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# Neutralization Properties of Simian Immunodeficiency Viruses Infecting Chimpanzees and Gorillas

Hannah J. Barbian,<sup>a</sup> Julie M. Decker,<sup>b</sup> Frederic Bibollet-Ruche,<sup>a</sup> Rachel P. Galimidi,<sup>c</sup> Anthony P. West Jr.,<sup>c</sup> Gerald H. Learn,<sup>a</sup> Nicholas F. Parrish,<sup>a</sup> Shilpa S. Iyer,<sup>a</sup> Yingying Li,<sup>a</sup> Craig S. Pace,<sup>d</sup> Ruijiang Song,<sup>e</sup> Yaoxing Huang,<sup>e</sup> Thomas N. Denny,<sup>f</sup> Hugo Mouquet,<sup>g,h</sup> Loic Martin,<sup>i</sup> Priyamvada Acharya,<sup>j</sup> Baoshan Zhang,<sup>j</sup> Peter D. Kwong,<sup>j</sup> John R. Mascola,<sup>j</sup> C. Theo Verrips,<sup>k</sup> Nika M. Strokappe,<sup>l</sup> Lucy Rutten,<sup>l</sup> Laura E. McCoy,<sup>m</sup> Robin A. Weiss,<sup>m</sup> Corrine S. Brown,<sup>n</sup> Raven Jackson,<sup>n</sup> Guido Silvestri,<sup>o</sup> Mark Connors,<sup>p</sup> Dennis R. Burton,<sup>q</sup> George M. Shaw,<sup>a</sup> Michel C. Nussenzweig,<sup>g</sup> Pamela J. Bjorkman,<sup>c</sup> David D. Ho,<sup>e</sup> Michael Farzan,<sup>r</sup> Beatrice H. Hahn<sup>a</sup>

Departments of Medicine and Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania, USA<sup>a</sup>; Department of Microbiology, The University of Alabama at Birmingham, Birmingham, Alabama, USA<sup>b</sup>; Division of Biology and Biological Engineering and Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California, USA<sup>c</sup>; Gilead Sciences, Foster City, California, USA<sup>d</sup>; Aaron Diamond AIDS Research Center, The Rockefeller University, New York, New York, USA<sup>e</sup>; Duke Human Vaccine Institute, Duke University School of Medicine, Durham, North Carolina, USA<sup>f</sup>; Laboratory of Molecular Immunology and Howard Hughes Medical Institute, The Rockefeller University, New York, New York, USA<sup>g</sup>; Laboratory of Humoral Response to Pathogens, Department of Immunology, Institut Pasteur, Paris, France<sup>h</sup>; CEA, iBiTECS, Service d'Ingénierie Moléculaire des Protéines, Gif-sur-Yvette, France<sup>i</sup>; Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA<sup>j</sup>; QVQ BV, Utrecht, the Netherlands<sup>k</sup>; Biomolecular Imaging (BMI), Faculty of Science, Utrecht University, Utrecht, the Netherlands<sup>l</sup>; Division of Infection and Immunity, University College London, London, United Kingdom<sup>m</sup>; Chimp Haven, Inc., Keithville, Louisiana, USA<sup>n</sup>; Yerkes Regional Primate Research Center, Emory University, Atlanta, Georgia, USA<sup>o</sup>; Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA<sup>p</sup>; Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California, USA<sup>q</sup>; Department of Immunology and Microbial Science, The Scripps Research Institute, Jupiter, Florida, USA<sup>r</sup>

**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 ( $n = 10$ ), peptidoglycans 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-Ig<sup>mim2</sup>, CD4-218.3-E51, and CD4-218.3-E51-mim2), 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 Cf2Th cells expressing chimpanzee CD4 and CCR5 and neutralized SIVcpz in chimpanzee CD4<sup>+</sup> T cells, with 50% inhibitory concentrations (IC<sub>50</sub>s) 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 immunoprophylaxis 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<sup>+</sup> 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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Address correspondence to Beatrice H. Hahn, bhahn@upenn.edu.

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Simian 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<sup>+</sup> 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). 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 pathogens (19, 25). In this context, broad and potent neutralizing antibodies (bNabs) may be of utility (26–29). Although immunogens capable of eliciting such antibodies do not yet exist, several studies have shown that bNabs can prevent the acquisition and/or suppress the replication of HIV-1 and simian-human 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 exterior (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-

TABLE 1 Clinical history of the chimpanzees studied

Ape	Code	Date of birth (mo/day/yr)	Virus strain	Yr infected	Duration of infection (yr)	Plasma sampling date(s) (mo/day/yr)	CD4 <sup>+</sup> T cell count (cells/ $\mu$ l) <sup>a</sup>	Viral load (RNA copies/ml)	SIVcpz-ANT infection <sup>c</sup>	Virus-specific antibodies <sup>d</sup>	HIV vaccine history	Reference
Marc	C487	9/6/81	HIV-1/LAV	1984	30	11/8/11	1,154	<50		+	None	21
Artica	C544	2/13/79	HIV-1/LAV	1986	28	11/10/11	1,153	4,794		+	None	23
Joye	C542	2/13/79	HIV-1/LAV	1986	28	11/10/11	2,529	337		ND <sup>e</sup>	LAV <i>env</i>	23
Tika	C534	10/3/78	HIV-1/NC	1997	17	11/10/11	4	244,324		+	None	22
Debbie	X0284	9/23/85	SIVcpzANT	1996	18	1/21/10	NA	ND	ND	ND	None	20
						10/26/10	NA	26,397	+	+		
						6/28/11	NA	ND	ND	ND		
Cotton	X0115	4/10/77	HIV-1/IIIB SIVcpzANT	NA <sup>f</sup> 1996	>18	5/21/02	NA	77,142	ND	ND	NA	20
						9/25/12	229	1,440,622	+	+		
						11/25/14	220 <sup>b</sup>	861,000	ND	ND		
Bucky	NA	NA	NA	NA	NA	11/10/11	1,016	<50		+	NA	NA
Josie	NA	5/18/81	NA	NA	NA	11/10/11	516	<50		+	NA	NA

<sup>a</sup> CD4<sup>+</sup> T cell counts were performed using blood collected in September 2010 unless otherwise noted.

<sup>b</sup> The CD4<sup>+</sup> T cell count was performed using blood collected in November 2014.

<sup>c</sup> +, SIVcpzANT-specific amplification and sequence confirmation.

<sup>d</sup> +, antibody reactivity determined using Western blot analysis (see Fig. S1 in the supplemental material).

<sup>e</sup> ND, not done.

<sup>f</sup> NA, not available.

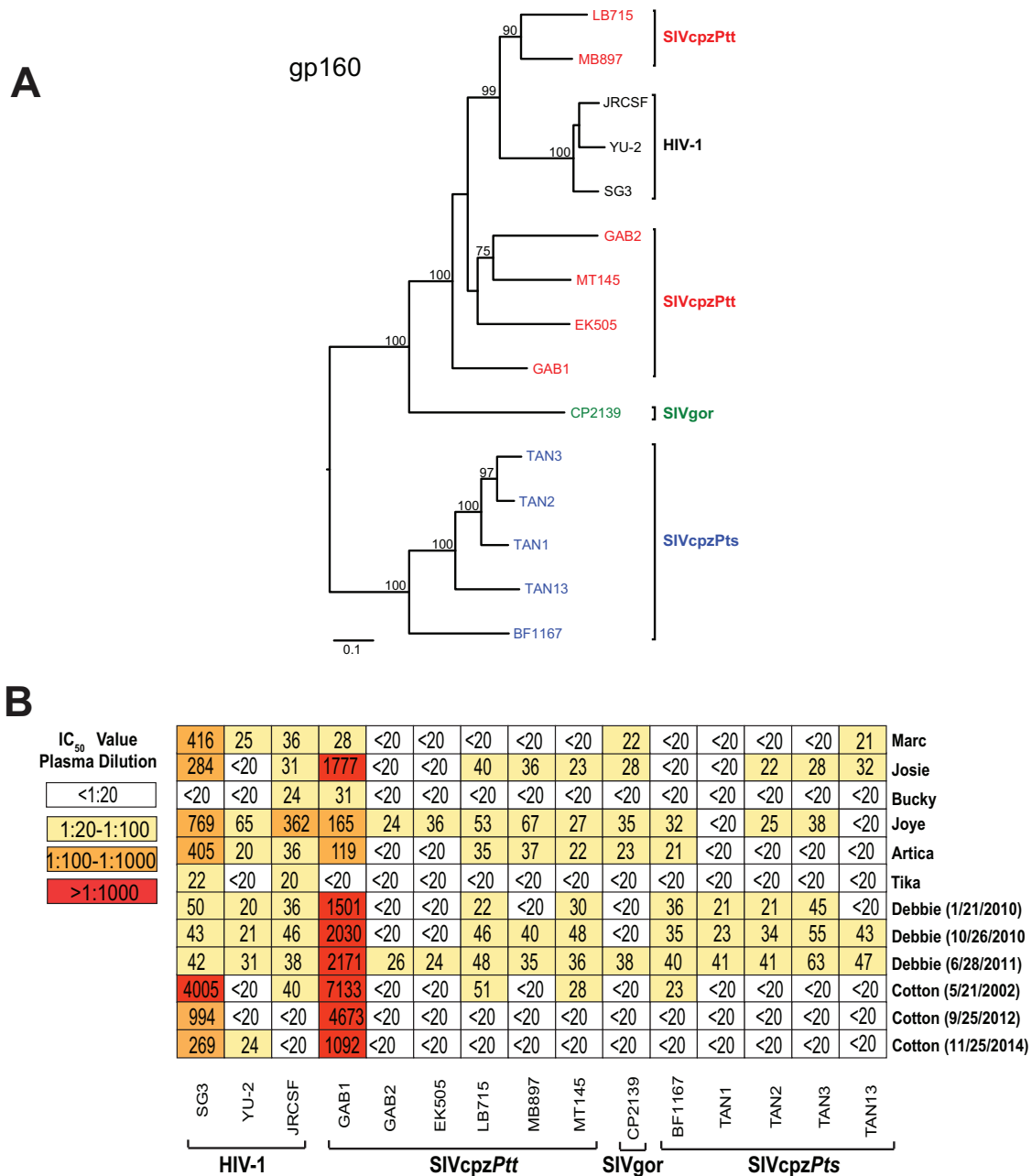
inally isolated from a wild-caught chimpanzee from the Democratic Republic of the Congo (67). Although Cotton was also exposed to HIV-1/LAV (Table 1), reverse transcriptase PCR (RT-PCR) analysis identified SIVcpzANT as the only replicating virus in his plasma. Thus, the latter two animals represent rare 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 SIVcpz*Ptt* lineage and SIVcpz*Pts* 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 IMCs, except for the T cell line-adapted, CXCR4-tropic HIV-1 SG3 strain, used CCR5 as the coreceptor and replicated efficiently in primary human and chimpanzee CD4<sup>+</sup> T cells (6, 7, 11, 15, 68–70). Upon testing of the available plasma samples in the TZM-bl neutralization assay, we found that seven of eight chimpanzees, including the two SIVcpzANT-infected individuals, had activity against the easy-to-neutralize (tier 1) HIV-1 SG3 strain (Fig. 1B). All chimpanzee plasma samples, except for one (Tika), also neutralized SIVcpzGAB1, with IC<sub>50</sub> titers exceeding 1:1,000 in three animals. Since SIVcpzGAB1 was cloned from a viral isolate that was extensively propagated in human peripheral blood mononuclear cells (PBMCs) (68), it likely also represents an easy-to-neutralize (tier 1) chimpanzee virus. In contrast, little cross-reactivity was observed against the remaining primary (tier 2) HIV-1 and SIVcpz strains, with most plasma samples containing very low-level (<1:50) or no neutralizing activity (Fig. 1B). Longitudinal plasma samples were available for two chimpanzees, one of whom (Cotton) showed no neutralization breadth after more than 12 years of infection. The second animal (Debbie) developed antibodies that neutralized all SIVcpz strains but with very low titers (<1:70). Thus, despite the long duration of their infection (Table 1), none of the chronically infected chimpanzees, including the two SIVcpzANT-infected animals, developed appreciable neutralization potency against heterologous HIV-1, SIVcpz, and SIVgor strains (Fig. 1B).

**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), F105 (73), b13 (74), 45-46<sup>G54W</sup> (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<sub>50</sub>s ranging from 0.004 to 1.2  $\mu$ g/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  $\mu$ g/ml (Fig. 2A). This was the case despite the fact that the panel included some of the most potent CD4bs bNabs, such as 45-46<sup>G54W</sup>, which is known to neutralize highly diverse HIV-1 strains, with IC<sub>50</sub>s of <0.05  $\mu$ g/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.

**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 antibodies.



**FIG 1** Neutralizing antibody responses in long-term HIV-1- and SIVcpz-infected chimpanzees. (A) Phylogenetic relationship of HIV-1, SIVcpz, and SIVgor infectious molecular clones (IMCs). A maximum likelihood phylogenetic tree of Env (gp160) protein sequences is depicted, with sequences color coded to differentiate HIV-1 (black), SIVgor (green), SIVcpzPtt (red), and SIVcpzPts (blue) strains. Bootstrap values of  $\geq 70\%$  are shown; the scale bar represents 0.1 amino acid replacement per site. (B) Plasma samples from eight long-term-infected chimpanzees (listed on the right and shown in Table 1) were tested against HIV-1 ( $n = 3$ ), SIVcpzPtt ( $n = 6$ ), SIVgor ( $n = 1$ ), and SIVcpzPts ( $n = 5$ ) strains (bottom) in the TZM-bl neutralization assay. (Collection dates are indicated for samples from the same individual.) IC<sub>50</sub>s (expressed as plasma dilutions) averaged from three replicate experiments are shown, with a heat map indicating the relative neutralization potency.

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  $\mu\text{g/ml}$ , except for PGT128, which neutralized a single SIVcpz-

Ptt (EK505) virus, with an IC<sub>50</sub> of 0.02  $\mu\text{g/ml}$  (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

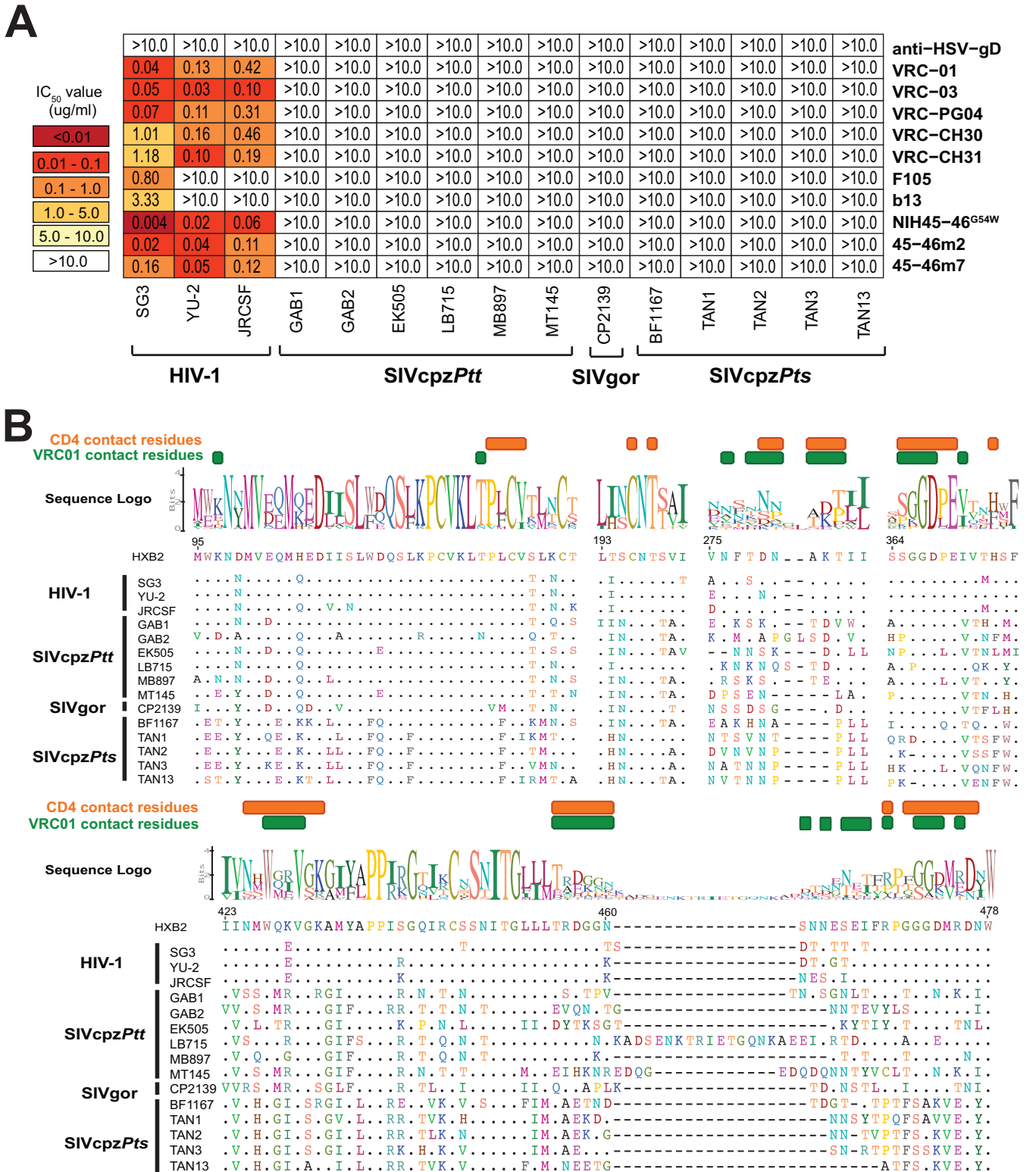
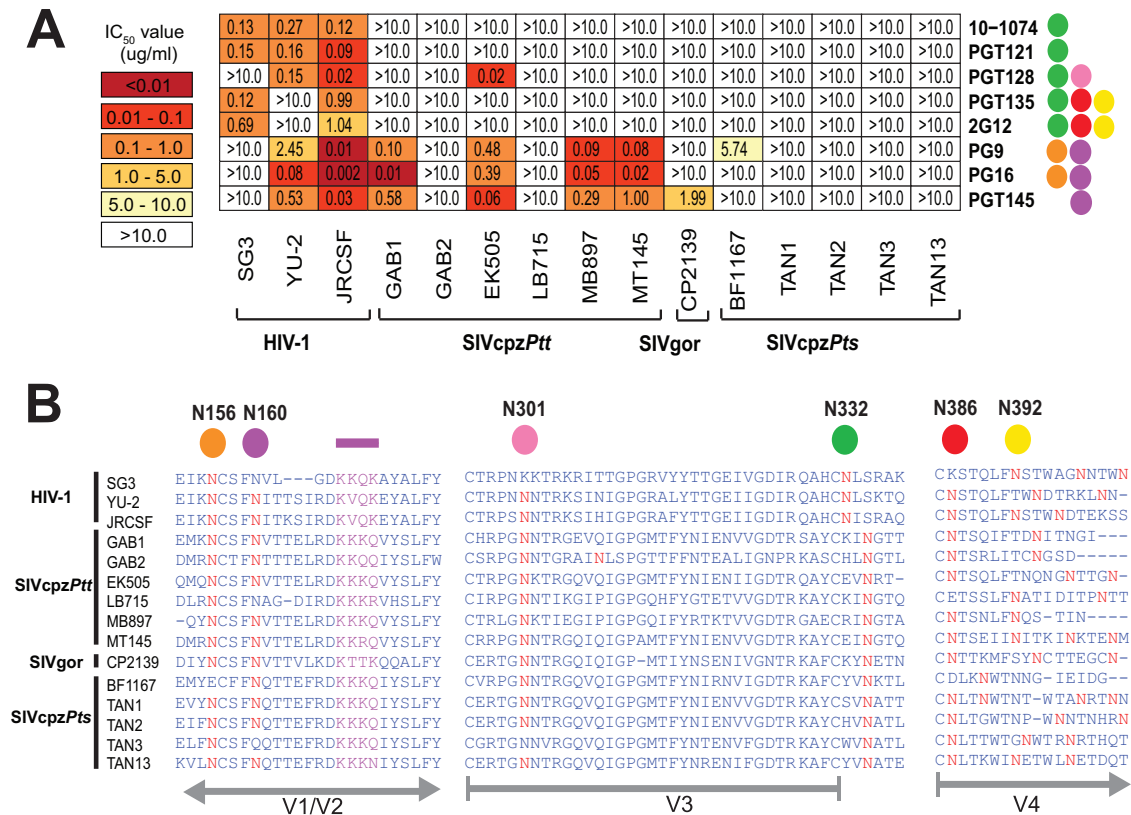


FIG 2 Neutralizing capacity of CD4 binding site (CD4bs) antibodies. (A) The ability of CD4bs monoclonal antibodies (listed on the right) to neutralize HIV-1, SIVcpz, and SIVgor strains (listed on the bottom) is shown. Numbers indicate IC<sub>50</sub>s (in micrograms per milliliter) in TZM-bl cells, averaged 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 residues (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.

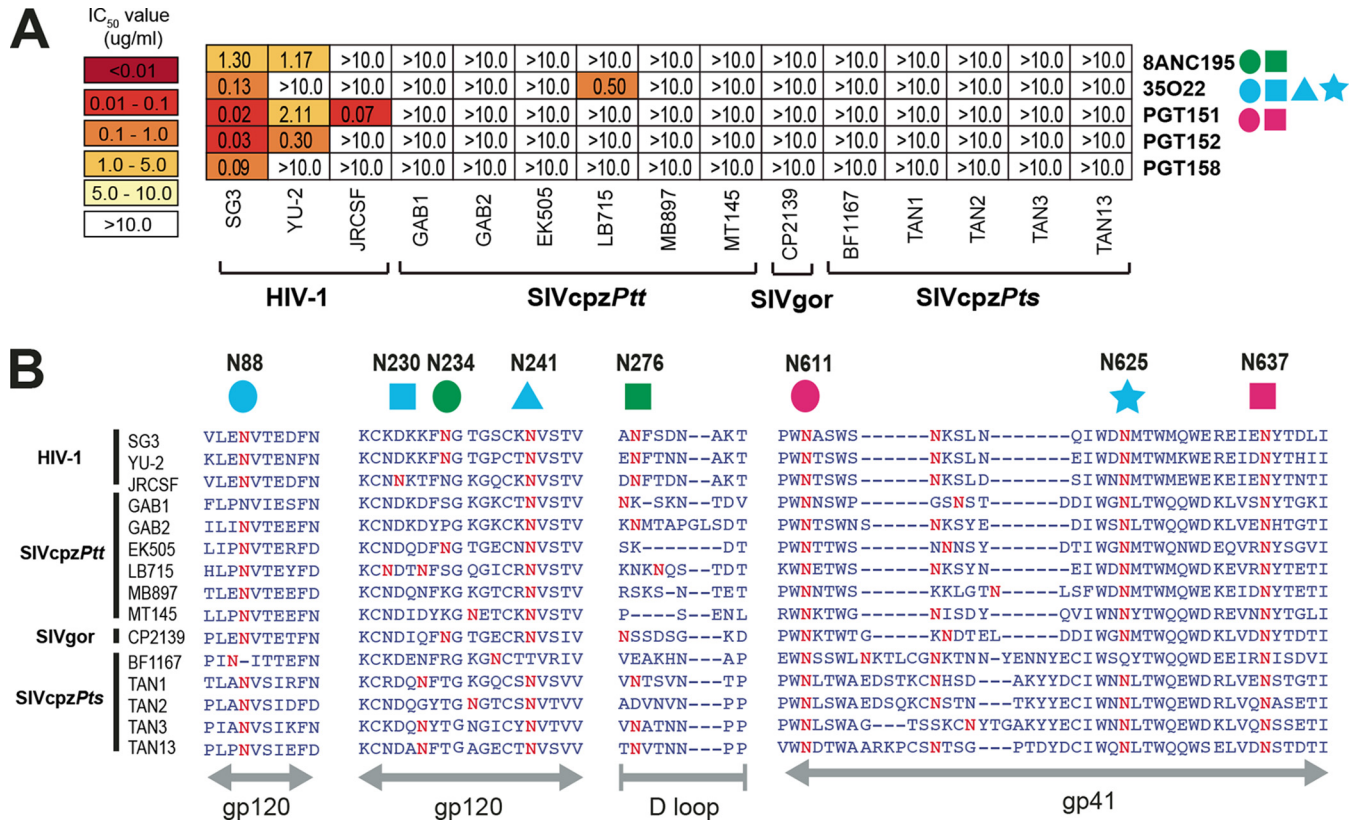


**FIG 3** Neutralizing capacity of high-mannose-patch- and apex V2-directed antibodies. (A) The ability of peptidoglycan-associated monoclonal antibodies (listed on the right) to neutralize HIV-1, SIVcpz, and SIVgor strains (bottom) is shown. Numbers indicate IC<sub>50</sub>s (in micrograms per milliliter) 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 N-linked glycans that are important for their neutralizing activity (orange, N156; purple, N160; pink, N301; green, N332; red, N386; yellow, N392) (27, 28, 52, 81). The highest antibody concentration used was 10 μg/ml. (B) Conservation of glycans associated with bNab activity. An alignment of HIV-1, SIVcpz, and SIVgor Env protein sequences is shown, with predicted N-linked glycans (NXS/T) highlighted in red. Four residues comprising a lysine-rich motif in the V1/V2 strand (C), which together with glycans N156 and N160 form the core epitope for PG9, PG16, and PGT145, are highlighted in purple. N-linked glycans known to influence bNab binding are highlighted above the alignment, with HXB2 numbering in black. The positions of variable loops (V1/V2, V3, and V4) are shown in gray below the alignment.

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 PGT145 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 Envs but absent from all SIVcpz and SIVgor Envs, potentially explaining the lack of cross-neutralization of 10-1074, PGT121, PGT128, PGT135, and 2G12. However, SIVcpzEK505 was potentially neutralized by PGT128 (Fig. 3A), despite the absence of the N332 glycan. (EK505 is also the only strain with a deletion at position 337.) Moreover, all SIVcpz and SIVgor strains encode an NXS/T sequon at position 334, which could potentially compensate for the absence of N332 (82). Thus, the lack of N332 is unlikely to be the sole reason for the inability of glycan V3-directed bNabs to cross-neutralize SIVcpz and SIVgor. In contrast, the core epitopes of PG9, PG16, and PGT145 were surprisingly well conserved among ape viruses (Fig. 3B, left panel), with many SIVcpz and SIVgor Envs contain-

ing the essential N156 and N160 glycans as well as a lysine-rich (KKKQ) motif in strand C of V1/V2 (83, 84). Given this degree of conservation, it is not surprising that SIVcpzPtt strains GAB1, EK505, MB897, and MT145 were exquisitely sensitive to neutralization by PG9, PG16, and PGT145 (Fig. 3A). However, an intact 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 SIVcpzPtt 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 4** Neutralizing capacity of antibodies targeting the interface of HIV-1 gp120 and gp41 regions. (A) The ability of glycan-associated antibodies (right) to neutralize HIV-1, SIVcpz, and SIVgor strains (bottom) is shown. Numbers indicate IC<sub>50</sub>s (in micrograms per milliliter) from TZM-bl cells, averaged from three different experiments, with a heat map indicating the relative neutralization potency. Colored shapes to the right of each antibody indicate the N-linked glycans that are associated with antibody neutralizing activity. Antibody 8ANC195 contacts N234 (green circle) and N276 (green square), 35O22 utilizes N88 (blue circle), N230 (blue square), N241 (blue triangle), and N625 (blue square), and PGT151 requires N611 (pink circle) and N637 (pink square) for optimal neutralization (53–56). The highest antibody concentration used was 10 μg/ml. (B) Conservation of glycans associated with antibody neutralizing activity. An alignment of HIV-1, SIVcpz, and SIVgor Env protein sequences is shown, with predicted N-linked glycans (NXS/T) highlighted in red. N-linked glycans known to influence bNab binding are highlighted above the alignment, with HXB2 numbering in black. The positions of various Env regions are shown in gray below the alignment.

single SIVcpz strain (LB715) at an IC<sub>50</sub> of 0.5 μg/ml (3.3 nM), all other antibodies failed to block SIVcpz and SIVgor infection at concentrations of up to 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 camelid 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 SIVcpzPtt strains at IC<sub>50</sub>s of <5 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 SIVcpzPtt strains and one SIVcpzPts strain at IC<sub>50</sub>s of <10 μg/ml (<40 nM). However, this breadth did not extend to the more divergent SIVcpzPts and SIVgor viruses, and the bivalent MPER antibody Bi-2H10 was unable to neutralize any SIVcpz and SIVgor strains (Fig. 5A).

Since the epitopes of 2H10, 2E7, 11F1F, and JM4 have been



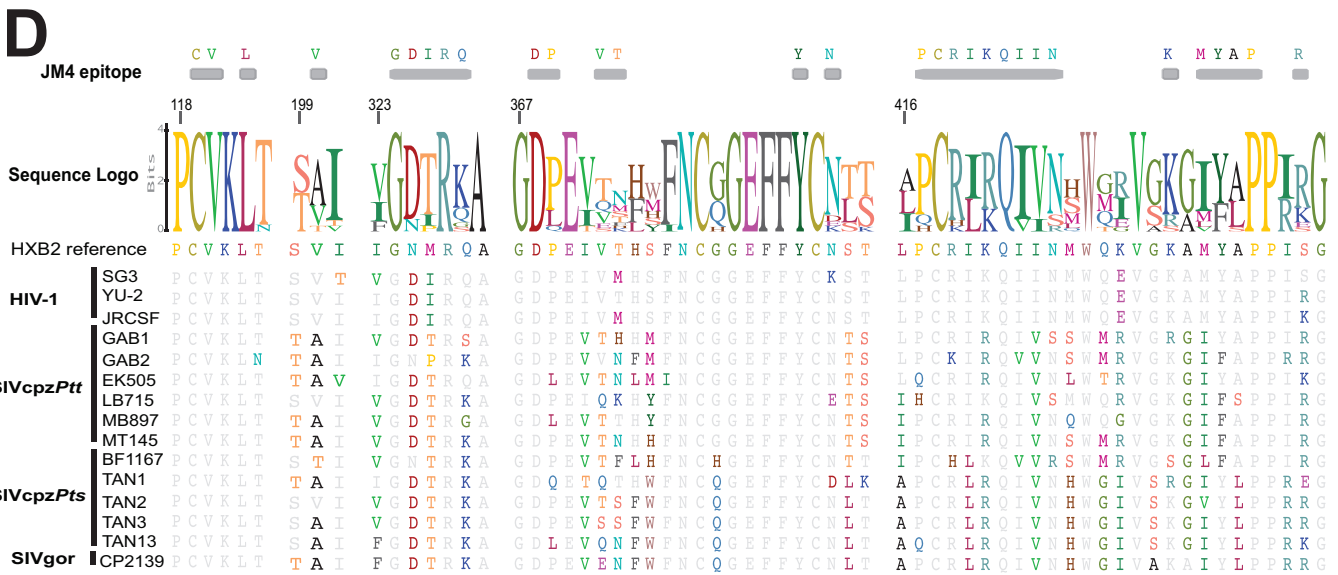
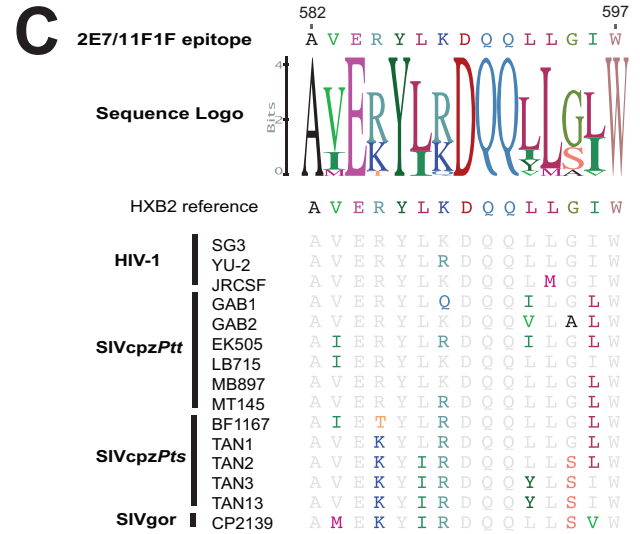
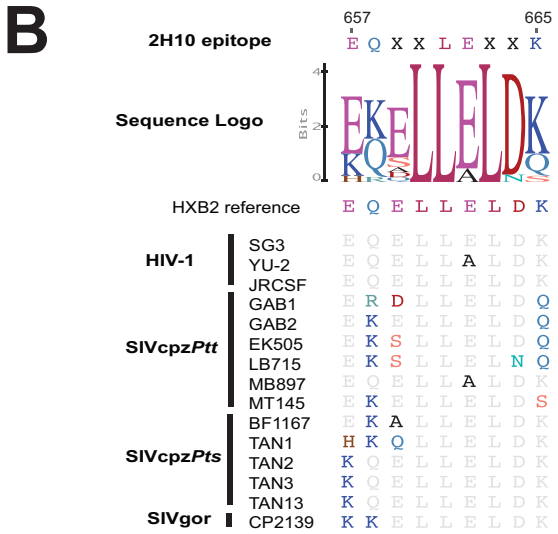
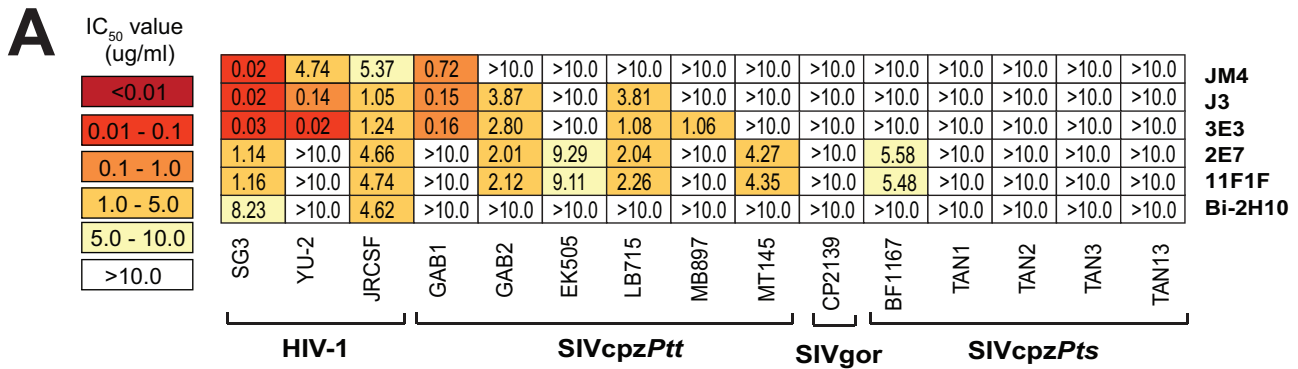
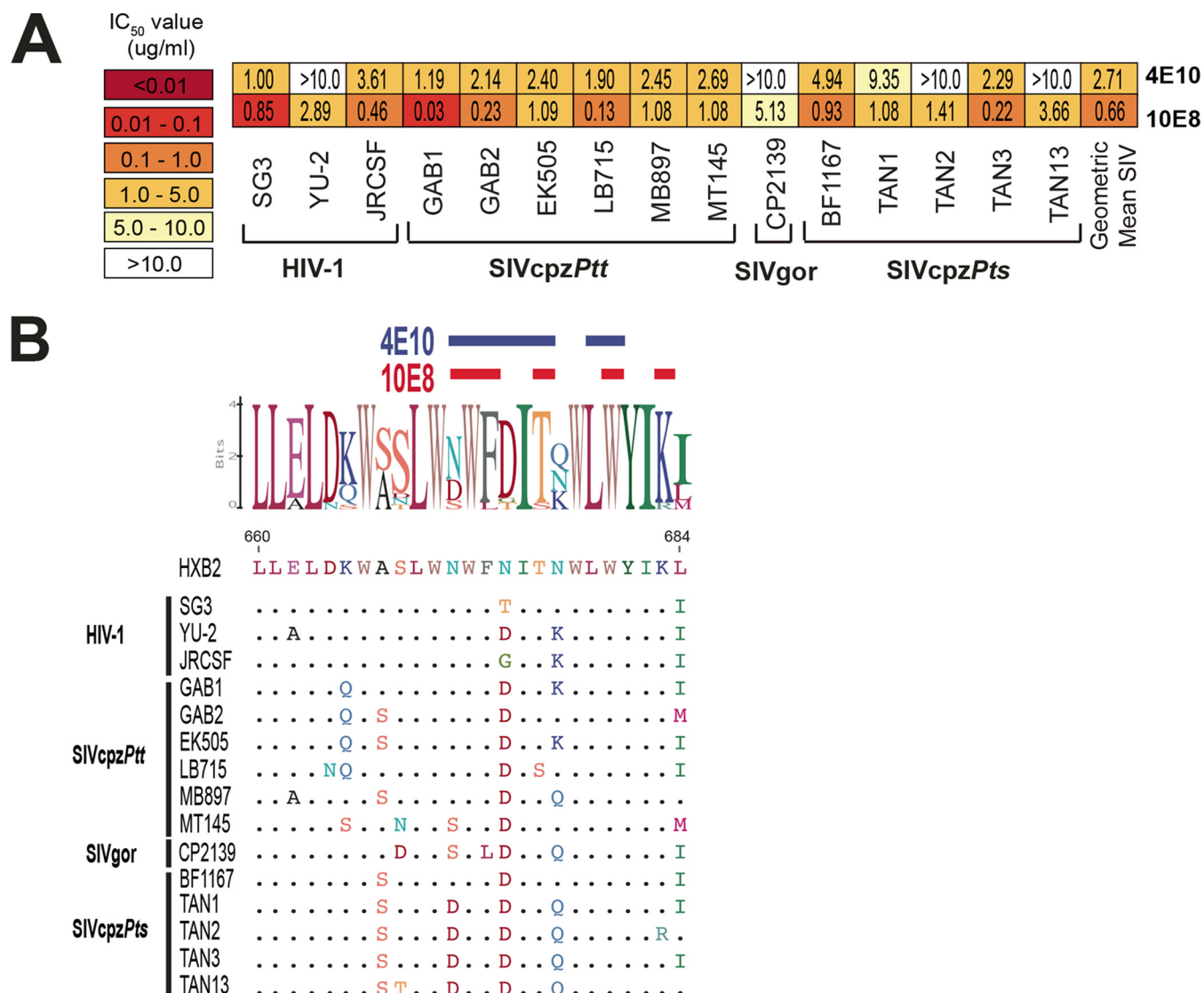


FIG 5 Neutralizing capacity of camelid antibodies. (A) The ability of llama-derived (heavy-chain-only) antibodies (listed on the right) to neutralize HIV-1, SIVcpz, and SIVgor strains (bottom) is shown. Numbers indicate IC<sub>50</sub>s (in micrograms per milliliter) from TZM-bl cells, averaged from 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.

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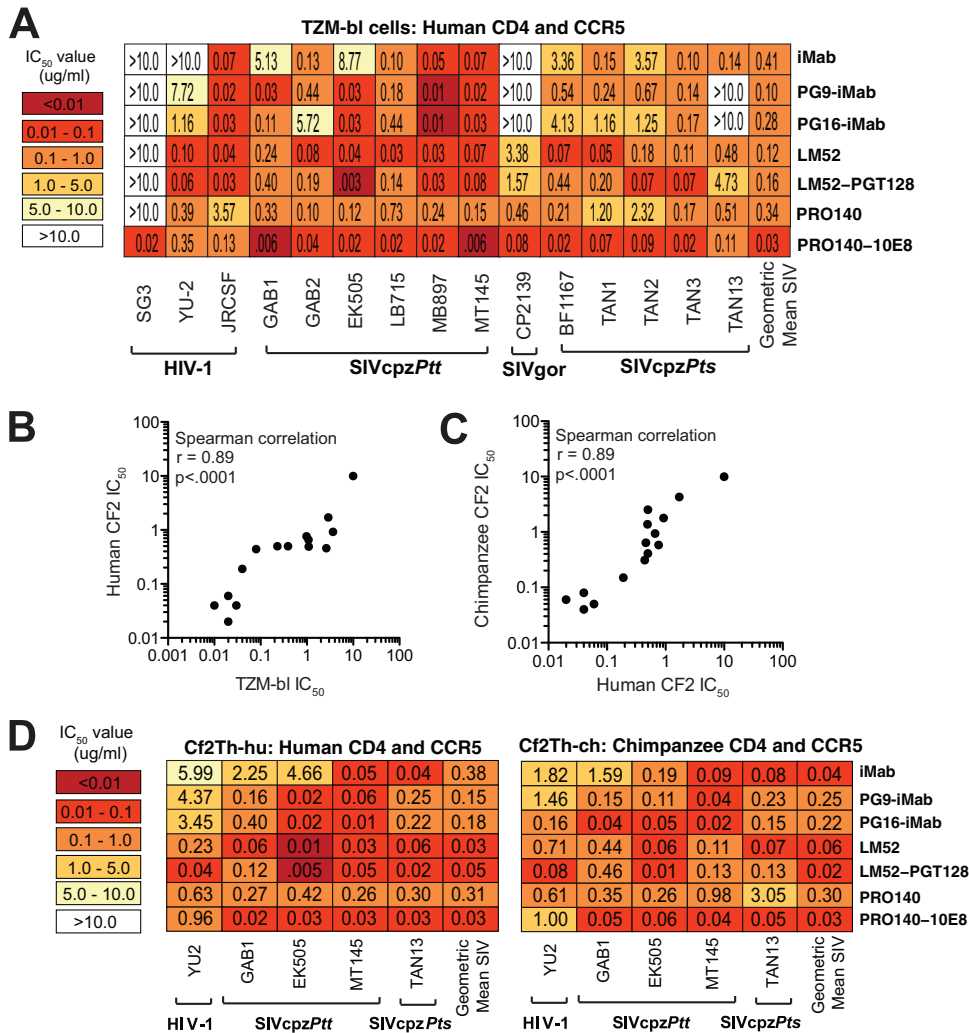


**FIG 6** Neutralizing capacity of MPER antibodies. (A) The ability of two MPER antibodies (listed on the right) to neutralize a panel of HIV-1, SIVcpz, and SIVgor strains (bottom) is shown. Numbers indicate IC<sub>50</sub>s (in micrograms per milliliter) from TZM-bl cells, averaged from three different experiments, with a heat map indicating the relative neutralization potency. The highest antibody concentration used was 10  $\mu$ g/ml. (B) Conservation of 4E10 and 10E8 epitopes. An alignment of HIV-1, SIVcpz, and SIVgor Env protein sequences is depicted, with dots indicating identity to the HXB2 reference sequence. A logo plot denotes the conservation of individual amino acids, with the height of each letter indicating the proportion of sequences that contain the residue at that site. Contact residues of 4E10 (blue) and 10E8 (red) are highlighted above the alignment.

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 ( $n = 181$ ) 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<sub>50</sub> of 2.7  $\mu$ g/ml (18.1 nM), while 10E8 neutralized all ape viruses, with a mean IC<sub>50</sub> 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-



**FIG 7** Neutralizing capacity of anti-host receptor antibodies (A) The ability of monospecific anti-human CD4 (iMab and LM52) and anti-CCR5 (PRO140) antibodies as well as their bispecific derivatives (PG9-iMab, PG16-iMab, LM52-PGT128, and PRO140-10E8) (listed on the right) to neutralize a panel of HIV-1, SIVcpz, and SIVgor strains (bottom) is shown. Numbers indicate IC<sub>50</sub>s (in micrograms per milliliter) from TZM-bl cells, averaged from three different experiments, with a heat map indicating the relative neutralization potency. The geometric mean IC<sub>50</sub> is shown for each antibody. (Only values for SIVcpz and SIVgor strains that were below 10 μg/ml were included in the calculation.) (B) Correlation of TZM-bl- and Cf2Th-hu-derived neutralization data. Three bNabs (10E8, PG16, and eCD4-Ig<sup>gmm2</sup>) were used to neutralize a subset of HIV-1 and SIVcpz strains (YU-2, GAB1, EK505, MT145, and TAN13) in TZM-bl and Cf2Th cells expressing human CD4 and CCR5 receptors (Cf2Th-hu). IC<sub>50</sub>s from TZM-bl (x axis) and Cf2Th-hu (y axis) cells were plotted and analyzed using the Spearman correlation test. (C) Correlation of Cf2Th-hu and Cf2Th-ch neutralization data. The same three bNabs shown in panel B were used to neutralize the same subset of HIV-1 and SIVcpz strains in Cf2Th cells expressing human (Cf2Th-hu) and chimpanzee (Cf2Th-ch) CD4 and CCR5 receptors. IC<sub>50</sub>s from Cf2Th-hu (x axis) and Cf2Th-ch (y axis) cells were plotted and analyzed using the Spearman correlation. (D) The ability of antireceptor antibodies (listed on the right) to neutralize a subset of HIV-1 and SIVcpz strains (bottom) in Cf2Th cells expressing either human (left panel) or chimpanzee (right panel) CD4 and CCR5 receptors is shown. Numbers indicate IC<sub>50</sub>s (in micrograms per milliliter) averaged from three different experiments, with heat maps indicating relative neutralizing potencies. The geometric mean IC<sub>50</sub> is shown for each antibody. (Only values from SIVcpz strains were included.)

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 IC<sub>50</sub>s 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 IC<sub>50</sub> 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 PG9, PG16, PGT128, and 10E8 (60), were potent inhibitors, especially when the fusion partner was also an effective neutralizer. For example, the bispecific PG9-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  $IC_{50}$  of 0.03  $\mu\text{g/ml}$  (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  $IC_{50}$ s from Cf2Th-hu cells were highly correlated with those from TZM-bl cultures (Fig. 7B), and this was also true for  $IC_{50}$ s 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  $IC_{50}$ s ranging from 0.30  $\mu\text{g/ml}$  (PRO140) to 0.02  $\mu\text{g/ml}$  (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  $IC_{50}$ s of 2.0  $\mu\text{g/ml}$  (47.7 nM) and 2.1  $\mu\text{g/ml}$  (23.1 nM), respectively (Fig. 8B). The tetravalent PRO542, in which D1/D2 domains are linked to both the constant heavy ( $C_H$ ) and light ( $C_L$ ) chains of IgG1 (Fig. 8A), was slightly more potent and neutralized 10 of 12 ape viruses, with a mean  $IC_{50}$  of 1.1  $\mu\text{g/ml}$  (5.2 nM) (Fig. 8B). Finally, CD4-218.3-E51, a CD4-induced (CD4i) neutralizing antibody (E51) linked to D1/D2 via the  $V_H$  chain (Fig. 8A), neutralized all SIVcpz and SIVgor strains, with a geometric mean  $IC_{50}$  of 1.68  $\mu\text{g/ml}$  (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).

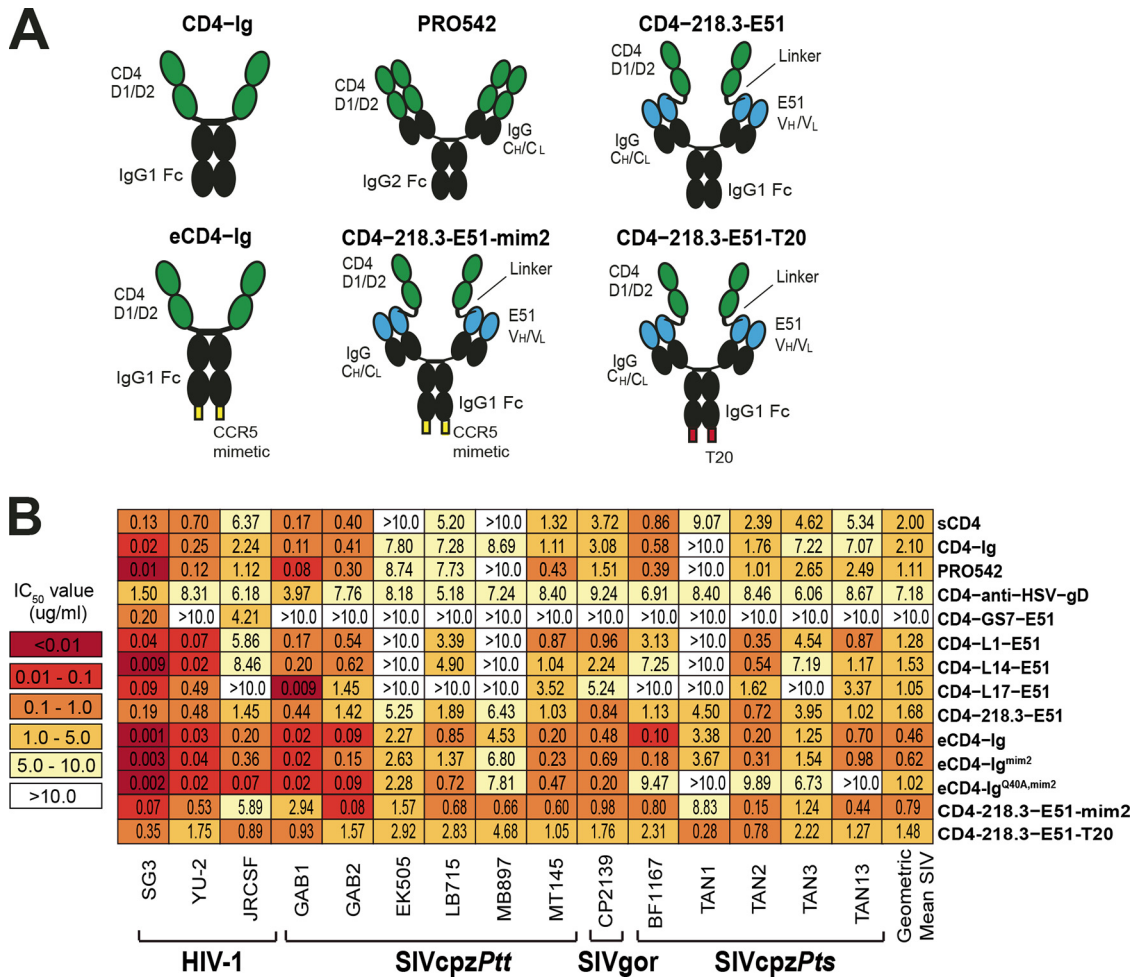
Some HIV-1 bNabs use tyrosine sulfation of their complementarity-determining regions (CDRs) to mimic CCR5 sulfation and thereby inhibit virus entry (103). Sulfopeptides derived from such antibodies, including a 15-amino-acid tyrosine-sulfated peptide derived from the CD4i antibody E51, have been shown 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 macques (SIVmac) and HIV-2 isolates, with  $IC_{50}$ s of  $<0.05 \mu\text{g/ml}$  (63). Using TZM-bl cells, we found that eCD4-Ig neutralized SIVcpz and SIVgor strains also more potently than CD4-Ig, with a mean  $IC_{50}$  of 0.46  $\mu\text{g/ml}$  (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<sup>mim2</sup> (63) was slightly less potent than eCD4-Ig ( $IC_{50}$  of 0.62  $\mu\text{g/ml}$ ), and eCD4-Ig<sup>Q40A,mim2</sup> (63) failed to neutralize two SIVcpzPts strains (Fig. 8B). Addition of CCR5mim2 to the carboxy terminus of CD4-218.3-E51 (CD4-218.3-E51-mim2) 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<sup>+</sup> 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<sup>+</sup> 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<sup>mim2</sup>), 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<sup>+</sup> 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  $IC_{50}$ s of 0.64 (4.3 nM), 0.68 (4.5 nM), and 0.75  $\mu\text{g/ml}$  (3.6 nM), respectively (Table 2). Immunoadhesins were roughly 10-fold less potent, with mean  $IC_{50}$ s ranging from 3.7  $\mu\text{g/ml}$  (38.3 nM) for eCD4-Ig to 6  $\mu\text{g/ml}$  (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<sup>+</sup> T cells (see Fig. S2B), although the observed  $IC_{50}$ s 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



**FIG 8** Neutralizing capacity of human CD4 D1/D2 domain containing immuno-adhesins. (A) Schematic representation of six constructs. Human CD4 D1 and D2 domains are shown in green, immunoglobulin (IgG) Fc and constant heavy- and light-chain (C<sub>H</sub>/C<sub>L</sub>) regions are shown in black, E51 variable heavy and light (V<sub>H</sub>/V<sub>L</sub>) regions are shown in blue, CCR5 mimetic peptides are shown in yellow, and the T-20 fusion inhibitor is shown in red. (B) The ability of human CD4 containing antibody-like constructs (listed on the right) to neutralize a panel of HIV-1, SIVcpz, and SIVgor strains (bottom) is shown. Numbers indicate IC<sub>50</sub>s (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. The geometric mean IC<sub>50</sub> is shown for each construct. (Only values for SIVcpz and SIVgor strains that were below 10 μg/ml were included in the calculation.)

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-Ig<sup>mim2</sup>, 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 for evidence of neutralization breadth (Table 1). Although all animals tested had high-titer Env binding antibodies (see Fig. S1 in the supplemental material), their plasma samples neutralized only the easy-to-neutralize (tier 1) HIV-1 SG3 and SIVcpzGAB1 strains (Fig. 1B). Four animals had very low (Joye) or undetectable (Marc, Bucky, and Josie) plasma viral loads, suggesting insufficient antigenic stimulation as a reason for the lack of bNab development (Table 1). In addition, one animal (Tika) had severe CD4<sup>+</sup> T cell depletion and thus may have suffered from immune exhaustion (Table 1). However, two chimpanzees (Debbie and Cotton) had plasma viral titers exceeding 20,000 RNA copies/ml, suggesting sustained productive infection for almost two decades (Table 1). Both were experimentally infected with SIVcpzANT, a

TABLE 2 Neutralization potency in primary chimpanzee CD4<sup>+</sup> T cells

Antibody	IC <sub>50</sub> for <sup>a</sup> :			
	HIV-1 SG3		SIVcpz MT145	
	μg/ml	nM	μg/ml	nM
CD4-Ig	0.02 ± 0.02	0.22 ± 0.22	6.02 ± 3.5	65.4 ± 38.0
CD4-218.3-E51	0.04 ± 0.04	0.20 ± 0.20	3.74 ± 1.4	18.7 ± 7.0
eCD4-Ig	<0.01 ± 0.0	<0.10 ± 0.0	3.68 ± 1.1	38.3 ± 11.5
eCD4-Igmim2	<0.01 <sup>b</sup>	<0.10 ± 0.0	5.86 <sup>b</sup>	61.0 <sup>b</sup>
CD4-218.3-E51-mim2	<0.01 ± 0.0	<0.05 ± 0.0	3.93 ± 1.5	19.7 ± 7.5
CD4-218.3-E51-T20	<0.01 ± 0.0	<0.05 ± 0.0	4.90 ± 4.4	24.5 ± 22.0
VRC01	0.03 ± 0.03	0.20 ± 0.20	>10 ± 0.0	>66.7 ± 0.0
10E8	ND	ND	6.07 ± 1.82	40.5 ± 12.1
iMab	ND	ND	0.64 ± 0.41	4.3 ± 2.7
LM52	ND	ND	0.68 ± 0.18	4.5 ± 1.2
PRO140	ND	ND	2.33 ± 1.99	15.5 ± 13.3
PRO140-10E8	ND	ND	0.75 ± 0.68	3.6 ± 3.2

<sup>a</sup> Shown are average values from 3 donors ± standard deviations, except as noted. ND, not done.

<sup>b</sup> Not applicable (only performed for 1 donor).

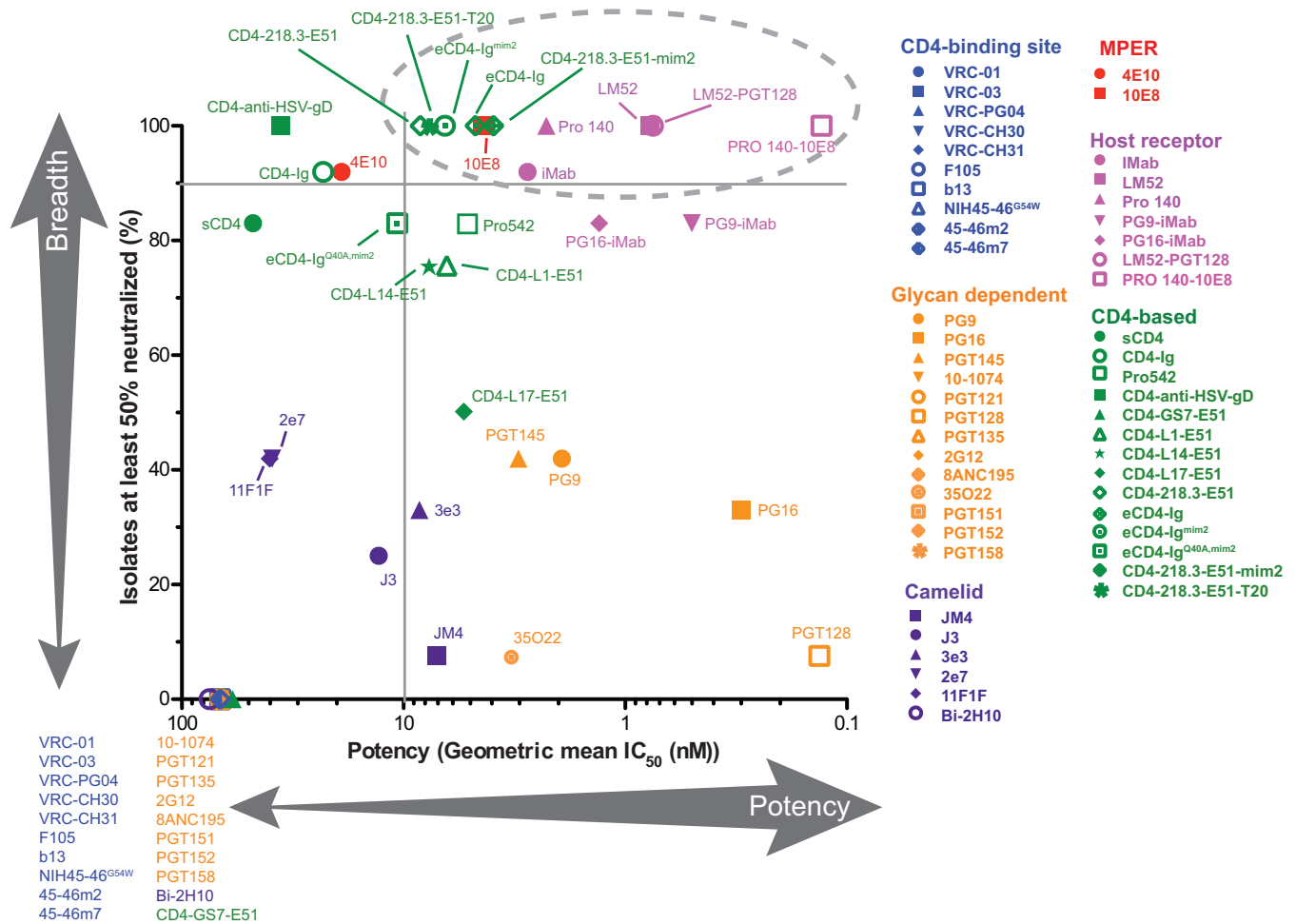
naturally occurring SIVcpzPtt strain that was administered by mucosal routes using plasma and/or peripheral blood mononuclear cells from a third chimpanzee (20). Despite this “near-natural” infection history, only one of these animals (Debbie) exhibited cross-reactive neutralizing activity, albeit with 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 SIVcpzPtt strains, these bNabs lacked activity against SIVgor and SIVcpzPtt 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 SIVcpzPtt strains, with a mean IC<sub>50</sub> of 2.7 μg/ml (18.1 nM), but lacked activity against SIVgor and some SIVcpzPtt strains. In contrast, 10E8 neutralized all 12 SIVcpz and SIVgor strains, with an IC<sub>50</sub> 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 SIVcpzPtt 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 as well as primary chimpanzee CD4<sup>+</sup> T cells (Table 2; see Table S1 in the supplemental material). This was also true for LM52, a derivative of iMab with increased neutralization breadth (59), as well as PRO140, which binds a complex epitope spanning multiple extracellular CCR5 domains (114), both of which neutralized all ape viruses in all cell types tested (Table 2; see Table S1). 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



**FIG 9** Breadth and potency of antibodies and antibody-like constructs with anti-SIVcpz and anti-SIVgor neutralizing activity. For each bNab, the percentage of SIVcpz and SIVgor strains neutralized with  $IC_{50}$ s of  $<10 \mu\text{g/ml}$  (y axis) is plotted against the corresponding geometric mean  $IC_{50}$  (nanomolar concentration) (x axis). Antibodies and antibody-like constructs are color coded, with those that exhibited no anti-SIVcpz and SIVgor activity listed in the left lower corner. Horizontal and vertical bars denote 90% breadth and 10 nM potency, respectively, and the most potent and cross-reactive reagents are circled.

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  $IC_{50}$ s ranging from  $0.03 \mu\text{g/ml}$  (0.13 nM) in TZM-bl cells to  $0.75 \mu\text{g/ml}$  (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/maaque model to determine whether its *in vitro* 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-Ig-based reagents that contain short CCR5-mimetic sulfopeptides on their C terminus (Fig. 8A). Although both CD4-CD4i and eCD4-Ig constructs neutralized human and ape viruses with nanomolar potency (see Table S1 in the supplemental material), we were particularly interested in eCD4-Ig, because of its smaller size as well as extensive *in vitro* and *in vivo* characterization. Recent studies demonstrated that eCD4-Ig not only neutralizes difficult-to-neutralize (tier 2 and 3) strains of HIV-1 but also inhibits HIV-2 and SIVmac viruses, with  $IC_{50}$ s of less than  $0.01 \mu\text{g/ml}$  (0.1 nM). Moreover, a rhesus macaque-adapted form of eCD4-Ig (rh-eCD4-Ig) 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-Ig to neutralize ape viruses with similar breadth and potency. However, this was only partially the case. Although eCD4-Ig neutralized 100% of SIVcpz and SIVgor strains, the  $IC_{50}$ s were  $\sim 50$ -fold higher than those previously reported for HIV-1, HIV-2, and SIVmac strains (63). Moreover, this potency was not

improved when modified versions of eCD4-Ig (eCD4-Ig<sup>mim2</sup> and eCD4-Ig<sup>Q40Amim2</sup>) were used (63), which exhibit enhanced anti-HIV-1 activity (Fig. 8B; see Table S1). Overall, eCD4-Ig neutralized SIVcpz and SIVgor strains only 5-fold more potently than CD4-Ig, compared to a 20- to 200-fold improvement for HIV-1 strains (63). Thus, eCD4-Ig neutralized ape viruses with the desired breadth but not with the desired potency.

Although a crystal structure of eCD4-Ig in complex with the HIV-1 Env is not available, modeling suggests that eCD4-Ig binds the HIV-1 Env trimer in a “claw-like” fashion, with two sulfopeptides and one CD4 moiety engaging two protomers within the same Env spike (63). Cross-linking of spike protomers has recently been shown to increase neutralization potency of antibody-like molecules by more than 100-fold (117). It is thus tempting to speculate that the architecture of the SIVcpz/SIVgor envelope spike differs from that of HIV-1, HIV-2, and SIVmac strains in such a way that one or more of the eCD4-Ig interaction sites are altered or occluded. It is also possible that the human CD4i antibody-derived E51 peptide does not properly mimic the binding of the chimpanzee and gorilla CCR5 coreceptors, which differ from human and macaque CCR5 molecules in one or two amino acids near the N terminus (94). It should be noted that replacing the human CD4 D1/D2 domain with the corresponding chimpanzee CD4 D1/D2 region in eCD4-Ig did not increase its potency against ape viruses (data not shown). Moreover, while SIVcpz and SIVgor Envs tend to have longer V1/V2 loops and more associated glycans than HIV-1 strains, this is also true for SIVmac and HIV-2 strains. Thus, V1/V2 loop length cannot explain the difference in eCD4-Ig neutralization susceptibility of ape viruses. Since there are no SIVcpz/SIVgor specific bNabs whose Fab fragments could be used for intraspine cross-linking, it will be important to determine whether the ape virus neutralizing capacity of eCD4-Ig can be improved by altering the length and/or flexibility of the IgG Fc hinge region and/or by improving the binding of the CCR5 mimetic peptide.

**Vectored antibody gene transfer to combat ape pathogens.** Wild ape populations will go extinct unless there is a comprehensive approach to their conservation, which—under certain circumstances—may have to include medical interventions (19, 25). We have shown in the past that SIVcpz infection can have a substantial negative impact on the health, reproductive success, and longevity of chimpanzees in Gombe (2, 3). Because infected chimpanzees in Gombe are individually known, it would be possible to use vectored antibody gene transfer to administer a cocktail of neutralizing antibodies. This may not only benefit the infected chimpanzees but may also reduce their ability to transmit SIVcpz, which in an isolated population such as Gombe could lead to virus extinction. Vectored antibody gene delivery, alone or in combination with long-acting antiretrovirals (118, 119), could also be used to treat SIVcpz-infected chimpanzees in African sanctuaries (120) and/or to vaccinate orphaned chimpanzees prior to releasing them into the wild (121). Finally, vectored antibody gene delivery may be effective against other pathogens, such as anthrax and Ebola virus (122, 123). Obviously, delivered antibodies must be sufficiently potent and expression levels must be sufficiently high to be effective *in vivo*, but this seems achievable. As shown recently, AAV-delivered rh-eCD4-Ig protected macaques from repeated low-dose intravenous SHIV challenge at serum concentrations as low as 17  $\mu\text{g/ml}$  (63). Moreover, AAV-rh-eCD4-Ig was much less immunogenic than other antibody-based AAV con-

structs, and functional rh-eCD4-Ig was stably expressed for 40 weeks at concentrations ranging between 17 and 77  $\mu\text{g/ml}$  (63). Obviously, efforts to treat and/or vaccinate wild ape communities would only be contemplated if their survival was in serious jeopardy and if the safety and efficacy of the particular intervention were first demonstrated in captivity. However, given the increasing threat of infectious diseases to ape survival, there is an urgent need to explore new ways to curb the spread of pathogens in wild populations. Although further improvements will be necessary, the finding of several antibody-like constructs that are capable of neutralizing SIVcpz and SIVgor strains with nanomolar potency suggests that this goal is achievable.

## MATERIALS AND METHODS

**IMCs.** Full-length infectious molecular clones (IMCs) of HIV-1 strains SG3 (124), YU2 (125), and JRCSF (126), SIVcpzPtt strains EK505 (71), GAB1 (68), GAB2 (69), MB897 (71), and MT145 (71), SIVcpzPts 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 SIVcpzPtt (LB715) and SIVcpzPts (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 (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. M93258; JRCSF, accession no. M38429; and SG3, accession no. L02317), SIVgor (CP2139.287, accession no. FJ424866), and SIVcpz (LB715, accession no. KP861923; MB897, accession no. EF535994; GAB2, accession no. AF382828; MT145, accession no. DQ373066; EK505, accession no. DQ373065; GAB1, 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 + I + 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-order trees 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 in Keithville, 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), Joye (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 vRNA 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  $\mu$ g) 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  $\beta$ -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 and incubated 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  $\beta$ -galactosidase-expressing cells.

**TZM-bl cell-based neutralization assay.** The neutralizing capacity of chimpanzee plasma, anti-HIV-1 monoclonal antibodies, and immunoadhesins 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 immunoadhesins (10, 2, 0.4, 0.08, and 0.016  $\mu$ g/ml) were incubated with 4,800 infectious units (IU) of transfection-derived virus in a total volume of 100  $\mu$ l in the presence of DEAE-dextran (40  $\mu$ g/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. To test the neutralization capacity of anti-CD4 and anti-CCR5 receptor antibodies, 5-fold serial dilutions (10, 2, 0.4, 0.08, and 0.016  $\mu$ g/ml) were incubated with TZM-bl cells in a volume of 50  $\mu$ l for 1 h at 37°C, followed by the addition of virus (4,800 IU in 50  $\mu$ l) in the presence of DEAE-dextran (40  $\mu$ g/ml) and further incubation for 48 h at 37°C. Controls included untreated cells and virus pretreated with normal human plasma or no antibody. Relative infectivity was calculated by dividing the number of luciferase units at each plasma and antibody/immunoadhesin dilution with values obtained for wells that contained normal human plasma or no antibody. Half-maximal inhibitory concentrations (IC<sub>50</sub>s) were determined by linear regression. All monoclonal antibodies were tested in duplicate on three independent occasions. Since available amounts of chimpanzee plasma were limited, samples were tested only once in duplicate.

**Cf2Th cell-based neutralization assay.** To test the neutralization capacity of anti-host receptor antibodies, we transiently transfected Cf2Th cells with plasmids expressing the chimpanzee CD4 and CCR5 genes. These canine thymus-derived cells do not naturally express CD4 or CCR5 but contain a firefly luciferase reporter gene stably integrated under the control of an HIV-1 long terminal repeat (LTR) (2, 3, 136). To generate a chimpanzee CD4 expression plasmid, we extracted RNA from chimpanzee peripheral blood mononuclear cells (PBMCs) and used RT-PCR to generate a cDNA clone of the entire CD4 coding region. Since the chimpanzee CD4 gene is polymorphic (72), we selected one allele that is most predominant in chimpanzees from Gombe National Park. Using site-directed mutagenesis, we then "humanized" this cDNA clone by changing 3 amino acids to match the (nonpolymorphic) human CD4 protein (T34I, V55A, and G88E). To generate a chimpanzee CCR5 expression

plasmid, we first amplified the human CCR5 gene from PBMC RNA and then introduced a single (chimpanzee-specific) amino acid substitution (N13D) by site-directed mutagenesis. These expression plasmids were then used to transfect Cf2Th cells in 10-cm dishes at 50% confluence to generate cells that expressed either chimpanzee or human CCR5 and CD4 receptors. 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 of viruses (10, 2, 0.4, 0.08, and 0.016  $\mu$ g/ml) of mono- and bispecific anti-CD4 (iMab, LM52, PG9-iMab, PG16-iMab, and LM52-PGT128) and anti-CCR5 (PRO-140 and PRO-140-10E8) antibodies in a volume of 50  $\mu$ l DMEM for 1 h, and then infected with 50  $\mu$ l 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 the luciferase units of wells lacking antibodies. Half-maximal inhibitory concentrations (IC<sub>50</sub>s) were determined by linear regression. HIV-1 SG3, which requires CXCR4 for entry, was used as a negative 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<sup>+</sup> T cells were enriched using nonhuman primate CD4 microbeads (MACS Miltenyi Biotec) and magnetic cell sorting (Miltenyi Biotec), stimulated with staphylococcal enterotoxin B (Sigma-Aldridge) for 12 to 15 h (3  $\mu$ g/ml), and subsequently cocultivated with autologous monocyte-derived macrophages for optimal activation. After 5 to 6 days, CD4<sup>+</sup> T cells were removed from the macrophage feeder layer, placed into DMEM with 10% FBS, and incubated with 30 U/ml interleukin-2 (IL-2). To test the neutralizing capacity of anti-HIV-1 monoclonal antibodies and immunoadhesins, serial dilutions (10, 3.3, 1.1, 0.36, and 0  $\mu$ g/ml) were incubated with SIVcpzMT145 and HIV-1 SG3 viral stocks (10,000 IU) for 1 h at 37°C, and the mixture was then added to 5  $\times$  10<sup>5</sup> activated chimpanzee CD4<sup>+</sup> T cells in a total of 500  $\mu$ l DMEM containing 10% FBS and 30 U/ml IL-2. The CD4bs antibody VRC01 and uninfected cells were used as negative controls. To test the neutralizing capacity of antireceptor antibodies, serial dilutions (10, 3.3, 1.1, and 0.36  $\mu$ g/ml) were first incubated with 5  $\times$  10<sup>5</sup> activated chimpanzee CD4<sup>+</sup> T cells for 1 h at 37°C and then exposed to 10,000 IU of virus stocks. After an overnight incubation, cells were washed three times with PBS to remove antibody and non-cell-associated virus, suspended in DMEM with 10% FBS and 30 U/ml IL-2, and plated in triplicate in 96-well plates. Supernatant (50  $\mu$ l) was harvested every 48 h, starting at day 3 postinfection, and replaced with fresh media. To monitor viral replication, supernatants were tested for reverse transcriptase (RT) activity using a colorimetric assay (Roche). Neutralization was calculated by dividing the infectivity (RT activity) of wells with antibody dilutions by that of the untreated control wells. IC<sub>50</sub>s were calculated by linear regression. All monoclonal antibodies were tested in CD4<sup>+</sup> T cells from three different chimpanzee donors.

**Statistical analyses.** Statistical analyses were performed using Prism version 5.0d software (GraphPad), and correlations were assessed using Spearman tests. Geometric mean IC<sub>50</sub>s were calculated using Microsoft Excel version 14.3.9 software.

**Accession numbers.** The nucleotide sequences of SIVcpz strains LB715 and TAN13 as well as the chimpanzee CD4 and CCR5 genes used for transfection are available under GenBank accession no. KP861923, JQ768416, KP235488, and KP235489, respectively.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00296-15/-/DCSupplemental>.

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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