Neutralization Properties of Simian Immunodeficiency Viruses Infecting Chimpanzees and Gorillas

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Neuralization Properties of Simian Immunodeficiency Viruses Infecting Chimpanzees and Gorillas


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ABSTRACT

Broadly cross-reactive neutralizing antibodies (bNabs) represent powerful tools to combat human immunodeficiency virus type 1 (HIV-1) infection. Here, we examined whether HIV-1-specific bNabs are capable of cross-neutralizing distantly related simian immunodeficiency viruses (SIVs) infecting central (Pan troglodytes troglodytes) (SIVcpzPtt) and eastern (Pan troglodytes schweinfurthii) (SIVcpzPts) chimpanzees (n = 11) as well as western gorillas (Gorilla gorilla gorilla) (SIVgor) (n = 1). We found that bNabs directed against the CD4 binding site (n = 10), peptideglycans at the base of variable loop 3 (V3) (n = 5), and epitopes at the interface of surface (gp120) and membrane-bound (gp41) envelope glycoproteins (n = 5) failed to neutralize SIVcpz and SIVgor strains. In addition, apex V2-directed bNabs (n = 3) as well as llama-derived (heavy chain only) antibodies (n = 6) recognizing both the CD4 binding site and gp41 epitopes were either completely inactive or neutralized only a fraction of SIVcpzPtt strains. In contrast, one antibody targeting the membrane-proximal external region (MPER) of gp41 (10E8), functional CD4 and CCR5 receptor mimetics (eCD4-Ig, eCD4-Ig10E8, CD4L-218.3-E51, and CD4L-218.5-E51-m1m2), as well as mono- and bispecific anti-human CD4 (iMab and LM52) and CCR5 (PRO140, PRO140-10E8) receptor antibodies neutralized >90% of SIVcpz and SIVgor strains with low-nanomolar (0.13 to 8.4 nM) potency. Importantly, the latter antibodies blocked virus entry not only in TZM-bl cells but also in C2Th cells expressing chimpanzee CD4 and CCR5 and neutralized SIVcpz in chimpanzee CD4+ T cells, with 50% inhibitory concentrations (IC50s) ranging from 3.6 to 40.5 nM. These findings provide new insight into the protective capacity of anti-HIV-1 bNabs and identify candidates for further development to combat SIVcpz infection.

IMPORTANCE SIVcpz is widespread in wild-living chimpanzees and can cause AIDS-like immunopathology and clinical disease. HIV-1 infection of humans can be controlled by antiretroviral therapy; however, treatment of wild-living African apes with current drug regimens is not feasible. Nonetheless, it may be possible to curb the spread of SIVcpz in select ape communities using vectored immunophylaxis and/or therapy. Here, we show that antibodies and antibody-like inhibitors developed to combat HIV-1 infection in humans are capable of neutralizing genetically diverse SIVcpz and SIVgor strains with considerable breadth and potency, including in primary chimpanzee CD4+ T cells. These reagents provide an important first step toward translating intervention strategies currently developed to treat and prevent AIDS in humans to SIV-infected apes.

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S


imian immunodeficiency virus of chimpanzees (Pan troglodytes) (SIVcpz) is the precursor of human immunodeficiency virus type 1 (HIV-1), the causative agent of the AIDS pandemic (1). Like HIV-1 in humans, SIVcpz is pathogenic in chimpanzees and can cause substantial morbidity and mortality in its natural host (2, 3). Long-term observational health studies in Gombe National Park, Tanzania, revealed that SIVcpz-infected chimpanzees have a 10- to 16-fold increased risk of death compared to uninfected chimpanzees and that infected females are less likely to give birth and have a much higher infant mortality rate than uninfected females (2). Necropsy studies showed that SIVcpz-infected chimpanzees can develop severe CD4+ T lymphocyte depletion and a histopathology consistent with end-stage AIDS (2, 4). Most importantly, the Gombe community with the highest SIVcpz prevalence rate suffered a catastrophic population decline (3). Thus, SIVcpz infection has a substantial negative impact on the health, reproduction, and life span of wild-living chimpanzees.

Chimpanzees acquired SIVcpz by cross-species transmission and recombination of SIVs that infect monkeys (5). Although only the central (P. t. troglodytes) and eastern (P. t. schweinfurthii) subspecies are naturally infected, SIVcpz is widespread throughout their habitats in west-central and eastern Africa, with overall prevalence rates of 6% and 14%, respectively (6–11). Transmission of SIVcpz occurs by sexual routes, from infected mothers to their infants as well as between chimpanzee communities through the migration of infected females (2, 3). Thus, SIVcpz has the potential to disperse over long distances and penetrate uninfected chimpanzee populations (7, 10, 12). SIVcpz is also the source of SIVgor, which emerged in western lowland gorillas (Gorilla gorilla gorilla) following the cross-species transmission of an SIVcpz strain from sympatric P. t. troglodytes apes (SIVcpzPtt) (13). Although less prevalent than SIVcpz, SIVgor has been identified at several locations throughout southern Cameroon, with some gorilla troops exhibiting high infection rates (14–16). Transmission of SIVcpz occurs by sexual routes, from infected mothers to their infants as well as between chimpanzee communities through the migration of infected females (2, 3). Thus, SIVcpz has the potential to disperse over long distances and penetrate uninfected chimpanzee populations (7, 10, 12). SIVcpz is also the source of SIVgor, which emerged in western lowland gorillas (Gorilla gorilla gorilla) following the cross-species transmission of an SIVcpz strain from sympatric P. t. troglodytes apes (SIVcpzPtt) (13). Although less prevalent than SIVcpz, SIVgor has been identified at several locations throughout southern Cameroon, with some gorilla troops exhibiting high infection rates (14–16). Given its recent emergence from SIVcpz, SIVgor may share some of the same pathogenic properties. Thus, SIVcpz, and possibly also SIVgor, contributes to the infectious disease burden of wild apes, which are already highly endangered due to extensive habitat destruction and relentless poaching (7, 10, 16–19).

Historically, chimpanzees have been used as an animal model to test new therapies and vaccines for humans, including AIDS vaccines (20–23), a practice that is no longer considered ethical (24). However, the opposite has not been explored, i.e., whether treatment and prevention strategies developed for HIV-1-infected humans could benefit SIVcpz-infected chimpanzees. As chimpanzee and gorilla populations are dwindling in the wild, primatologists and conservation groups have become increasingly interested in exploring novel avenues to curb the spread of ape immunodeficiency viruses (SHIVs) in humanized mice and rhesus macaques, respectively (30–37). For example, antibody-mediated immunotherapy was effective in reducing systemic viral loads and improving immune responses in chronically SHIV-infected rhesus macaques (36, 37). Administration of bNabs as purified proteins or transgenes also prevented virus infection in animal models (30–34, 38). Since antibody infusions in wild settings are not feasible, delivery through recombinant vectors, such as adeno-associated virus (AAV), may represent an alternative. AAV has an outstanding safety record in humans as well as other animal species (39–42). Moreover, a single administration by dart or an equivalent has the potential to induce long-lasting antibody expression (30, 31, 38, 43). Recombinant AAV (rAAV) vectors could also be used to deliver cocktails of potent neutralizing antibodies, which may be able to reduce systemic viral loads.

First-generation bNabs failed to control HIV-1 replication when administered to infected patients, suggesting that they were of little or no clinical value (44, 45). However, recent advances in HIV-1-specific B cell isolation and antibody cloning techniques have led to the discovery of a large number of additional bNabs (46–48), many of which neutralize HIV-1 with much improved breadth and potency (27–29, 49–52). All bNabs target structurally conserved regions on the HIV-1 envelope (Env) spike, including (i) the CD4 binding site (CD4bs), (ii) peptidoglycans surrounding N160 at the trimer apex in variable loops 1 and 2 (V1/V2), (iii) a high-mannose patch surrounding N332 at the base of variable region 3 (V3), (iv) the membrane-proximal external region (MPER), and (v) glycan-associated epitopes at the interface of envelope (gp120) and membrane-bound (gp41) Env glycoproteins (46, 47, 53–56). In addition, antibodies directed against the host receptors CD4 and CCR5 (57–60), as well as immunoadhesins containing domains 1 and 2 (D1/D2) of human CD4 (61–63), have been shown to have substantial anti-HIV-1 activity. While these reagents inhibit a large number of pandemic (group M) HIV-1 strains (64), their capacity to neutralize related lentiviruses from chimpanzees and gorillas has not been examined. Here, we used a panel of SIVcpz and SIVgor infectious molecular clones (IMCs) to show that some but not all anti-HIV-1 bNabs and immunoadhesins are capable of neutralizing these viruses. Since the Env proteins of SIVcpz and SIVgor strains are nearly twice as divergent as those of the various HIV-1 group M subtypes, these data yield new insight into the breadth and protective efficacy of anti-HIV-1 antibodies.

**RESULTS**

Plasma samples from long-term HIV-1- and SIVcpz-infected chimpanzees lack neutralization breadth. Almost 50% of HIV-1-positive humans develop some degree of heterologous neutralization breadth within 2 to 4 years postinfection (65), indicating that the human immune system is capable of targeting conserved epitopes in the HIV-1 envelope (Env) glycoprotein. To determine whether this is also true for chronically infected apes, we tested plasma samples from eight chimpanzees, who had been inoculated with HIV-1 and/or SIVcpz 17 to 30 years earlier as part of vaccine and/or pathogenesis studies (Table 1). Although three of these chimpanzees (Marc, Bucky, and Josie) had undetectable plasma virus at the time of sampling, they had high-titer antibodies that reacted with HIV-1 Gag, Pol, and/or Env proteins on Western immunoblots, suggesting that they were once productively infected (see Fig. S1 in the supplemental material). The remaining five chimpanzees were viremic, with plasma viral loads ranging from 337 to >1,000,000 RNA copies/ml (Table 1). Among the animals with the highest viral loads, Tika (>200,000 RNA copies/ml) was infected with HIV-1/NC, a pathogenic chimpanzee-adapted strain of HIV-1 (22, 66). In contrast, Debbie (>20,000 RNA copies/ml) and Cotton (>1,000,000 RNA copies/ml) were infected with SIVcpzANT (20), an SIVcpzPtt strain orig-
inally isolated from a wild-caught chimpanzee from the Democratic Republic of the Congo (67). Although Cotton was also exposed to HIV-1/LAV (Table 1), reverse transcriptase PCR (RT-PCR) analysis identified SIVcpzANT as the only replicating virus in his plasma. Thus, the latter two animals represent rare examples of captive chimpanzees with chronic SIVcpz infection.

To screen available plasma samples for neutralization breadth, we generated a panel of infectious molecular clones (IMCs) of SIVcpz and SIVgor strains by amplifying viral consensus sequences from fecal samples of wild apes (Fig. 1A). Members of both the SIVcpzPtt lineage and SIVcpzPts lineage were included, which differed in up to 48% of their Env protein sequence. (Three previously reported strains of HIV-1 were used as controls.) All which differed in up to 48% of their Env protein sequence. (Three neutralization potency against heterologous HIV-1, SIVcpz, and SIVgor strains included, some of the most potent CD4bs antibodies, such as 45-46G54W, which is known to neutralize highly diverse HIV-1 strains, with IC_{50} ranging from 0.004 to 1.2 μg/ml. (A monoclonal antibody directed against herpes simplex virus glycoprotein D served as a negative control.) The two exceptions were F105 and b13, which are known to have only limited neutralization breadth and potency (77). In contrast, none of these CD4bs antibodies neutralized any SIVcpz or SIVgor strain at concentrations of up to 10 μg/ml (Fig. 2A). This was the case despite the fact that the panel included some of the most potent CD4bs bNabs, such as 45-46G54W, which is known to neutralize highly diverse HIV-1 strains, with IC_{50} of <0.05 μg/ml (75).

To examine the reasons for this resistance, we compared viral Env sequences for conservation of previously identified CD4 and VRC01 contact residues (78, 79). Not surprisingly, most amino acid residues required for CD4 binding were relatively well conserved, but this was not the case for many VRC01 contact residues (Fig. 2B). For example, residues 461 to 467 in variable loop 5 (V5), which are known to be critical for VRC01 binding (78), were present in all HIV-1 strains but exhibited considerable length variations in SIVcpz and SIVgor strains (Fig. 2B). Although the CD4 binding site of SIVcpz and SIVgor strains must be conserved to maintain function, amino acid diversity in neighboring Env regions likely causes clashes with anti-HIV-1 CD4bs antibodies.

**Anti-HIV-1 CD4 binding site bNabs fail to neutralize SIVcpz and SIVgor strains.** Since all primate lentiviruses identified to date can use the human CD4 receptor to gain entry into target cells (7, 15, 70, 71) and since the CD4 molecules from humans, chimpanzees, and gorillas are closely related (72), we asked whether CD4 binding site (CD4bs) antibodies from HIV-1-infected humans could cross-neutralize SIVcpz and SIVgor strains. Testing VRC01 (29), VCR03 (29), VRC-PG04 (51), VRC-CH30 (51), VRC-CH31 (51), FI05 (73), b13 (74), 45-46G54W (75), 45-46m2 (76), and 45-46m7 (76) in the TZM-bl assay, we found that most of these antibodies neutralized the three HIV-1 Env controls potently, with IC_{50}s ranging from 0.004 to 1.2 μg/ml. (A monoclonal antibody directed against herpes simplex virus glycoprotein D served as a negative control.) The two exceptions were F105 and b13, which are known to have only limited neutralization breadth and potency (77). In contrast, none of these CD4bs antibodies neutralized any SIVcpz or SIVgor strain at concentrations of up to 10 μg/ml (Fig. 2A). This was the case despite the fact that the panel included some of the most potent CD4bs bNabs, such as 45-46G54W, which is known to neutralize highly diverse HIV-1 strains, with IC_{50} of <0.05 μg/ml (75).

**Glycan-dependent variable loop bNabs lack neutralization breadth against SIVcpz and SIVgor.** In addition to the CD4 binding site, HIV-1 envelope glycoproteins contain conserved peptidoglycans in variable loops V1 and V2 and at the base of V3, which represent targets for broadly cross-reactive neutralizing antibo
ies (46, 47). To examine the ability of these anti-HIV-1 bNabs to neutralize SIVcpz and SIVgor strains, we tested apex V2-directed antibodies PG9 (27), PG16 (27), and PGT145 (28) as well as the V3-directed antibodies 10-1074 (52), PGT121 (28), PGT128 (28), PGT135 (28), and 2G12 (80) in the TZM-bl neutralization assay. Like the CD4bs bNabs, the glycan V3-associated bNabs failed to cross-neutralize SIVcpz and SIVgor strains at concentrations of 10 μg/ml, except for PGT128, which neutralized a single SIVcpz-
Ptt (EK505) virus, with an IC50 of 0.02 μg/ml (0.13 nM) (Fig. 3A). The apex V2-directed quaternary bNabs PG9 (27), PG16 (27), and PGT145 (28) were considerably more cross-reactive and potently neutralized four of six SIVcpzPtt strains (GAB1, EK505, MB897, and MT145) (Fig. 3A). However, SIVgor and SIVcpzPts strains were either poorly sensitive or completely resistant to neutralization by the same bNabs.

Quaternary and glycan V3 bNabs neutralize by engaging the...
### FIG 2
Neutralizing capacity of CD4 binding site (CD4bs) antibodies. (A) The ability of CD4bs monoclonal antibodies (listed on the right) to neutralize HIV-1, SIVcpz, and SIVgor strains (listed on the bottom) is shown. Numbers indicate IC50s (in micrograms per milliliter) in TZM-bl cells, averaged from three different experiments, with a heat map indicating the relative neutralization potency. The highest antibody concentration was 10 μg/ml. A herpesvirus antibody (anti-HSV-gD) was used as a negative control. (B) Conservation of HIV-1, SIVcpz, and SIVgor strains in the CD4 binding region. An alignment of Env protein sequences (left) in regions surrounding the CD4 binding site is shown. CD4 and VRC01 contact resides (indicated in the HXB2 reference strain) are highlighted in orange and green, respectively. A logo plot above the alignment denotes the conservation of each amino acid, with the height of each letter indicating the proportion of the sequences that contain the residue at that site. Dots indicate identity to the HXB2 reference sequence, and dashes represent gaps introduced to improve the alignment.

#### Table: Neutralization IC50 Values (μg/ml)

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#### Alignment of Env Protein Sequences

![Alignment of Env Protein Sequences](alignment.png)

HXB2

**CD4 contact residues**

**VRC01 contact residues**

**Sequence Logo**

**HIV-1**
- SG3
- YU-2
- JRCSF
- GAB1
- GAB2
- EK505
- LB715
- MB897
- MT145
- CP2139
- BF1167
- TAN1
- TAN2
- TAN3
- TAN13

**SIVcpz Pts**
- SG3
- YU-2
- JRCSF
- GAB1
- GAB2
- EK505
- LB715
- MB897
- MT145
- CP2139
- BF1167
- TAN1
- TAN2
- TAN3
- TAN13

**SIVgor**
- SG3
- YU-2
- JRCSF
- GAB1
- GAB2
- EK505
- LB715
- MB897
- MT145
- CP2139
- BF1167
- TAN1
- TAN2
- TAN3
- TAN13

**CD4 contact residues**

**VRC01 contact residues**

**Sequence Logo**

**HXB2**
- SG3
- YU-2
- JRCSF
- GAB1
- GAB2
- EK505
- LB715
- MB897
- MT145
- CP2139
- BF1167
- TAN1
- TAN2
- TAN3
- TAN13

**SIVcpz Pts**
- SG3
- YU-2
- JRCSF
- GAB1
- GAB2
- EK505
- LB715
- MB897
- MT145
- CP2139
- BF1167
- TAN1
- TAN2
- TAN3
- TAN13

**SIVgor**
- SG3
- YU-2
- JRCSF
- GAB1
- GAB2
- EK505
- LB715
- MB897
- MT145
- CP2139
- BF1167
- TAN1
- TAN2
- TAN3
- TAN13
glycan shield, and removal of key glycans can abrogate this neutralization (28, 81). For example, 10−1074, PGT121, PGT128, PGT135, and 2G12 all bind a high-mannose patch that is centered around the conserved N332 glycan, while PG9, PG16, and PG145 contact the N156 and N160 glycans and insert between them to interact with glycan-obscured protein regions (27, 28, 52, 81). Examining SIVcpz and SIVgor Env sequences for NXS/T sequons, we found various degrees of conservation of these and other relevant N-linked glycosylation sites (Fig. 3B). For example, N332 was conserved in all three HIV-1 Env sequences, with predicted N-linked glycans (NXS/T) highlighted in red. Four residues comprising a lysine-rich motif in the V1/V2 core epitope was not always associated with neutralization susceptibility. Despite the presence of both N156 and N160 and the conserved lysine motif, TAN1, TAN2, and TAN13 were completely resistant to PG9, PG16, and PGT145 neutralization. Since these viruses encoded V1 loops that were almost twice as long as those of susceptible SIVcpzPts strains and since such insertions have been shown to confer neutralization escape to HIV-1 (85, 86), it is likely that this explains the resistance of SIVcpzPts strains to apex V2-directed bNabs.

HIV-1 bNabs directed against the interface of gp120 and gp41 fail to cross-neutralize SIVcpz and SIVgor. The most recently discovered class of anti-HIV-1 bNabs targets glycan-associated epitopes at the interface of gp120 and gp41 (53–56). Testing several representatives of this bNab group, including 8ANC195 (54), 35022 (53), PGT151 (56), PGT152 (56), and PGT158 (56), we found that none had cross-neutralization potential (Fig. 4A). With the exception of 35022, which neutralized a

![FIG 3 Neutralizing capacity of high-mannose-patch- and apex V2-directed antibodies. (A) The ability of peptidoglycan-associated monoclonal antibodies (listed on the right) to neutralize HIV-1, SIVcpz, and SIVgor strains (bottom) is shown. Numbers indicate IC$_{50}$ (in micrograms per milliliter) in TZM-bl cells, averaged from three different experiments, with a heat map indicating the relative neutralization potency. Colored circles to the right of each antibody indicate the ability of glycan V3-directed bNabs to cross-neutralize SIVcpz and SIVgor strains to apex V2-directed bNabs. (B) Conservation of glycans associated with neutralizing activity (orange, N156; purple, N160; pink, N301; green, N332; red, N386; yellow, N392) (27, 28, 81). The highest antibody concentration used was 10 $\mu$g/ml. (B) Conservation of glycans associated with neutralizing activity (orange, N156; purple, N160; pink, N301; green, N332; red, N386; yellow, N392) (27, 28, 81). The highest antibody concentration used was 10 $\mu$g/ml. (B) Conservation of glycans associated with neutralizing activity (orange, N156; purple, N160; pink, N301; green, N332; red, N386; yellow, N392) (27, 28, 81). The highest antibody concentration used was 10 $\mu$g/ml.
single SIVcpz strain (LB715) at an IC\textsubscript{50} of 0.5 µg/ml (3.3 nM), all other antibodies failed to block SIVcpz and SIVgor infection at concentrations of up to 10\textsuperscript{10} µg/ml (Fig. 4A). Since these bNabs are also dependent on the presence of key glycans, we examined the extent of sequence conservation in the corresponding Env regions (Fig. 4B). For example, 8ANC195 requires N-linked glycosylation sites at positions 234 and 276 (54), both of which are highly variable in SIVcpz and SIVgor strains (Fig. 4B). Similarly, 35O22 utilizes N-linked glycosylation sites at positions 88, 230, 241, and 625 (Fig. 4B), one of which (N230) is absent from all SIVcpz and SIVgor strains. Thus, the absence of key N-linked glycosylation sites may be one reason for the lack of cross-neutralizing capacity of this class of bNabs. However, variation in other Env regions must also be involved, since the glycosylation site contacts for PGT151 (N611 and N637) were conserved in all viruses, yet this monoclonal antibody also failed to cross-neutralize.

Camelid antibodies have limited cross-neutralization breadth. Members of the Camelidae family produce antibodies that lack light chains and thus comprise much smaller (heavy-chain-only) antibodies with long complementarity-determining regions (CDRs). It has thus been proposed that these single-chain antibodies may be better equipped to bind to small occluded sites on the HIV/SIV Env trimer than full-size antibodies (87). Indeed, several cameland single-domain antibodies derived by immunization with HIV-1 antigens have been shown to potently neutralize genetically divergent strains of HIV-1 (76, 88, 89). To examine whether this cross-reactivity extends to SIVcpz and SIVgor, we tested a panel of llama-derived antibodies, including single-domain antibodies JM4 (90), J3 (76), 3E3 (91), 2E7 (88), and 11F1F (92), as well as the bivalent antibody Bi-2H10, which contains two molecules of 2H10 joined by a glycine-serine linker (93). Of these, JM4 recognizes a region in gp120 that overlaps both the CD4 and CCR5 binding sites, J3 and 3E3 target the CD4 binding site, 2E7 and 11F1F recognize epitopes in the ectodomain of gp41 (76, 88, 90, 92), and 2H10 targets the membrane proximal external region (MPER) (93). Interestingly, J3 and 3E3 were able to neutralize a limited number of SIVcpz\textsubscript{Ptt} strains at IC\textsubscript{50} of \textasciitilde5 µg/ml (<13 nM) (Fig. 5A), thus exhibiting greater activity than conventional CD4bs antibodies, which were completely inactive (Fig. 2A). This was also true for the anti-gp41 antibodies 2E7 and 11F1F, which neutralized four SIVcpz\textsubscript{Ptt} strains and one SIVcpz\textsubscript{Pts} strain at IC\textsubscript{50} of \textasciitilde10 µg/ml (<40 nM). However, this breadth did not extend to the more divergent SIVcpz\textsubscript{Pts} and SIVgor viruses, and the bivalent MPER antibody Bi-2H10 was unable to neutralize any SIVcpz and SIVgor strains (Fig. 5A).
FIG 5 Neutralizing capacity of camelid antibodies. (A) The ability of llama-derived (heavy-chain-only) antibodies (listed on the right) to neutralize HIV-1, SIVcpz, and SIVgor strains (bottom) is shown. Numbers indicate IC50s (in micrograms per milliliter) from TZM-bl cells, averaged from three different experiments, with a heat map indicating the relative neutralization potency. The highest antibody concentration used was 10 μg/ml. (B to D) Conservation of antibody binding epitopes shown in panel A. Alignments of HIV-1, SIVcpz, and SIVgor Env protein sequences are depicted, with residues identical to the HXB2 reference shown in gray. Logo plots denote the conservation of individual amino acids within each epitope, with the height of each letter indicating the proportion of sequences that contain the residue at that site. The letter “X” indicates residues within the 2H10 epitope that do not impact neutralization. JM4 contact residues are indicated on top of the alignment. Sequences are numbered according to the HXB2 reference.
mapped, we examined the corresponding amino acid sequences in SIVcpz and SIVgor strains (Fig. 5B to D). This analysis showed that none was particularly highly conserved among the ape viruses. All SIVcpz and SIVgor strains differed by one or more substitutions in the 2H10 epitope (Fig. 5B). The partially overlapping 2E7 and 11F1F epitopes were also variable in ape viruses, with all SIVcpz and SIVgor sequences differing from the HIV-1 consensus (Fig. 5C). Finally, there was considerable variation in the JM4 epitope (Fig. 5D), with contact residues I326, V372, and M434 conserved in HIV-1 but mutated in most (V372) or all (I326 and M434) SIVcpz and SIVgor strains. Thus, epitope variation may explain at least some of the neutralization resistance of SIVcpz and SIVgor strains. However, in contrast to conventional CD4bs and most glycan-dependent antibodies, llama-derived antibodies neutralized a subset of SIVcpzPtt strains, possibly because of their smaller size.

HIV-1 MPER bNabs neutralize SIVcpz and SIVgor strains. Antibodies targeting the MPER of gp41 represent still another class of potent anti-HIV-1 bNabs (49, 50). Among those, 4E10 and 10E8 exhibit the greatest breadth, having been shown to neutralize 98% of a large panel \( \times \) of HIV-1 pseudoviruses (49, 50).

To examine their ability to cross-neutralize ape viruses, we tested 4E10 and 10E8 against our panel of SIVcpz and SIVgor strains. In contrast to all other anti-HIV-1 bNabs, 4E10 neutralized 9 of 12 SIVcpz and SIVgor strains, with a mean IC\textsubscript{50} of 2.7 \( \mu \)g/ml (18.1 nM), while 10E8 neutralized all ape viruses, with a mean IC\textsubscript{50} of 0.7 \( \mu \)g/ml (4.4 nM) (Fig. 6A). An amino acid alignment of the epitopes of these antibodies revealed considerable sequence con-
servation (Fig. 6B), likely explaining their remarkable breadth compared to all other anti-HIV-1 bNabs.

Anti-CD4 and CCR5 receptor antibodies potently neutralize SIVcpz and SIVgor strains. Since CD4 and CCR5 protein sequences are highly conserved between humans and apes (72, 94), we asked whether antibodies raised against the human receptors could block SIVcpz and SIVgor infection. Both anti-CD4 and anti-CCR5 antibodies were examined, including ibalizumab (iMab) and PRO140, which have previously been shown to be safe in human clinical trials (95–97). Using TZM-bl cells, which express human CD4 and CCR5, we found that both the anti-CD4 antibody iMab (96, 98, 99) and its improved version, LM52 (59), neutralized most or all ape viruses, with geometric mean IC50s of 0.41 μg/ml (2.8 nM) and 0.12 μg/ml (0.8 nM), respectively (Fig. 7A). PRO140, which targets the CCR5 coreceptor (57), also neutralized all SIVcpz and SIVgor strains, with an IC50 of 0.34 μg/ml (2.3 nM) (Fig. 7A). Finally, bispecific versions of iMab, LM52 and PRO140, in which these antibodies were linked to single-chain variable fragments (scFv) of P9G, PG16, PGT128, and 10E8 (60), were potent inhibitors, especially when the fusion partner was also an effective neutralizer. For example, the bispecific P9G-iMab and PG16-iMab antibodies neutralized the PG9/
PG16-sensitive GAB1 and EK505 strains with 2 orders of magnitude greater potency than iMab alone, but this enhancement was not observed for viruses that were resistant to PG9 and PG16 (Fig. 3A and 7A). By far the most potent antireceptor antibody was PRO140-10E8, which neutralized SIVcpz and SIVgor strains, with a mean IC50 of 0.03 μg/ml (0.13 nM) (Fig. 7A).

To determine whether the anti-human receptor antibodies would block virus entry in cells expressing chimpanzee CD4 and CCR5, we developed a neutralization assay that utilized transiently transfected Cf2Th cells. This canine thymus cell line lacks CD4 and CCR5 but expresses a tat-inducible luciferase reporter. Cf2Th cells were cotransfected with either human (Cf2Th-hu) or chimpanzee (Cf2Th-ch) CD4 and CCR5 expression plasmids and then validated by testing the neutralizing activity of anti-gp120 bNabs (n = 3), which should not be affected by entry molecules, against a subset of HIV-1 (YU2) and SIVcpz (GAB1, EK505, MT145, and TAN13) strains (Fig. 7B and C). The results showed that IC50s from Cf2Th-hu cells were highly correlated with those from TZM-bl cultures (Fig. 7B), and this was also true for IC50s from Cf2Th-hu and Cf2Th-ch cells (Fig. 7C). Having validated the Cf2Th-ch cell assay, we next examined the neutralizing capacity of the antireceptor antibodies using a subset of SIVcpz strains (Fig. 7D). Both mono- and bispecific antibodies blocked these viruses in chimpanzee CD4- and CCR5-expressing cells, with mean IC50s ranging from 0.30 μg/ml (PRO140) to 0.02 μg/ml (LM52-PGT128). Thus, several anti-human receptor antibodies that potently neutralized SIVcpz strains in cells expressing human CD4 and CCR5 also neutralized these viruses in cells expressing chimpanzee CD4 and CCR5.

CD4-containing antibodies and immunoadhesins potently neutralize SIVcpz and SIVgor strains. Soluble CD4 (sCD4), as well as immunoadhesins that contain the first two immunoglobulin-like domains (D1 and D2) of CD4 linked to antibody constant regions, are known to have broad anti-HIV-1 activity in vitro and in vivo (61, 62, 100). To examine their breadth and potency against ape viruses, we tested several members of this inhibitor class. Soluble CD4 (101) and the more bioavailable CD4-Ig (102), in which the D1/D2 domains of CD4 are linked to an IgG Fc region (Fig. 8A), neutralized nearly all ape viruses, with mean IC50s of 2.0 μg/ml (47.7 nM) and 2.1 μg/ml (23.1 nM), respectively (Fig. 8B). The tetravalent PR0542, in which D1/D2 domains are linked to both the constant heavy (ClH) and light (ClL) chains of IgG1 (Fig. 8A), was slightly more potent and neutralized 10 of 12 ape viruses, with a mean IC50 of 1.1 μg/ml (5.2 nM) (Fig. 8B). Finally, CD4-218.3-E51, a CD4-induced (CD4i) neutralizing antibody (E51) linked to D1/D2 via the VH chain (Fig. 8A), neutralized all SIVcpz and SIVgor strains, with a geometric mean IC50 of 1.68 μg/ml (8.4 nM) (Fig. 8B). Interestingly, other E51-based constructs that differed in the length or amino acid composition of their linkers (CD4-GS7-E51 [62], CD4-L1-E51, CD4-L14-E51, and CD4-L17-E51) were much less broadly acting and/or potent (Fig. 8B), and this was also true for a construct in which the D1/D2 domains were linked to an anti-herpes simplex virus antibody (CD4-anti-HSV-gD).

Some HIV-1 bNabs use tyrosine sulfation of their complementarity-determining regions (CDRs) to mimic CCR5 sulfation and thereby inhibit virus entry (103). Sulfopeptides derived from such antibodies, including a 15-amino-acid tyrosine-sulfated peptide derived from the CD4i antibody E51, have been shown to reproduce this effect (104). When linked to an antibody Fc domain, this peptide (CCR5mim) was able to block infection of diverse HIV-1 strains (105). However, its neutralization capacity was most improved when fused to the carboxy terminus of CD4-Ig (Fig. 8A). In fact, this enhanced CD4-Ig (eCD4-Ig) not only neutralized a diverse panel of HIV-1 strains but also neutralized SIV of macaques (SIVmac) and HIV-2 isolates, with IC50s of <0.05 μg/ml (63). Using TZM-bl cells, we found that eCD4-Ig neutralized SIVcpz and SIVgor strains also more potently than CD4-Ig, with a mean IC50 of 0.46 μg/ml (4.8 nM). However, this 5-fold enhancement was modest compared to the 20- to 200-fold enhancement observed for HIV-1 strains (63). Moreover, the use of CCR5mim peptide variants previously shown to bind HIV-1 Envs with greater affinity did not improve neutralization: eCD4-Ig-CCR5mim (63) was slightly less potent than eCD4-Ig (IC50 of 0.62 μg/ml), and eCD4-Ig-CCR5mim2 (63) failed to neutralize two SIVcpzPs strains (Fig. 8B). Addition of CCR5mim2 to the carboxy terminus of CD4-218.3-E51 (CD4-218.3-E51-CCR5mim2) resulted in a 2-fold increased neutralization potency, while addition of the fusion-inhibiting peptide T-20 (106) (CD4-218.3-E51-T20) had very little effect (Fig. 8B). Thus, as previously shown for HIV-1, HIV-2, and SIVmac (63), addition of entry-inhibiting peptides to the carboxy terminus of D1/D2-containing antibody constructs enhanced their ability to neutralize SIVcpz and SIVgor strains, although the magnitude of this enhancement was much less pronounced.

Neutralization of SIVcpz in primary chimpanzee CD4+ T cells. TZM-bl and Cf2Th-ch cells identified a number of antiviral and antireceptor bNabs that neutralized ape viruses with both breadth and potency. To confirm this phenotype in a physiologically more relevant culture system, we tested these antibodies in primary chimpanzee lymphocytes. Chimpanzee CD4+ T cells were isolated from the blood of three animals, activated using autologous macrophages, and used to test the neutralizing capacity of immunoadhesins (CD4-Ig, eCD4-Ig, and eCD4-Ig-CCR5mim2), the MPER antibody 10E8, CD4-CD4i constructs (CD4-218.3-E51, CD4-218.3-E51-mim2, and CD4-218.3-E51-T20), and antireceptor antibodies (iMab, LM52, PRO140, and PRO140-10E8) (see Fig. S2A in the supplemental material). Since chimpanzee blood samples were limited, we were able to test only a single SIVcpz strain (MT145), which was selected based on its ability to replicate efficiently in CD4+ T cells from multiple chimpanzee donors (71). Using serial antibody dilutions (see Fig. S2A), we found that MT145 was most potently neutralized by iMab, LM52, and PRO140-10E8, with mean IC50s of 0.64 (4.3 nM), 0.68 (4.5 nM), and 0.75 μg/ml (3.6 nM), respectively (Table 2). Immunoadhesins were roughly 10-fold less potent, with mean IC50s ranging from 3.7 μg/ml (38.3 nM) for eCD4-Ig to 6 μg/ml (65.4 nM) for CD4-Ig. As expected, the CD4bs antibody VRC01 had no inhibitory activity (Table 2; see Fig. S2A). Thus, all antibody constructs that had broad and potent anti-SIVcpz activity in the TZM-bl assay also inhibited SIVcpz infection in chimpanzee CD4+ T cells (see Fig. S2B), although the observed IC50 were 10- to 100-fold higher in the primary T cell cultures that permitted multiple rounds of replication (see Fig. S2B).

DISCUSSION

In this study, we examined whether antibodies and immunoadhesins known to potently neutralize diverse strains of HIV-1 can cross-neutralize related lentiviruses naturally infecting chimpanzees and gorillas to assess their utility for antibody-based strategies.
to combat ape SIV infections. We found that the great majority of HIV-1-specific bNabs, including those directed against highly conserved Env epitopes, such as the CD4 binding site, failed to neutralize SIVcpz and SIVgor strains. However, one antibody directed against the MPER region (10E8) as well as bispecific CD4 and CCR5 receptor mimetics (eCD4-Ig, eCD4-Igmm2, CD4-218.3-E51, and CD4-218.3-E51-mim2) neutralized 100% of ape viruses with low-nanomolar potency (Fig. 9; see Table S1 in the supplemental material). Anti-host receptor antibodies (iMab and PRO140) were also effective neutralizers, especially when fused to the antigen binding regions of potent bNabs, such as 10E8 (Fig. 9; see Table S1). These data indicate that pandemic HIV-1 shares an extremely limited number of cross-reactive epitopes with its immediate ape precursors. However, the sites of vulnerability that were identified represent attractive targets across the entire HIV-1/SIVcpz/SIVgor clade and may be of utility to combat other HIV-1 lineages, including group O viruses, which are estimated to have infected ~100,000 people in west-central Africa (16).

Neutralizing antibody responses in long-term HIV-1- and SIVcpz-infected chimpanzees. Since ape-derived monoclonal antibodies may be more appropriate for vector-mediated antibody gene delivery than human bNabs, we tested plasma samples from eight long-term HIV-1- and SIVcpz-infected chimpanzees (see Fig. S1 in the supplemental material). Anti-host receptor antibodies (iMab and PRO140) were also effective neutralizers, especially when fused to the antigen binding regions of potent bNabs, such as 10E8 (Fig. 9; see Table S1). These data indicate that pandemic HIV-1 shares an extremely limited number of cross-reactive epitopes with its immediate ape precursors. However, the sites of vulnerability that were identified represent attractive targets across the entire HIV-1/SIVcpz/SIVgor clade and may be of utility to combat other HIV-1 lineages, including group O viruses, which are estimated to have infected ~100,000 people in west-central Africa (16).
naturally occurring SIVcpzPts strain that was administered by mucosal routes using plasma and/or peripheral blood mononuclear cells from a third chimpanzee (20). Despite this “near-natural” infection history, only one of these animals (Debbie) exhibited cross-reactive neutralizing activity, albeit with very low titers (<1:70) (Fig. 1B). Since the kinetics of bNab development may vary in chimpanzees and humans, it will be important to follow this individual to examine whether neutralizing titers will increase and to map the corresponding antibody specificities. It may also be informative to molecularly clone the SIVcpzANT env gene and study autologous neutralization responses and associated virus escape in Cotton and Debbie. Since very few apes and humans can be studied decades after their infection in the absence of antiretroviral therapy, this may provide an opportunity to uncover common pathways, as well as roadblocks, to bNab development (107–109).

**HIV-1 shares a very limited number of cross-reactive epitopes with SIVcpz and SIVgor strains.** The envelope glycoprotein of HIV-1 group M viruses contains five regions of vulnerability that are targeted by broadly cross-reactive neutralizing antibodies. All of these, except for the MPER region, contain conformational epitopes that span heavily glycosylated and/or structurally flexible protein domains. Since even the most cross-reactive CD4bs bNabs bind an area that extends beyond the CD4 binding pocket (110), it is possible that variation in adjacent Env regions obstructs antibody binding and neutralization (77). SIVcpz and SIVgor Envs vary extensively in regions surrounding the CD4 binding site (Fig. 2B), thus providing a possible explanation for their resistance to CD4bs bNabs (Fig. 2 and 5). In addition, the SIVcpz/SIVgor Env spike may be more compact, such that CD4bs bNab epitopes, although present, are less exposed. The fact that some camelid (heavy-chain-only) CD4bs bNabs neutralize a subset of SIVcpz strains is consistent with these hypotheses. Ape viruses also vary in Env regions that are targeted by apex and high-mannose-patch bNabs, which are typically associated with key N-linked glycosylation sites (Fig. 3 and 4). Although apex V2-directed antibodies neutralized a large fraction (>60%) of SIVcpzPts strains, these bNabs lacked activity against SIVgor and SIVcpzPts viruses, most likely because of substitutions within the core epitope and/or exceedingly long V1 loops. Nonetheless, antibodies directed against the MPER region neutralized even the most divergent ape viruses (Fig. 6). 4E10 neutralized all SIVcpzPts strains, with a mean IC_{50} of 2.7 μg/ml (18.1 nM), but lacked activity against SIVgor and some SIVcpzPts strains. In contrast, 10E8 neutralized all 12 SIVcpz and SIVgor strains, with an IC_{50} of 0.7 μg/ml (4.4 nM). The latter finding may be of interest for “neutralization fingerprinting” studies (111), since inclusion of SIVgor and select SIVcpzPts Envs into existing pseudovirus panels should permit the differentiation of 4E10-like and 10E8-like bNab specificities in polyclonal patient plasma samples.

**Antireceptor antibodies circumvent SIVcpz and SIVgor Env diversity.** Antibodies raised against human CD4 and CCR5 receptors are known to inhibit HIV-1 in vitro (112) as well as reduce viral loads in infected patients in vivo (96). Here, we show that these antibodies also neutralize SIVcpz and SIVgor strains in cells expressing human as well as chimpanzee CD4 and CCR5 receptors (Fig. 7). For example, iMab, which blocks HIV-1 infection by binding to the second domain (D2) of human CD4 (113), neutralized nearly all ape viruses with low-nanomolar potency in TZM-bl infected cells (Table 2; see Table S1 in the supplemental material). Thus, antireceptor antibodies neutralized even the most divergent ape viruses with remarkable potency, including in physiologically relevant target cells (Fig. 7; see Fig. S2 in the supplemental material). Although iMab achieved a 1-log reduction in virus titers in chronically HIV-1-infected humans (96, 112), a second anti-CD4 antibody (2D5) afforded only partial protection in rhesus macaques challenged with a simian/human immunodeficiency virus (SHIV) (115). Because antireceptor antibodies have to block all susceptible target cells, they may require antibody concentrations at the site of virus entry that are difficult to achieve in vivo (115). We thus tested bispecific constructs, in which iMab, LM52, and PRO140 were linked to the antigen binding domains of potent anti-HIV-1 bNabs. Indeed, PG9-iMab, PG16-iMab, and LM52-PGT128 were able to outperform iMab and LM52 (Fig. 7; see Table S1 in the supplemental material) but only when used to...
neutralize the few ape viruses that were sensitive to PG9, PG16, and PGT128 (Fig. 3). However, the bispecific PRO140-10E8 neutralized all SIVcpz and SIVgor strains with up to 20-fold increased potency, regardless of whether TZM-bl, Cf2Th-ch, or primary chimpanzee CD4+ T cells were used as target cells (Fig. 7 and 9 and Table 2; see Fig S2 and Table S1 in the supplemental material). Of all antireceptor antibodies tested, PRO140-10E8 was by far the most potent, neutralizing 100% of ape viruses, with IC_{50} ranging from 0.03 μg/ml (0.13 nM) in TZM-bl cells to 0.75 μg/ml (3.6 nM) in chimpanzee CD4+ T cells (Fig. 9). These findings are consistent with the idea that bispecific antireceptor antibodies exhibit enhanced neutralizing capacity because they concentrate anti-Env bNabs at the site of virus entry (60). It will be important to test PRO140-10E8 in the SHIV/macaque model to determine whether its in vivo potency will translate into in vivo protection.

**Bispecific receptor mimetics confer broad anti-SIVcpz and anti-SIVgor activity.** Several studies have shown that CD4-containing immunoadhesins exhibit considerable anti-HIV-1 activity and even protect a subset of macaques from SIVmac infection (63, 116). Among these, constructs that use receptor mimicry to simultaneously engage both the CD4 and CCR5 binding sites are the most potent (62). These include CD4-CD4i reagents, in which the D1/D2 region of CD4 is linked to the heavy-chain variable region of a CD4-induced (CD4i) antibody, as well as CD4-lg-based reagents that contain short CCR5-mimetic sulfopeptides on their C terminus (Fig. 8A). Although both CD4-CD4i and eCD4-lg constructs neutralized human and ape viruses with nanomolar potency (see Table S1 in the supplemental material), we were particularly interested in eCD4-lg, because of its smaller size as well as extensive in vitro and in vivo characterization. Recent studies demonstrated that eCD4-lg not only neutralizes difficult-to-neutralize (tier 2 and 3) strains of HIV-1 but also inhibits HIV-2 and SIVmac viruses, with IC_{50} of less than 0.01 μg/ml (0.1 nM). Moreover, a rhesus macaque-adapted form of eCD4-lg (rh-eCD4-lg) was able to protect monkeys from multiple low-dose intravenous SHIV challenges when expressed from a recombinant AAV vector (63). Given these findings, we expected eCD4-lg to neutralize ape viruses with similar breadth and potency. However, this was only partially the case. Although eCD4-lg neutralized 100% of SIVcpz and SIVgor strains, the IC_{50} were ~50-fold higher than those previously reported for HIV-1, HIV-2, and SIVmac strains (63). Moreover, this potency was not
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IMCs. Full-length infectious molecular clones (IMCs) of HIV-1 strains SG3 (124), YU2 (125), and JRCSF (126), SIVcpzPrt strains EK505 (71), GABI (68), GAB2 (69), MB897 (71), and MT145 (71), SIVcpzPrt strains BF1167 (7), TAN1 (70), TAN2 (70), and TAN3 (70), and the SIVgor strain CP2139 (15) have previously been reported. To generate additional IMCs, consensus SIVcpzPrt (LB715) and SIVcpzPrt (TAN13) sequences were generated from two additional fecal samples (70). Briefly, partially overlapping subgenomic fragments were amplified from fecal RNA, gel purified, and sequenced directly. Chromatograms were examined for positions of base mixtures, and ambiguous sites were resolved as previously reported (70). The resulting proviral consensus sequences were synthesized in 3 nonoverlapping fragments (Blue Heron Biotechnology) and ligated using internal restriction enzyme enzymes. In addition, NotI and MluI restriction enzyme sites were added to the 5’ and 3’ termini to enable directional cloning into a modified (low-copy-number) pBR322 vector (15). Since full-length SIVcpz and SIVgor molecular clones are notoriously unstable, plasmids were grown in MAX Efficiency Stbl2 competent cells in 3 nonoverlapping fragments (Blue Heron Biotechnology) and ligated using internal restriction enzyme enzymes. In addition, NotI and MluI restriction enzyme sites were added to the 5’ and 3’ termini to enable directional cloning into a modified (low-copy-number) pBR322 vector (15). Since full-length SIVcpz and SIVgor molecular clones are notoriously unstable, plasmids were grown in MAX Efficiency Stbl2 competent cells (Invitrogen) at 30°C and harvested before reaching saturating density, and each IMC was completely sequenced prior to biological analyses to confirm its integrity. The newly derived SIVcpz clones LB715 and TAN13 have been submitted to the National Institutes of Health Research and Reference Program (Rockville, MD), and their nucleotide sequences are available at GenBank.

Phylogenetic analysis. Amino acid sequences inferred from env genes of HIV-1 (YU2, GenBank accession no. M38429; and SG3, accession no. L02317), SIVgor (CP2139.287, accession no. FJ424866), and SIVcpz (LB715, accession no. K861923; MB897, accession no. EF535994; GABI, accession no. AF382828; MT145, accession no. DQ373066; EK505, accession no. DQ373065; GABI, accession no. X52154; TAN2, accession no. DQ374657; TAN3, accession no. DQ374658; TAN1, accession no. EF394356; TAN13, accession no. JQ768416; BF1167, accession no. JQ866001) strains were aligned using ClustalW (127), with regions that could not be unambiguously aligned excluded from the analysis. PhyML (version 3) was used to estimate the phylogeny based on a WAG + 1 + G + F model of amino acid replacement chosen using ProtTest (version 2.4) and a second-order Akaike information criterion (AIC) framework (128–130). Ten random-addition-tree and a neighbor-joining tree were likelihood optimized using subtree pruning-regrafting (SPR) searches. Bayesian posterior probabilities were estimated with MrBayes using a mixed prior model (131).

Chimpanzee plasma. Plasma samples were obtained from eight chimpanzees that had been experimentally infected with HIV-1 and/or SIVcpzANT decades earlier as part of AIDS pathogenesis and/or vaccine studies (Table 1). Samples were obtained from seven of these chimpanzees at the National Chimpanzee Sanctuary Chimp Haven inKeithville, LA, while samples were obtained from the remaining animal (Debbie) at the Southwest National Primate Research Center (SNPRC) in San Antonio, Texas. For Marc (21), Joyce (23), Tika (22), Artica (23), Debbie (20), and Cotton (20), detailed infection histories and clinical follow-up studies have been reported; however, such information was not available for the
remaining two animals. Blood samples were collected for veterinary purposes in the context of health examinations. Plasma samples from seven animals were analyzed for the presence of virus-specific antibodies using an enhanced chemiluminescent Western blot assay as previously described (9). Plasma samples were also tested for viral loads: for HIV-1-infected chimpanzees, this was done using the COBAS AmpliPrep/COBAS TaqMan HIV-1 test, v2.0 (Roche). For SIVcpzANT-infected chimpanzees, a previously reported quantitative PCR (qPCR) designed to detect both HIV-1 and SIVcpz RNA was used (132). In addition, SIVcpzANT viremia was confirmed by amplifying and sequencing a virus-specific 1.3-kb gag-pol fragment from plasma viral RNA (Table 1). Sample collection was approved by the respective Institutional Animal Care and Use Committees.

**Virus stocks.** HIV-1, SIVcpz, and SIVgor infectious molecular clones (8 µg) were transfected into 293T cells, and culture supernatants were harvested 72 h later. Viral stocks were tested for infectivity using TZM-bl cells, a HeLa-derived line that constitutively expresses CD4, CCR5, and CXCR4 receptors and contains integrated luciferase and β-galactosidase reporter genes under the control of an HIV-1 long terminal repeat (LTR) (133). TZM-bl cells (8,300 cells per well) were seeded in 96-well plates overnight with 10-fold serial dilutions of transfection stocks for 48 h to allow single round infection, and infectious units (IU) were determined by counting the number of β-galactosidase-expressing cells.

TZM-bl cell-based neutralization assay. The neutralizing capacity of chimpanzee plasma, anti-HIV-1 monoclonal antibodies, and immunomodulins was assessed using the TZM-bl assay as previously described (134, 135). Briefly, 96-well plates were seeded with TZM-bl cells (8,300 cells per well) overnight in Dulbecco’s modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Serial (5-fold) dilutions of chimpanzee plasma (1:20, 1:100, 1:500, 1:2,500, and 1:12,500) or anti-HIV-1 monoclonal antibodies and immunomodulins (10, 2, 0.4, 0.08, and 0.016 µg/ml) were incubated with 4,800 infectious units (IU) of transfection-derived virus in a total volume of 100 µl in the presence of DEAE-dextran (40 µg/ml) for 1 h at 37°C, and this mixture was then added to TZM-bl cells in 96-well plates. After 48 h, TZM-bl cells were analyzed for luciferase expression using a Synergy H4 Hybrid microplate reader (Bio-Tek) with Gen5 version 1.11 software. Relative infectivity was calculated by dividing the luciferase units of wells containing antibody dilutions by that of the untreated control. All monoclonal antibodies were tested in triplicate, and the average from 3 replicates is reported.

**CD4 T cell-based neutralization assay.** Leftover blood samples from health examinations of uninfected chimpanzees housed at the Yerkes Regional Primate Center were shipped at room temperature (137), and peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Life Sciences). Chimpanzee CD4+ T cells were enriched using nonhuman primate CD4 microbeads (MACS Miltenyi Biotec) and magnetic cell sorting (Milteniy Biotec). These canine thymus-derived cells do not naturally express CD4 or CCR5 (72). We transiently transfected Cf2Th cells with plasmids expressing the chimpanzee CD4 and CCR5 genes. Twenty-four hours posttransfection, Cf2Th cells were trypsinized and plated in 96-well plates at a concentration of 6,000 cells per well in DMEM containing 3.5% FBS. Transfected cells were cultured overnight, incubated with serial (5-fold) dilutions (10, 2, 0.4, 0.08, and 0.016 µg/ml) of mono- and bispecific anti-CD4 (iMab, LM52, PG9-iMab, PG16-iMab, and LM52-PGT128) and anti-CCR5 (PRO-140 and PRO-140-108E) antibodies in a volume of 50 µl DMEM for 1 h, and then infected with 50 µl of virus stock (5,000 IU) in the absence of DEAE-dextran, which is toxic to Cf2Th cells. After 48 h, Cf2Th cells were lysed and analyzed for luciferase expression using a Synergy H4 Hybrid microplate reader (Bio-Tek) with Gen5 version 1.11 software. Relative infectivity was calculated by dividing the luciferase units of wells containing antibodies by that of the untreated control. All monoclonal antibodies were tested in triplicate, and the average from 3 replicates is reported.

**Statistical analyses.** Statistical analyses were performed using Prism version 5.0d software (GraphPad), and correlations were assessed using Spearman tests. Geometric mean IC50s were calculated using Microsoft Excel version 14.3.9 software.
SUPPLEMENTAL MATERIAL


Figure S1, PDF file, 1.1 MB.
Figure S2, PDF file, 0.4 MB.
Table S1, PDF file, 0.5 MB.

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