification of antibodies impacted the observed neutralization activity of the antibody (Fig. 4). A side-by-side comparison of a D5_AR antibody prep that was purified via only protein A chromatography with D5_AR that had the subsequent SEC purification step revealed that there was indeed a difference in neutralization for D5_AR (Fig. 4C); however, neutralization was still enhanced compared to that of D5. Reviewing the UV traces from SEC performed after affinity chromatography, we observed and removed aggregates for both D5 and D5_AR IgG (Fig. 4A and B). We note that the aggregate fraction in the SEC traces (Fig. 4A and B) accounted for 8.2% and 11% of the total protein for D5 and
D5_AR, respectively; this is in comparison to the 90% and 83% in the major IgG fraction for D5 and D5_AR, respectively. We hypothesize that the presence of aggregates reduced the neutralization potencies of D5 and D5_AR, possibly because aggregated D5 has less neutralizing activity. Once aggregates were removed, D5 and D5_AR IgG preparations did not form aggregates readily. SEC-purified D5_AR samples frozen in 10% glycerol-1/2 phosphate-buffered saline (PBS) solution at −220°C retain similar neutralization activity after thawing (Fig. 4D).

Experiments in Table 1 were the only experiments reported here that used non-SEC-purified antibody preparations, which accounts for the difference in ID50 values for HXB2 reported in Table 1 (and see Fig. 6) (SEC-purified preparations had a 1.5-fold decrease in ID50 for D5 and a 2.3-fold decrease in ID50 for D5_AR).

**D5 and D5_AR neutralize with similar potency as an scFv, Fab, and IgG.** In the first description of D5, the IgG (~150 kDa) and single-chain variable-fragment (scFv) (~25 kDa) constructs neutralized similarly to one another (53). However, more recent studies reported that D5 scFv is more potent than D5 Fab (~50 kDa) and that both were more potent than D5 IgG (59, 67, 80); in addition, there are reports that increasing the size of NHR inhibitors reduces neutralization potency (55, 67, 80–82). These size-dependent findings would imply steric hindrance in accessing the PHI. Given our finding that SEC purification impacted the neutralization potency of D5 and D5_AR IgG (Fig.

**FIG 3** D5_AR Fab has a greater binding affinity to CCIZN17, an NHR-mimetic, than D5 Fab. The binding affinity (reported numerically as the K_D) of D5 (red) and D5_AR (blue) Fab for CCIZN17, a variation of a previously described NHR mimetic (58, 79), is presented as association and dissociation curves using biolayer interferometry. The K_Ds are reported as the means from fitted values across multiple concentrations from at least two independent experiments and are reported with the standard errors of the means.
we decided to reinvestigate the question of size-dependent neutralization for D5 using antibody preparations free of aggregates. Notably, in agreement with the initial report (53) and in contrast to other reports (59, 67, 80), we found that D5 as an IgG, Fab, and scFv (all SEC purified) did not exhibit a size-dependent pattern of neutralization (Fig. 5A). This difference may be due to our final size exclusion chromatography step, which, to our knowledge, was not performed in other studies in which size dependence was observed. Additionally, we detected comparable neutralization potency for D5_AR as an scFv, Fab, and IgG (Fig. 5B). These results demonstrate that neither D5 nor D5_AR is impacted by steric hindrance and suggest the presence of protein aggregates could explain previous reports of size-dependent neutralization for D5 scFv, Fab, and IgG.

**D5_AR exhibits cross-clade tier-2 neutralization of HIV-1 viruses.** We next investigated the potency of D5_AR IgG in neutralizing a diverse panel of 19 tier-1 and tier-2 pseudotyped viruses across eight viral clades (A, AC, B, C, G, CRF01, CRF02, and CRF07) (77, 83–98). Ten of these strains originated from a 12-virus panel designed to capture the sequence diversity of the HIV-1 epidemic globally (77). Tier-1A and tier-1B contain viruses that are most sensitive to neutralization by antibodies, whereas tier-2 viruses have modest sensitivity to neutralizing antibodies (90). D5_AR IgG neutralized virus more effectively than D5 IgG across all tiers and clades (Fig. 6A). Notably, D5_AR neutralized more viruses with an ID50 of \( <50 \mu g/ml \) (63%) than D5 (11%) (Fig. 6B).

**D5_AR neutralization potency is enhanced >1,000-fold in FcRI-expressing cells.** Recently, we reported the neutralization potency of D5 IgG to be greatly increased in TZM-bl cells expressing the cell surface receptor FcRI (TZM-bl/FcRI cells) (71). FcRI is the only known IgG receptor in humans capable of binding monomeric IgG with high affinity (99). To determine whether D5_AR IgG is similarly potentiated, we tested its neutralization of an additional panel of tier-2 HIV-1 viruses and SHIV
challenge viruses in TZM-bl/FcγRI cells (Fig. 7). As recently reported for D5 (71), and as previously characterized for MPER mAbs (72, 73), the neutralization potency of D5_AR was enhanced at least 1,000-fold in TZM-bl/FcγRI cells compared to that in TZM-bl cells without FcγRI (Fig. 7A and B). In the presence of FcγRI, D5_AR had potent neutralization activity against a panel of tier-2 HIV-1 viruses, with ID$_{50}$ values of <0.1 μg/ml (Fig. 7C and D). Consistent with an Fc-dependent mechanism, the Fab form of D5_AR did not

<table>
<thead>
<tr>
<th>Env Pseudotype</th>
<th>Tier</th>
<th>Clade</th>
<th>$ID_{50}$ (μg/mL)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF162</td>
<td>1A</td>
<td>B</td>
<td>120 ± 81</td>
<td>42 ± 17</td>
</tr>
<tr>
<td>MW965</td>
<td>1A</td>
<td>C</td>
<td>9.8 ± 3.6</td>
<td>2.5 ± 0.041</td>
</tr>
<tr>
<td>Q23</td>
<td>1B</td>
<td>A</td>
<td>92 ± 4.8</td>
<td>26 ± 8.4</td>
</tr>
<tr>
<td>92RW020.5</td>
<td>1B</td>
<td>A</td>
<td>180 ± 42</td>
<td>21 ± 14</td>
</tr>
<tr>
<td>X2278</td>
<td>1B</td>
<td>B</td>
<td>130 ± 36</td>
<td>9.4 ± 4.1</td>
</tr>
<tr>
<td>HXB2</td>
<td>1B</td>
<td>B</td>
<td>31 ± 5.5</td>
<td>5.3 ± 1.2</td>
</tr>
<tr>
<td>BaL</td>
<td>1B</td>
<td>B</td>
<td>&gt;200</td>
<td>28 ± 8.3</td>
</tr>
<tr>
<td>SS1106</td>
<td>1B</td>
<td>B</td>
<td>130 ± 46</td>
<td>23 ± 12</td>
</tr>
<tr>
<td>ZM197M.PB7</td>
<td>1B</td>
<td>C</td>
<td>140 ± 66</td>
<td>52 ± 28</td>
</tr>
<tr>
<td>DJ263.8</td>
<td>1B</td>
<td>CRF02</td>
<td>&gt;200</td>
<td>45 ± 13</td>
</tr>
<tr>
<td>246-F3</td>
<td>2</td>
<td>AC</td>
<td>&gt;200</td>
<td>140 ± 27</td>
</tr>
<tr>
<td>TRO.11</td>
<td>2</td>
<td>B</td>
<td>180 ± 1.4</td>
<td>54 ± 14</td>
</tr>
<tr>
<td>25710</td>
<td>2</td>
<td>C</td>
<td>200 ± 4.1</td>
<td>22 ± 11</td>
</tr>
<tr>
<td>Ce1176</td>
<td>2</td>
<td>C</td>
<td>&gt;200</td>
<td>140 ± 23</td>
</tr>
<tr>
<td>Ce0217</td>
<td>2</td>
<td>C</td>
<td>180 ± 11</td>
<td>85 ± 26</td>
</tr>
<tr>
<td>CNE55</td>
<td>2</td>
<td>CRF01</td>
<td>&gt;200</td>
<td>89 ± 32</td>
</tr>
<tr>
<td>BJOX2000</td>
<td>2</td>
<td>CRF07</td>
<td>&gt;200</td>
<td>48 ± 5.5</td>
</tr>
<tr>
<td>CH119</td>
<td>2</td>
<td>CRF07</td>
<td>&gt;200</td>
<td>40 ± 28</td>
</tr>
<tr>
<td>X1632</td>
<td>2</td>
<td>G</td>
<td>&gt;200</td>
<td>66 ± 7.8</td>
</tr>
</tbody>
</table>

**FIG 5** D5 and D5_AR neutralize HIV-1 in vitro in a size-independent manner. scFv, Fab, and IgG constructs of D5 (A) and D5_AR (B) are similarly effective in vitro at neutralizing lentivirus pseudotyped with HIV-1 HXB2. Data points and error bars are the mean percent infections and standard errors of the means, respectively ($n$ = 2). Antibody construct images used in this figure were generated with BioRender.

**FIG 6** D5_AR IgG has higher neutralization potency than D5 across tiers and clades of HIV-1. (A) D5_AR IgG has an enhanced neutralization profile versus D5 IgG, as indicated by ID$_{50}$ values (geometric mean ± standard error of the mean) against a panel of tier-1 and tier-2 HIV-1 viruses from multiple clades. (B) D5_AR IgG neutralizes a greater percentage of HIV-1 viruses than D5 IgG within the <10 μg/ml and 10 to 50 μg/ml ID$_{50}$ ranges.
exhibit potentiation (Fig. 8A). This observed potentiation was specific to FcγRI: enhanced neutralization of D5_AR IgG was minimal or not observed in cell lines expressing FcγRIIa, FcγRIIb, and FcγRIIIa (Fig. 8B). It is noteworthy that the ID₅₀ values of D5_AR IgG in TZM-bl cells were approximately linearly related to the ID₅₀ values in TZM-bl/FcγRI cells (Fig. 9).

DISCUSSION

The PHI of HIV-1 is a validated drug target in humans (50–52), and antibodies that bind the NHR of gp41 that is exposed in the PHI can inhibit HIV-1 infection in vitro (53–56, 67). The first of these NHR-binding antibodies, D5, has weak neutralization potency against tier-1 HIV-1 strains (53). We hypothesized that combining multiple CDR mutations from enhanced D5 variants previously described (67) would create a more-effective neutralizing mAb against HIV-1. To test this hypothesis, we evaluated a panel of 16 variants: wild-type D5, four previously described CDR variants (67), and 11 recombinant CDR variants (Fig. 2). Here we engineered and characterized a more potent D5 derivative, D5_AR. Using 10 global HIV-1 reference strains, we determined that at modest concentrations, D5_AR neutralizes the majority of tier-1 and tier-2 HIV-1 strains.
tested from a variety of clades (Fig. 6). Like other anti-NHR antibodies (Fig. 1), D5_AR demonstrates a lower level of somatic hypermutation than bNAbs, with 7.6% of amino acids mutated from germline across both heavy- and light-chain V genes. Taken together with the high sequence conservation of the NHR (https://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html), these proof-of-concept results suggest the PHI, and more specifically, the NHR, is a viable vaccine target.

Several previous reports had suggested that access to the D5 epitope was impacted by steric hindrance, as smaller antibody constructs were more potent than full-length IgG (55, 59, 67, 80). We reinvestigated this issue using antibody preparations that were free of observed protein aggregates (Fig. 5A). In contrast to earlier reports (59, 67, 80), here we confirm (53) that D5 and D5_AR are similarly potent when tested in neutralization assays in scFv, Fab, and IgG formats (Fig. 5). We hypothesize that the presence of higher-order protein aggregates (that can be removed by SEC) may explain the previous reports of size-dependent neutralization by D5. Given these findings for D5 and D5_AR, we conclude that steric hindrance is not an obstacle for at least some anti-PHI antibodies.

Previous work on antibodies targeting another epitope of gp41, the MPER, found that neutralization activity was potentiated as much as 5,000-fold in cells expressing FcγRI, an integral membrane protein that interacts with the Fc portion of immunoglobulins (72, 73, 100). Since the MPER is not fully exposed until after Env engages with cellular receptors (101, 102), these results suggest that by binding the Fc region of MPER mAbs, FcγRI provides a local concentration advantage at the cell surface that enhances neutralization (72, 73). Because the PHI, like the MPER, is fully exposed only during the viral membrane fusion

**FIG 8** D5_AR is not potentiated as a Fab and shows minimal or no potentiation in the presence of other Fc receptors. (A) Neutralization curves for D5_AR Fab against lentivirus pseudotyped with HIV-1 HXB2 show no neutralization enhancement between TZM-bl cells and TZM-bl/FcγRI cells. Data points and error bars are the means and standard errors of the means (n = 2), respectively. (B) The degree of observed neutralization enhancement is FcγRI specific, as demonstrated by the neutralization potency of D5_AR IgG against lentivirus pseudotyped with HIV-1 HXB2 in TZM-bl cells expressing various Fc receptors. Data points and error bars are the means and standard errors of the means (n = 3), respectively.

**FIG 9** Comparison of HIV-1-neutralizing activity of D5_AR IgG in TZM-bl versus TZM-bl/FcγRI cells. The ID₅₀ values of D5_AR IgG in TZM-bl and TZM-bl/FcγRI cells are approximately linearly related in a panel of tier-2 HIV-1 viruses from multiple clades. ID₅₀ values represented in this figure were reported previously in Fig. 6 and 7 and selected because they are the only viral isolates for which we conducted neutralization analyses in both TZM-bl and TZM-bl/FcγRI cells.
process, we previously investigated the effect of FcRRI on D5 and found neutralization by this anti-PHI mAb is also enhanced ~5,000-fold (71). Like D5, D5_AR IgG displayed ~1,000-fold enhancement in neutralization potency in TZM-bl/FcRRI cells (Fig. 7A and B). In particular, we have previously demonstrated that D5 potency against HIV-1 HXB2 is enhanced ~6,400-fold in the presence of FcRRI (71) compared to a range of ~1,500-fold (Fig. 7B) to 2,500-fold (Fig. 8B) enhancement for D5_AR against HIV-1 HXB2. We conclude that the enhancement in FcRRI-expressing cells is independent of the effect of the mutations introduced in D5_AR. The magnitude of the potentiation observed by D5_AR is in line with what was previously reported for MPER antibodies and is thus much greater than antibodies to the CD4bs, V2 or V3 loop, or gp41 cluster, which show little to negligible enhancement in cells expressing FcRRI (72, 73). Notably, this enhancement makes D5_AR IgG an extremely potent neutralizing antibody of tier-2 HIV-1 viruses in the TZM-bl/FcRRI cell line, with ID_{50} values of <0.1 µg/ml (Fig. 7C and D).

TZM-bl/FcRRI cells enable sensitive detection of neutralization activity from anti-NHR antibodies and could be used, in conjunction with TZM-bl cells, to monitor progress toward eliciting neutralizing antisera that target the PHI. We hypothesize that neutralizing activity detected by TZM-bl/FcRRI cells could be used as an indicator of low-affinity antibody precursors in serum that have the potential to mature to high-affinity neutralizing activity independent of FcRRI. This hypothesis is supported by our findings that the neutralizing activity of D5_AR in FcRRI-expressing cells was approximately linearly related to the neutralizing activity of D5_AR in cells without FcRRI (Fig. 9).

Although not expressed on CD4^+ T cells, FcRRI is expressed on macrophages and dendritic cells (100), which can be productively infected by HIV-1 (103–106), and can then mediate viral transmission to CD4^+ T cells (107–110). Importantly, studies of intra-vaginal inoculation of simian immunodeficiency virus (SIV) of nonhuman primates demonstrated that intraepithelial and submucosal dendritic cells are infected in the earliest stages (18 to 48 h) of SIV infection (111–114). More recent work has shown that at 48 h postinoculation, 25% of infected cells are dendritic cells and macrophages, with the remainder comprising CD4^+ T cells, primarily of the Th17 type (115, 116). Thus, it is plausible that inhibiting HIV-1 infection of these FcRRI-expressing cells at the mucosal surfaces by antibodies against the PHI could decrease the likelihood of sexual HIV-1 transmission (71). Indeed, in a vaginal challenge with SHIV in rhesus macaques, an MPER mAb (2F5) afforded dose-dependent protection when administered as an IgG but not when administered in its Fab form (117), suggesting an Fc-dependent mechanism of protection in vivo. Previous studies have also demonstrated that MPER mAbs are much more protective against SHIV challenge than other bNAbs when measured in vitro (118–121).

While D5_AR does not have the same breadth or potency as bNAb, D5_AR demonstrates cross-clade tier-2 HIV-1-neutralizing activity and extremely potent activity when measured in cells expressing FcRRI (Fig. 7). These results motivate future efforts to investigate the ability of passively transferred anti-PHI antibodies such as D5_AR to protect against HIV-1 transmission in nonhuman primates. Importantly, D5_AR presents proof of concept that an anti-PHI MAb can neutralize tier-2 HIV-1 viruses. These results will encourage novel vaccine designs against the PHI as an alternative and orthogonal approach for HIV-1 vaccines that is fundamentally different from the prevalent approaches in the field of bNAb and germline-targeting strategies.

MATERIALS AND METHODS

Mammalian expression of D5 constructs. The variable heavy (VH)-chain regions of the D5 variants were ordered as gene fragments from Twist Biosciences. Gene fragments were resuspended to 10 ng/µl in H2O and PCR amplified using the following two primers: (HC_forward) 5’-ACCGGTGATACATTCCA GTGCAAC-3’ and (HC_reverse) 5’-GCCCTTGGTCGACGCGCTTGATACG-3’. The mutated variable light (VL)-chain region, with only the third CDR mutated according to Montgomery et al. (67), was ordered as a gBlock gene fragment from Integrated DNA Technologies and PCR amplified using the following two primers: (LC_forward) 5’-ACCGGTGATACATTCCAATCTGATACG-3’ and (LC_reverse) 5’-TCCAGCCACCGTACGTTTG-3’. Purified VH and VL fragments were cloned into linearized pCMVR with either the human IgG heavy- or kappa light-constant regions, respectively (9, 122). The primers for linearizing the pCMVR

August 2021 Volume 95 Issue 15 e02350-20 jvi.asm.org

Downloaded from https://journals.asm.org/journal/jvi on 08 September 2021 by 2603:8001:440:4300:9035:1719:facc:eff0.
IgG heavy-chain plasmid were (HC_lin_forward) 5’-GGTTCAGACAAAGCCCATCGTGTTCT-3’ and (HC_lin_reverse) 5’-GGAATGTACCGGTTGCTGACTAGAAGAAC-3’. The primers for linearizing the pCMVR Igk kappa light-chain plasmid were (LC_lin_forward) 5’-CTGACGTGTCCTCACACTCTGCTCATCCCT-3’ and (LC_lin_reverse) 5’-TGAATGTACCGGTTGCTGACTAGAAGAACGATA-3’. The DS VH and VL segments were cloned into the linearized pCMVR backbones with 5’ In-Fusion HD enzyme premix (Takara Bio). Plasmids were transformed into Stellar competent cells (Takara Bio), and transformed cells were grown at 37°C. Colonies were sequence confirmed and then maxiprepared (NucleoBond Xtra Maxi; Macherey-Nagel). Plasmids were sterile filtered using a 0.22-μm syringe filter and stored at −20°C.

DS IgG variants used for neutralization assays were expressed in Exp293F cells (Thermo Fisher Scientific) using FectoPRO (Polyplus). VH and VL plasmids were cotransfected at a 1:2 ratio, respectively; cells were transfected at 3 × 10⁶ cells/ml. Cell cultures were incubated at 37°C and 8% CO₂, with shaking at 120 rpm. Cells were harvested 3 days posttransfection by spinning at 300 × g for 5 min and then filtered through a 0.22-μm filter. IgG supernatants were diluted 1:1 with 1× phosphate-buffered saline (PBS) and batch bound to Pierce protein A agarose (Thermo Fisher Scientific) overnight at 4°C. The supernatant-resin slurry was added to a column, and the resin was washed with 1× PBS and eluted with 100 mM glycine (pH 2.8) into one-tenth volume of 1 M Tris (pH 8.0).

D5 and D5_AR Fab used for neutralization assays were also produced in Exp293F cells. D5 and D5_AR Fab VH regions were cloned into a pCMVR heavy-chain linearized backbone with a portion (CH2 and CH3 domains) of the constant region removed. Fab VH and VL plasmids were cotransfected and harvested with the protocol for IgG described above. Fab supernatants were diluted 1:1 with 50 mM sodium acetate (pH 5.0), batch bound to Pierce protein G agarose (Thermo Fisher Scientific) over an acetonitrile (ACN) gradient in the presence of 0.1% trifluoroacetic acid (TFA). Fab protein was dissolved to 1 mg/ml in 100 mM Tris-HCl (pH 8.0) and oxidized by air at 37°C with gentle shaking for 48 h. The peptide mixture was then lyophilized, dissolved into 20% ACN-80% water, and purified via HPLC. Peptide product whose mass corresponded to that of three biotinylated-CCIZN17 de bridges was collected.

For all neutralization assays, elutions were further via size exclusion chromatography using the Superdex 200 Increase 10/300 GL column (GE HealthCare) in 1× PBS, and eluted with 250 mM imidazole in 1× PBS. Samples were spin concentrated using a 0.22-μm filter. IgG supernatants were diluted 1:1 with 1× PBS, and eluted with 100 mM glycine (pH 2.8) into one-tenth volume of 1 M Tris (pH 8.0).

For all other neutralization assays, elutions were further purified after affinity purification on an AKTA using a GE Superdex 200 Increase 10/300 GL column (GE HealthCare) in 1× PBS. After size exclusion chromatography, samples were spin concentrated using Amicon Ultra-15 10-kDa 15-ml spin filters. Fab and scFv constructs were eluted from affinity purification and then purified further via size exclusion chromatography using the Superdex 200 Increase 10/300 GL column (GE HealthCare) and 1× PBS. Samples were spin concentrated as described above.

For all samples, regardless of the purification procedure, concentrated elution samples were syringe filtered using a 0.22-μm filter and stored at 4°C prior to use.

Synthesis of covalent biotinylated CCIZN17. Biotinylated CCIZN17 (CCGKIKKEIEAIKKEQAIKK KIEAKRDLKLTVWGKQQRH) was synthesized using standard Fmoc-based solid-phase peptide synthesis on a CSBio instrument. The resin was 250 μmol NovaSyn TGR R resin (Novabiochem), and coupling was performed for 15 min at 60°C with 4-fold molar excess of amino acids. Biotin was installed on the N terminus via coupling with biotin-polyethylene glycol (PEG)_₉-proionic acid (ChemPep). Dry peptide resin was cleaved with 94% trifluoroacetic acid, 2.5% water, 2.5% 1,2-ethanediol, and 1% trisopropylsiline at room temperature for 3.5 h following precipitation in cold diethyl ether. The crude peptide was purified by reversed-phase high-pressure liquid chromatography (HPLC) on a C₈ semiprep column over an acetonitrile (ACN) gradient in the presence of 0.1% trifluoroacetic acid (TFA), and fractions were collected based on liquid chromatography-mass spectrometry (LC/MS) analysis. The pure monomeric protein was dissolved to 1 mg/ml in 100 mM Tris-HCl (pH 8.0) and oxidized by air at 37°C with gentle shaking for 48 h. The peptide mixture was then lyophilized, dissolved into 20% ACN-80% water, and repurified via HPLC. Peptide product whose mass corresponded to that of three biotinylated-CCIZN17 peptide chains linked by three-disulfide bridges was collected.

Bi-layer interferometry. Biotinylated CCIZN17 peptide (200 mM) was loaded on streptavidin biosensors (Pall ForteBio) to a load threshold of 0.4 nm using an Octet RED96 system (Pall ForteBio). Sensors were immediately regenerated in 100 mM glycine (pH 1.5) and neutralized to remove aggregates and nonspecific interactions. Ligand-loaded sensors were diluted in known concentrations of Fab for an association step (4 min) and returned to the baseline well for a dissociation step (4 min). All reactions
were run in PBS (pH 7.4) with 0.1% bovine serum albumin (BSA) and 0.05% Tween 20. All samples in all experiments were baseline subtracted to a well that loaded the tip with biotinylated peptide but did not go into the sample as a control for any buffer trends within the samples. Association/dissociation binding curves were fit in GraphPad Prism 8 using the “Association then Dissociation” analysis to calculate the equilibrium dissociation constant ($K_d$), $k_{on}$, and $k_{off}$. Averages from fitted values across multiple concentrations from at least two independent experiments are reported.

**Transfection to produce HIV-1 pseudotyped lentiviruses.** HEK293T cells were transiently cotransfected with a backbone plasmid as well as an HIV-1 Env plasmid for HIV-1 pseudotyped lentivirus production using the calcium phosphate transfection protocol previously described (123–125). HEK293T cells were passaged in T75 flasks and incubated at 37°C in 5% CO2. The growth medium used for passaging and transfections was Corning Dulbecco’s modified eagle medium (DMEM; with 4.5 g/liter glucose, L-glutamine, and sodium pyruvate) with 10% fetal bovine serum. 1% penicillin-streptomycin (Corning), and 1% L-glutamine (Corning).

The backbone plasmid psg3ΔEnv was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from John C. Kappes and Xiaoyun Wu: HIV-1 SG3 ΔEnv noninfectious molecular clone (catalog number 11051) (126, 127). The psg3ΔEnv plasmid was propagated in MAX Efficiency Stbl2 cells grown at 30°C with shaking, and Env plasmids were propagated in Stellar competent cells grown at 37°C with shaking. DNA was isolated using a maxiprep kit (NucleoBond Xtra Maxi; Macherey-Nagel) and sequence confirmed.

In brief, 6 x 10^6 HEK293T cells were plated in 10-cm petri dishes in a total volume of 10 ml of DMEM and incubated overnight at 37°C and 5% CO2 without shaking. Once the cells reached 50% to 80% confluence, they were transfected as follows. In a Falcon tube, 20 μg of psg3ΔEnv was mixed with 10 μg of Env plasmid and water for a final volume of 500 μl. Five hundred microliters of 2 x HEPES-buffered saline (pH 7) (Alfa Aesar) was added dropwise to the mixture, and 100 μl 2.5 M CaCl2 was added subsequently. The mixture was incubated at room temperature for 20 min and then dispersed onto the cells. Next, 12 to 18 h after transfection, the medium was aspirated from the dish and replaced with 10 ml of fresh DMEM with additives. Virus-containing medium was harvested 48 h after the medium swap and centrifuged at 300 x g for 5 min; the supernatant was sterile filtered with a 0.45-μm polyvinylidene difluoride filter and stored in 1-ml aliquots at −80°C.

**Neutralization assay.** The neutralization assay was adapted from the TZM-bl assay protocol using HIV-1 Env-pseudotyped viruses as described previously (75, 78). Briefly, TZM-bl cells, derived from the JCS3-bl parental cell line, were used as reporter cells in this assay and were obtained through the NIH AIDS Reagent Program (catalog number 8129) from John C. Kappes and Xiaoyun Wu (126, 128–131), TZM-bl cells are adherent HeLa cells that stably express CD4 and CCR5 and constitutively express CXCR4; they have integrated β-galactosidase and firefly luciferase reporter genes under the control of the HIV-1 long terminal repeat (LTR) promoter. TZM-bl cells transduced to stably express FcRⅢ (72, 73) were also used in these neutralization assays. TZM-bl cells were passaged in T25 flasks and incubated at 37°C at 5% CO2 without shaking. The growth medium used for passaging and neutralization assays was Corning DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin (Corning), and 1% L-glutamine (Corning).

In brief, 5 x 10^5 TZM-bl cells were plated in the internal 60 wells of white-walled, clear-bottom 96-well plates and incubated overnight at 37°C at 5% CO2 without shaking. All outside wells were filled with 200 μl PBS to minimize evaporation. On the next day, the medium was aspirated without disturbing the cells and replaced with a final mixture composed of one-half volume DMEM, one-fourth volume HIV-1 pseudotyped lentivirus, one-fourth volume DS buffer containing DEAE dextran (10 μg/ml). Forty-eight hours after infection, all medium was aspirated off the wells, cells were lysed, and either luciferase activity was determined using BriteLite Plus reagent (Perkin Elmer) or β-galactosidase activity was determined using Tropix Gal-Screen (Applied Biosystems) and buffer A (Applied Biosystems). β-Galactosidase readout was used for neutralizations shown in Table 1 and Fig. 4, 5, and 6. Luciferase readout was used for neutralizations shown in Fig. 7 and 8.

Relative luminescence unit (RLU) values were quantified using a Synergy HTX multimode reader (BioTek), normalized against cells-only reference wells, and averaged for technical replicates on the plate. Percent infectivity and propagated error values (see “Statistics and data analysis”) were entered into GraphPad Prism 8. Neutralization titers are reported as the antibody concentration at which RLU’s were reduced by 50% compared to RLU’s in virus control wells after subtraction of background RLU in cell control wells. ID_(50) was calculated using the inhibitor concentration versus response (three parameters) dose-response curve fit in GraphPad Prism 8. This assay was conducted in compliance with good clinical laboratory procedures (GCLP) (132), including participation in a formal TZM-bl assay proficiency program for GCLP-compliant laboratories (74).

**Statistics and data analysis.** Percent infectivity for the neutralization assays was calculated as follows:

$$\frac{(\text{sample RLU})_{\text{cells only RLU}}}{(\text{viruses only RLU})} \times 100.$$

Propagated error for the percent infectivity was calculated using the following formula:

$$\frac{(\% \text{infection})}{\sqrt{\text{STD of sample RLU}^2 + \frac{\text{STD of virus only RLU}^2}{\text{AVG of RLU}^2}}}.$$  

where STD is the standard deviation and AVG is the average. The ID_(50) values in Table 1 and Fig. 6 represent the geometric means from the biological replicates for the tested antibodies with the standard
errors of the means reported. Fold difference in ID<sub>50</sub> was calculated for each experiment by dividing the DS ID<sub>50</sub> by the DS variant ID<sub>50</sub>. Because fold difference was calculated for each experiment, the reported fold differences in Table 1 are the geometric means and the standard errors of the means from all replicates.

ACKNOWLEDGMENTS

We thank members of the Kim Lab for helpful discussions and A. E. Powell, S. Tang, and D. Xu for critical readings of this manuscript.

This research was supported by the National Institutes of Health under award numbers T32GM007276 (B.N.B.), T32GM007365 (M.V.F.I.), and DP1DA043893 (P.S.K.), the NSF GRFP (B.N.B.), NIH/NIAID contract HHSN272201800004C (C.C.L.), Bill and Melinda Gates Foundation award OPP1113682 (P.S.K.), the Virginia and D. K. Ludwig Fund for Cancer Research (P.S.K.), and the Chan Zuckerberg Biohub (P.S.K.).

A.A.R., M.V.F.I., C.L.B., and P.S.K. conceived the experiments. A.A.R. performed protein purification, antibody characterization, and neutralization assays. M.V.F.I. and B.N.B. performed protein purification and neutralization assays. T.U.J.B. performed protein purification. Additional neutralization assays were also conducted under the supervision of C.C.L. and D.C.M. All authors contributed to revising the manuscript.

REFERENCES


Rubio et al. Journal of Virology
https://doi.org/10.1089/088922202760072294


August 2021 Volume 95 Issue 15 e02350-20 jvi.asm.org

An HIV-1 gp41 mAb with Tier-2-Neutralizing Activity
Journal of Virology
Rubio et al.
Journal of Virology


100. Li Y, Sehgal K, Mathyl NL, Voss G, Mascola JR, Wyatt R. 2006. Characterization of antibody responses elicited by human immunodeficiency virus type 1 primary isolates trimeric and monomeric envelope glycoproteins
An HIV-1 gp41 mAb with Tier-2-Neutralizing Activity


