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Trispecific broadly neutralizing HIV antibodies mediate potent SHIV protection in macaques

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The development of an effective AIDS vaccine has been challenging due to viral genetic diversity and the difficulty in generating broadly neutralizing antibodies (bnAbs). Here, we engineered trispecific antibodies (Abs) that allow a single molecule to interact with three independent HIV-1 envelope determinants: 1) the CD4 binding site, 2) the membrane proximal external region (MPER) and 3) the V1V2 glycan site. Trispecific Abs exhibited higher potency and breadth than any previously described single bnAb, showed pharmacokinetics similar to human bnAbs, and conferred complete immunity against a mixture of SHIVs in non-human primates (NHP) in contrast to single bnAbs. Trispecific Abs thus constitute a platform to engage multiple therapeutic targets through a single protein, and could be applicable for diverse diseases, including infections, cancer and autoimmunity.

A variety of broadly neutralizing antibodies (bnAbs) have been isolated from HIV-1 infected individuals (*1–3*), but their potential to treat or prevent infection in humans may be limited by the potency or breadth of viruses neutralized (*4*, *5*). The targets of these antibodies have been defined based on an understanding of the HIV-1 envelope structure (*6–9*). While bnAbs occur in selected HIV-1 infected individuals, usually after several years of infection, it remains a challenge to elicit them by vaccination because broad and potent HIV-1 neutralization often requires unusual antibody characteristics, such as long hypervariable loops, interaction with glycans, as well as a substantial level of somatic mutation. Strategies have thus shifted from active to passive immunization to both protect against infection and to target latent virus (*10–14*). We and others have begun to explore combinations of bnAbs that optimize potency and breadth of protection, thus reducing the likelihood of resistance and viral escape (*15–17*). Antibodies directed to the CD4bs, MPER, and variable region glycans are among the combinations that so far provide optimal neutralization (*18*). In addition, alternative combinations have also been investigated for the immunotherapy of AIDS, by directing T lymphocytes to activate latent viral gene expression and enhance lysis of virally-infected cells (*19*, *20*). Given that multiple antibodies may help to reduce the viral replication that sustains chronic HIV-1 infection, we report here the generation of multispecific antibodies designed to increasing the efficacy of HIV therapy.

Design of bispecific antibodies and evaluation of neutralization breadth

Although individual anti-HIV-1 bnAbs can neutralize naturally occurring viral isolates with high potency, the percentage of strains inhibited by these mAbs varies (*21*, *22*). In addition, resistant viruses can be found in the same patients from whom bnAbs were isolated, suggesting that immune pressure against a single epitope may not optimally protect or treat HIV-1 infection. We hypothesized that the breadth and potency of HIV-1 neutralization by a single antibody could be increased by combining the specificities against different epitopes into a single molecule. This strategy would be expected to not only improve efficacy, but also simplify both treatment regimens and the regulatory issues required for clinical development. To test this concept, we

initially incorporated prototype bnAbs to the CD4bs and MPER sites into a modified bispecific Ab. When two variable regions are linked in tandem, the distal site typically retains its ability to bind antigen while the proximal binding is markedly diminished. We therefore utilized an alternative configuration, termed CODV-Ig, which introduced linkers and inverted the order of the antibody binding site in light and heavy chains to alter the orientation of the variable regions, allowing each region to interact with their target (*23*). Several known bnAbs were evaluated, including VRC01, 10E8, PGT121, and PGT128 [reviewed in (*1*)] for their ability to neutralize a select panel of viruses with known resistance or sensitivity to these antibodies (fig. S1). Initially, we determined whether the position of the variable regions from VRC01 and 10E8 in the proximal or distal positions (Fig. 1A) could affect neutralization breadth and potency. Inclusion of both variable regions in either orientation in the bispecific antibody reduced the number of resistant strains compared to the parental antibodies alone (Fig. 1B). Better potency was observed when VRC01 was proximal and 10E8 distal, though neither bispecific antibody was as potent as a mixture of the two antibodies alone.

To explore whether other bnAbs could perform better in the bispecific format, we evaluated two different combinations, namely VRC01 plus PGT121, or VRC01 plus PGT128. For PGT121, expression was observed only with VRC01 in the distal position. When this antibody was compared to the parental antibodies alone, it provided marginally better neutralization (Fig. 2A). In contrast, VRC01 could be expressed with PGT128 in both positions, with greater breadth observed when VRC01 was distal (Fig. 2B). Together, these data indicated that improvements in breadth could be achieved with a bispecific format; however, the potency was not consistently improved compared to each Ab alone. We therefore sought an alternative format to improve the potency and breadth of neutralization.

Generation and comparison of broad and potent trispecific antibodies

To achieve our goal, we used a previously undescribed trispecific Ab format. Three specificities were combined by using knob-in-hole heterodimerization (*24*) to pair a single arm derived from a normal immunoglobulin (IgG) with a double-arm generated in the CODV-Ig. A panel of bnAbs was evaluated, including those directed against the CD4bs that included VRC01 and N6, as well as PGT121, PGDM1400 and 10E8 (fig. S1). A modified version of the latter, termed 10E8v4, was used because of its greater solubility (*25*). We first determined which bispecific arms showed the best potency, breadth and yield. This screening analysis revealed that combinations which contained PGDM1400, CD4bs, and 10E8v4 showed the highest level of production and greatest potency of neutralization (fig. S2).

We then evaluated different combinations of single arm and double arm specificities from PGDM1400, CD4bs, and 10E8v4 Abs for their expression levels and activity against a small panel of viruses (fig. S3), leading ultimately to the identification of trispecific antibodies VRC01/PGDM1400-10E8v4 and N6/PGDM1400-10E8v4 as lead candidates. When analyzed against a panel of 208 viruses (*18*) and compared to the parental antibodies alone, the highest neutralization potency and breadth was observed with N6/PGDM1400-10E8v4, with only 1 of the 208 viruses showing neutralization resistance and a median IC50 of less than 0.02 μg/ml (Fig. 3A). VRC01/PGDM1400-10E8v4 also displayed high potency and breadth, and only 4 resistant viruses were found. While some parental mAbs displayed either high breadth (e.g., 10E8, N6) or high potency (PGDM1400), none displayed a combination of breadth and potency as optimal as the trispecific Abs (Fig. 3B). For example, the most potent and broad parental mAb, N6, was around 5-fold less potent than the N6/PGDM1400-10E8v4 trispecific Ab and targeted only a single epitope, which could increase the chance of viral escape mutations. Importantly, as a single recombinant protein, the trispecific Abs demonstrated potency and breath superior to any single antibody yet defined (Fig. 3 and fig. S4). We also determined the binding affinity of each component of the trispecific Ab and compared each to its parental Fab. The equilibrium binding constant, K_d, of each binding site in the trispecific Ab, determined by surface plasmon resonance (SPR), was comparable to the affinity of the parental Fab, with PGDM1400 showing a slight decrease (~3-fold), and VRC01 and 10E8v4 exhibiting approximately a log increase in affinity (fig. S5). In addition, the trispecific Ab was able to bind sequentially to each of the three antigens (Fig. 3C), indicating that there is independent binding of each epitope.

The N6 trispecific Ab also showed greater potency and breadth compared to three related bispecific Abs when tested against a panel of 20 viruses that were selected for resistance to bnAbs (table S1). This finding is consistent with previous studies comparing the efficacy of mixtures of two vs. three bnAbs (*18*) and provides additional support for the multi-targeting concept. In addition to their greater efficacy, the trispecific Abs also yielded higher protein levels and greater solubility than the bispecific model (see fig. S2A vs. fig. S3), facilitating large scale production and clinical translation.

Fc modification to extend half-life and crystal structure

To identify the optimal candidate for further development, we determined the half-life of the trispecific Abs in NHP. We previously showed that, in context of the VRC01 mAb, mutations that increased binding to the neonatal Fc

receptor (FcRn), which recycles IgG in intestinal epithelial cells and increases levels in the serum, extended half-life enhanced mucosal localization and conferred more efficient protection against lentivirus infection compared to wild type antibody (*26*). One such mutation was incorporated into the trispecific Abs as well as the parental VRC01 and N6 Abs. Abs were then infused into rhesus macaques and serum levels analyzed over a 14 day time frame. Ab VRC01 displayed a longer half-life over the more broad and potent N6, which was also directed to the CD4bs (Fig. 4, VRC01 vs. N6). Similarly, the trispecific Ab containing VRC01 showed greater persistence and a longer half-life (7.43 days, based on day 1-14 serum concentrations) than the N6 trispecific (4.79 days) in vivo (Fig. 4, VRC01/PGDM1400-10E8v4 and N6/PGDM1400-10E8v4). For this reason, and because the N6 trispecific Ab yielded less product with decreased solubility, we studied the VRC01/PGDM1400-10E8v4 trispecific Ab further.

Further characterization was performed by solving the crystal structure of the bispecific arm of the trispecific Ab, PGDM1400-10E8v4 CODV Fab, at 3.55Å resolution (Fig. 5, A and B). While the light chain was well resolved in the electron density (with the exception of the two most C-terminal residues), the heavy chain showed some regions of dynamic disorder. The most notable region consisted of part of PGDM1400 CDRH3 and the linker between PGDM1400 Fv and the heavy chain constant domain (residues 280-305). Similar to the anti-IL4/IL13 CODV Fab crystal structures (*23*), PGDM1400 and 10E8v4 Fvs opposed one another with the CDRs well exposed to the solvent. The distance between the CDRH3s of PGDM1400 and 10E8v4 is over 100Å. The PGDM1400 and 10E8v4 Fvs superposed very well with their respective parental Fv structures with RMSD (Cα) around 1 Å (fig. S6) (*25*, *27*), confirming that their antigen binding properties have been well preserved in the CODV format. Most importantly, the orientations of the CDRs in two Fv's were 180 degrees from each other, suggesting that each antibody combining site can independently engage it antigen without obstructing the other Fv structure. A model for the trispecific Ab was constructed by combining the PGDM1400-10E8v4 CODV Fab with VRC01 (*6*) and the intact b12 (*28*) IgG crystal structures (Fig. 5C). Similar to a natural IgG, the distance between the monovalent fragment of antigen binding (Fab) and CODV Fab is about 150Å. Two out of three antigens (gp120 core and gp41 MPER) were also included in the model, though we do not have direct evidence that all three HIV epitopes can be engaged simultaneously by a single trispecific Ab.

Enhanced cross-protection and decreased viral escape in vivo

The VRC01/PGDM1400-10E8v4 trispecific Ab was evaluated for its ability to protect against infection, using a mixture of two SHIVs that each differed in neutralization sensitivity to the parental bnAbs. In vitro assessment of the replication competent SHIV challenge stocks showed that SHIV BaL P4 was sensitive to VRC01 and the trispecific antibody, however was resistant to PGDM1400 (Fig. 6A). In contrast, SHIV 325C virus was sensitive to PGDM1400 and the trispecific Ab, yet resistant to VRC01 (Fig. 6A). In a neutralization assay with an equal mixture of SHIV BalP4 and SHIV 325c, we observed only the trispecific Ab could achieve complete neutralization of the viral mixture compared to either VRC01 or PGDM1400 (fig. S7). When naïve rhesus macaques were infused with the half-life extended VRC01, PGDM1400 or VRC01/PGDM1400-10E8v4 (5 mg/kg) respectively, serum concentrations were maintained at levels of ≥1 μg/ml for more than 14 days for all Abs (Fig. 6B). A decrease in serum levels at later time points for the trispecific Ab correlated with the development of monkey anti-human Abs but arose almost two weeks after the SHIV challenge.

To ensure an adequate challenge dose, naïve animals were first challenged with each virus independently. For SHIV 325c, 4 naïve rhesus macaques were inoculated one time intrarectally with 1 ml of undiluted viral stock. All four animals were infected and showed persistent viremia for up to 90 days (fig. S8). For SHIV BaLP4, the same stock and dose of virus were used as described in several of our prior publications (*26*, *29*, *30*). In total, 30 control animals were previously challenged with a single 1ml intrarectal inoculation of SHIV BaLP4 and all became infected.

To assess in vivo protection, NHP were challenged mucosally with a mixture of these differentially sensitive SHIVs, 5 days after Ab infusion in two separate experiments, with 4 animals in each group. In total, 6 of 8 macaques (75%) infused with VRC01 alone and 5 of 8 (62%) animals treated solely with PGDM1400 became infected. In contrast, none of the 8 animals in the trispecific-treated group were infected (Fig. 6C; p=0.0058 by two-tailed Fisher exact test). These data confirm that the improved breath and potency of the trispecific Ab conferred protection against viruses that otherwise show resistance to single bnAbs alone.

Discussion

Next generation HIV bnAbs

A hallmark of HIV infection is the remarkable genetic diversity of the virus. Since 2010, significant progress has been made in the identification of bnAbs that show exceptional breadth and potency [reviewed in (*1*)]. Several of these antibodies have progressed into clinical trials for prevention or treatment, and there is renewed interest in exploring their potential in the clinical management of HIV

infection (*5*, *12*, *14*). Here, we explored the potential of different bnAbs to combine into a single protein that confers protection against diverse HIV strains. Among the classes of bnAbs, we found that trispecific Abs derived from bnAbs with CD4bs, MPER, and V1V2 glycan specificities had broad specificity, were potent and could be produced in sufficient quantities to allow evaluation in NHP, and eventually in humans. When tested in NHPs with viruses resistant to individual parental bnAbs, the trispecific Ab demonstrated complete protection against both viruses whereas infection was established in most animals treated with individual parental antibodies VRC01 and PDGM1400. In addition, the ability of this trispecific Ab to target three independent epitopes may improve treatment efficacy in humans.

In HIV-1 infected patients, reductions in viral load have been observed after one infusion of a single bnAb, thus demonstrating biological activity of HIV bnAbs (*31*–*34*). A modest extension of viral rebound was also observed when individual bnAbs were infused after antiretroviral drugs were discontinued in previously suppressed HIV-infected subjects (*32*, *33*). NHP and human passive transfer studies have also suggested that such bnAbs can enhance anti-viral immunity that may contribute to improved viral control (*35*, *36*). In addition, NHP studies demonstrate the importance of mAb potency and prolonged antibody half-life in mediating protection against infection (*26*, *29*). The generation of trispecific Abs with improved potency and breadth may further enhance the efficacy of either passive immunity or passive-active immunization strategies.

Although bnAbs show exceptional breadth and potency, resistant viral strains have been detected in patients who make these Abs (*6*, *37*) and among natural viral isolates (*38*–*40*), raising the concern that resistance and escape mutations may arise. Such escape mutations are produced frequently with antiviral drug therapy (*41*), and countermeasures to reduce the likelihood of escape would increase the likelihood of developing a globally relevant therapy. Such breadth of coverage might alternatively be generated by administering multiple bnAbs, and protective efficacy in a NHP model has recently been demonstrated against a mixture of SHIV viruses using an antibody cocktail (*42*), providing further support for the multi-targeting concept. Combination mAb therapy increases the complexity, development pathway, cost, and regulatory burdens of their use for treatment or prevention, in contrast to a single biologic therapy. The potency of the trispecific Abs described here also exceeds that of a broad and potent recombinant form of CD4 (*43*), termed eCD4-Ig (fig. S4), and this latter molecule is also directed to a single, albeit highly conserved, HIV Env epitope. The availability of a single protein that targets multiple independent epitopes on virus also reduces the potential generation of escape mutations. This advantage in part could relate to the presence of three independent binding specificities at all times in contrast to mixtures of antibodies where selective pressure by individual mAbs with shorter half-lives may wane.

Clinical translation

The trispecific Abs have not yet been evaluated for safety and efficacy in humans. While initial characterization of their half-life in NHPs suggests that they behave similarly to conventional antibodies, the question remains as to whether they could be immunogenic in vivo. The administration of a bispecific antibody to the human cytokines IL-4 and IL 13, which uses a related format and linkers (*44*), may provide guidance in this regard. This bispecific antibody has been evaluated in humans where single subcutaneous doses of SAR156597, ranging from 10-300 mg/kg, were well tolerated in healthy subjects, with low titers of ADA in only 4 of 36 subjects (*44*). Importantly, it showed a mean half-life of about two weeks (*44*), similar to natural monoclonal antibodies. While further human trials are needed to assess the full potential of the trispecific Ab platform, the data from the NHP challenge study described here, as well as the previous experience in humans with bispecific Abs (*44*), suggests that the approach merits further clinical investigation. Studies in HIV-infected subjects, alone or in combination with other immune interventions, will address the potential of trispecific Abs to provide durable protective immunity against infection or sustained viral control in HIV infected subjects during drug holidays or in the absence of antiretroviral therapy. The recognition of independent target sites with multi-specific antibodies can also be applied to other infectious diseases, cancer, and autoimmunity. These antibodies can promote recognition and binding to critical antigenic determinants on target cells and simultaneously allow engagement of immune cells that can stimulate relevant effector function without the complications and expense of delivering multiple recombinant proteins.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S8
Tables S1 and S2
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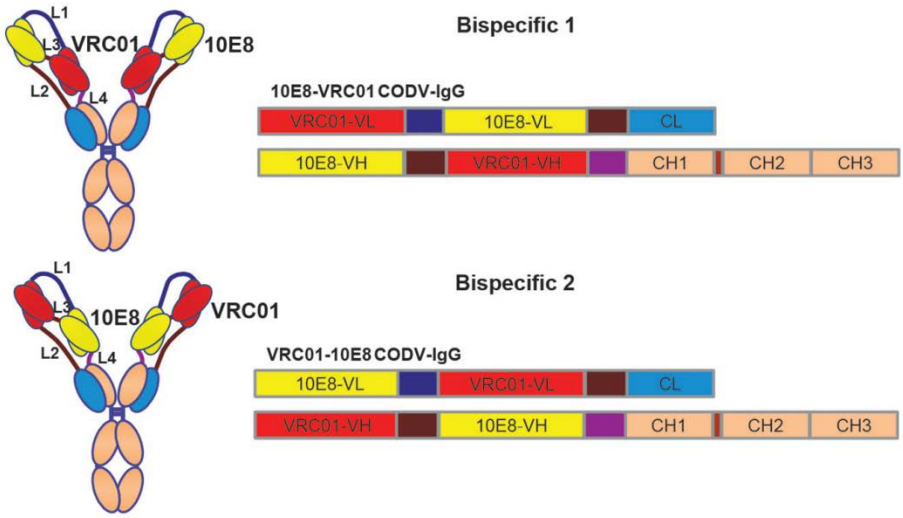
A



B

Virus	Clade	VRC01	10E8	VRC01 + 10E8	Bispecific 1	Bispecific 2
ZM106.9.SG3	C	0.192	>50	0.238	41.4	2.90
T266-60.SG3	AG	0.286	>50	0.279	4.01	1.17
H086.8.SG3	B	>50	0.010	0.014	0.016	1.97
T250-4.SG3	AG	>50	0.071	0.066	0.343	5.41
620345.c1.SG3	AE	>50	0.097	0.140	0.474	14.9
BL01.DG.SG3	B	>50	0.365	0.184	0.578	5.42
DU422.01.SG3	C	>50	0.139	0.188	1.58	5.35
6545.v4.c1.SG3	AC	>50	1.10	1.66	4.05	14.5
6540.v4.c1.SG3	AC	>50	1.54	2.08	4.25	38.0
6471.V1.C16.SG3	C	>50	6.96	4.60	29.9	>50
231965.c1.SG3	D	0.210	4.93	0.522	13.2	7.04
PVO.04.SG3	B	0.278	1.70	0.355	1.30	>50
CNE3.SG3	AE	0.336	0.358	0.325	1.10	18.3
CNE21.SG3	BC	0.363	0.552	0.159	1.015	2.94
0260.v5.c36.SG3	A	0.422	8.25	0.662	5.80	1.27
KER2008.12.SG3	A	0.572	16.8	0.788	4.01	6.28
ZM53.12.SG3	C	0.733	2.99	1.28	12.2	7.54
247-23.SG3	D	1.03	0.158	0.235	0.411	4.35
CH70.1.SG3	BC	2.23	1.81	1.59	0.441	12.8

Fig. 1. CODV-Ig bispecific antibody design and neutralization titers of the VRC01/10E8 bispecific antibodies. (**A**) CODV-Ig bispecific antibody design with two different orientations of 10E8 and VRC01. (**B**) Neutralization titers (IC_{50}) in μg/ml of VRC01/10E8 bispecific Abs and parental Abs against a select panel of 19 previously circulating HIV-1 strains highlighted in red, yellow and grey indicating highest, medium and lowest potency respectively.

Virus	Clade	A: VRC01	A: PGT121	A: VRC01 + PGT121	A: VRC01-PGT121	B: VRC01	B: PGT128	B: VRC01 + PGT128	B: PGT128-VRC01	B: VRC01-PGT128
0260.v5.c36.SG3	A	0.880	0.121	0.275	0.177	0.880	0.073	0.197	0.287	0.330
KER2008.12.SG3	A	0.727	1.98	0.748	0.339	0.727	>17.5	0.809	12.0	0.746
6540.v4.c1.SG3	AC	>17.5	>17.5	>35	>50	>17.5	0.402	0.893	21.1	>50
6545.v4.c1.SG3	AC	>17.5	>17.5	>35	>50	>17.5	>17.5	>35	>50	>50
620345.c1.SG3	AE	>17.5	>17.5	>35	>50	>17.5	>17.5	>35	>50	>50
CNE3.SG3	AE	1.60	>17.5	4.54	>50	1.60	>17.5	2.89	>50	>50
T250-4.SG3	AG	>17.5	0.0005	0.006	0.0003	>17.5	0.002	0.007	0.011	0.011
T266-60.SG3	AG	0.254	0.321	2.69	0.188	0.254	0.002	0.010	0.016	0.012
T278-50.SG3	AG	>17.5	>17.5	>35	>50	>17.5	0.014	0.065	0.078	0.099
BL01.DG.SG3	B	>17.5	>17.5	>35	>50	>17.5	>17.5	>35	>50	>50
H086.8.SG3	B	>17.5	>17.5	>35	>50	>17.5	>17.5	>35	>50	>50
PVO.04.SG3	B	0.454	0.129	0.236	0.156	0.454	0.004	0.011	0.044	0.029
CH070.1.SG3	BC	7.78	0.006	0.041	0.014	7.78	0.005	0.036	0.052	0.046
CNE21.SG3	BC	0.568	0.012	0.049	0.019	0.68	0.006	0.027	0.026	0.029
6471.V1.C16.SG3	C	>17.5	>17.5	>35	>50	>17.5	>17.5	>35	>50	>50
DU422.01.SG3	C	>17.5	0.069	0.224	0.129	>17.5	0.053	0.167	0.218	0.215
ZM106.9.SG3	C	0.247	0.008	0.031	0.015	0.247	0.008	0.032	0.040	0.037
ZM53.12.SG3	C	1.46	0.0002	0.002	0.0006	1.46	>17.5	3.07	>50	0.205
231965.c1.SG3	D	0.299	>17.5	1.18	5.30	0.299	>17.5	0.743	11.1	0.927
247-23.SG3	D	5.34	>17.5	8.66	>50	5.34	>17.5	6.63	>50	>50

Fig. 2. Neutralization titers of VRC01/PGT121 and VRC01/PGT128 based bispecific antibodies. Neutralization titers (IC_{50}) in μg/ml of the VRC01/PGT121 (**A**) and VRC01/PGT128 (**B**) bispecific Abs against a select panel of 20 circulating HIV-1 strains, with highlights as in Fig. 1.

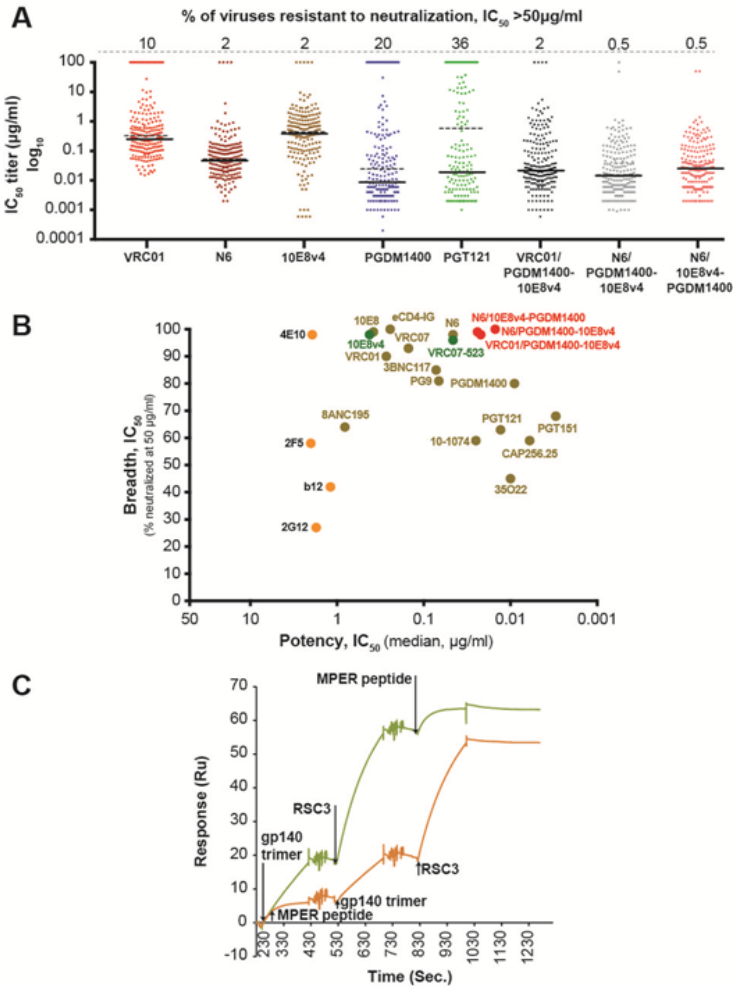


Fig. 3. Neutralization titers of trispecific antibodies and broadly neutralizing antibodies, and sequential binding of alternative Env epitopes. (**A**) The neutralization titers (IC_{50}) of different bnAbs and trispecific Abs against a genetically diverse panel of 208 circulating HIV-1 strains. The solid line denotes the median IC_{50} neutralization titer of sensitive viruses while the dotted line indicates median titers of all 208 viral strains. The percentage of resistant viruses are shown in the top line. (**B**) The breadth and potency of the trispecific Abs compared to other bnAbs. (**C**) Sequential binding of three antigens to the trispecific Ab, VRC01/PGDM1400-10E8v4 in the indicated order. The RSC3 (*45*) antigen represents monomeric gp120 optimized for the CD4 binding site ab VRC01. MPER peptide interacts with 10E8 (*7*), and gp140 trimer for PGDM1400 was derived from the gp140ΔN6 (BG505) protein.

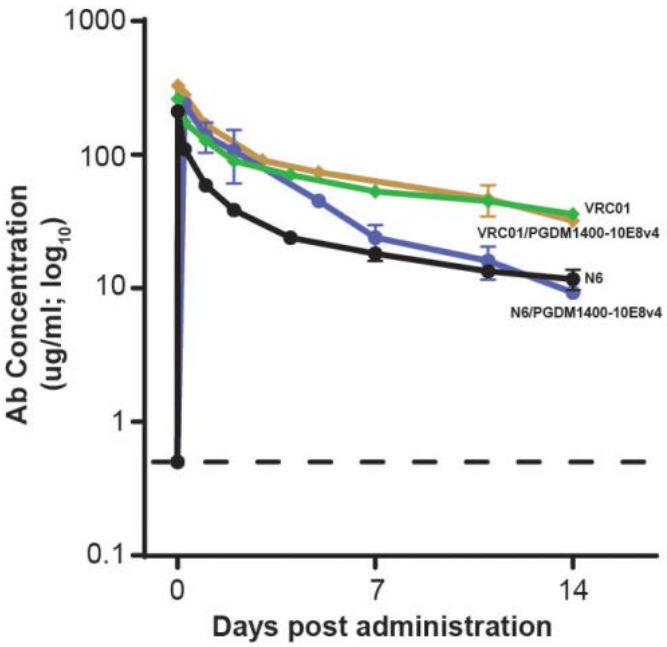


Fig. 4. Serum antibody levels in rhesus macaques infused with parental and trispecific antibodies. The concentration of VRC01, N6 and the two trispecific Abs containing a Fc mutation to extend half-life, were measured in serum over the course of 14 days after intravenous administration of a single 10 mg/kg dose of each antibody. Each data point represents the mean +/− SEM of the values from 2-6 animals per group (VRC01, n=6; N6, n=4; each trispecific Ab, n=2) and determined in replicates from two independent experiments.

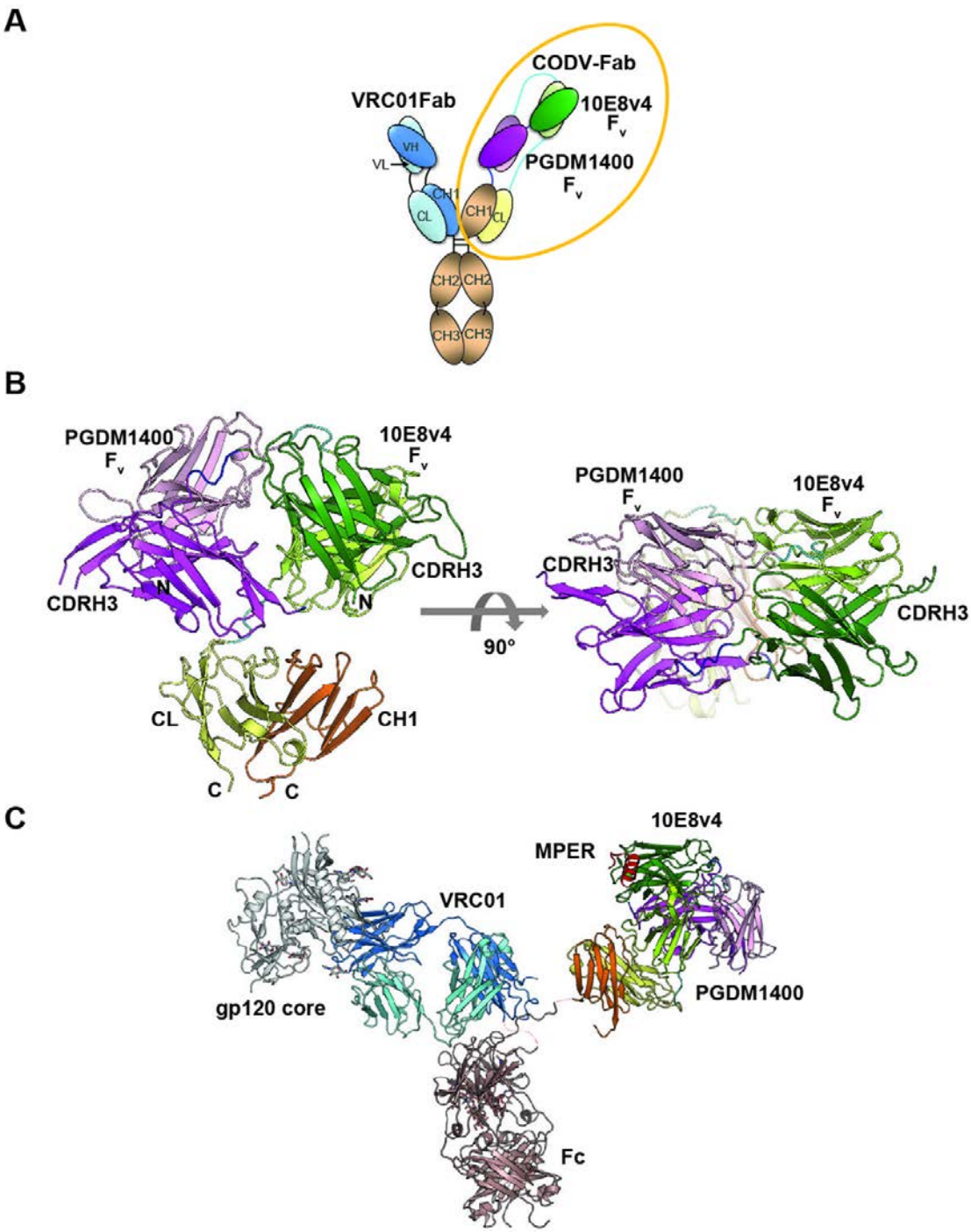


Fig. 5. Crystal structure of the CODV Fab and a structure model of the trispecific antibody. (**A**) Configuration of the trispecific antibody, color-coded by parental antibody. Dark shades (red or green) refer to heavy chain while pastels indicate light chain peptides. (**B**) Crystal structure of the PGDM1400-10E8v4 CODV Fab in side and top views. CDRH3s from the two Fvs are labeled to highlight the antigen binding region gp41 MPER was modeled in by superposing PDB 5IQ9 on to the 10E8v4 Fv. (**C**) VRC01/gp120 structure (PDB 4LST) and the CODV Fab were modeled onto the b12 structure (PDB 1HZH) by overlaying the CH1-CL domains. Color codes are matched in (A), (B), and (C).

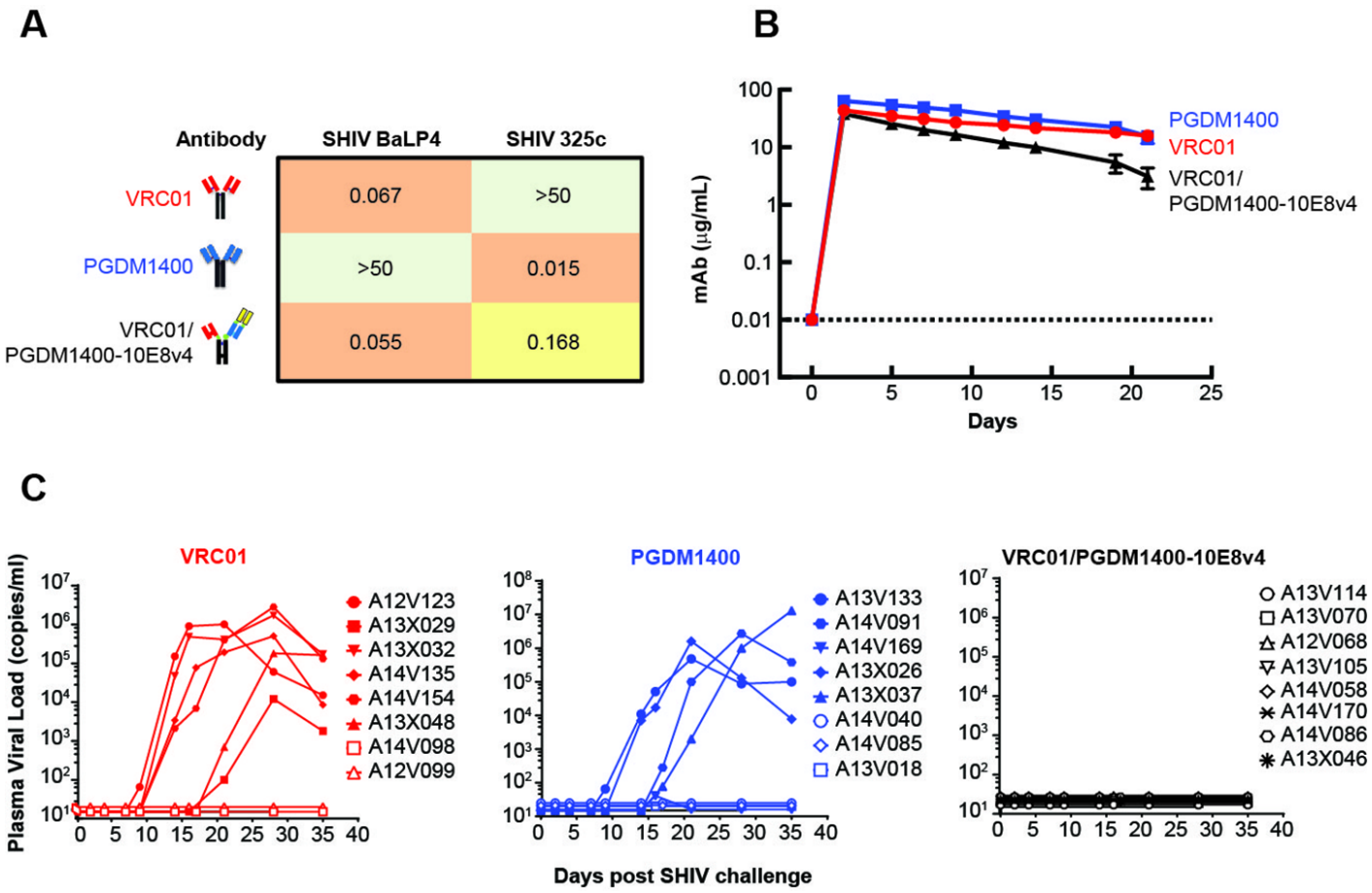


Fig. 6. Trispecific and broad neutralizing antibody sensitivity of SHIVs, plasma antibody levels and viremia in rhesus macaques. (**A**) The IC50 neutralizing titers (ug/ml) of VRC01, PGDM1400, and VRC01/10E8v4-PGDM1400 against replication competent SHIV BaLP4 or SHIV 325c. (**B**) Plasma levels of VRC01, PGDM1400 and VRC01/PGDM1400-10E8v4 in rhesus macaques (n=8 on each arm, done in two separate experiments with 4 animals each). All animals were administered 5 mg/kg of the indicated antibody intravenously. Each data point represents the mean +/− SEM of the values from all 8 animals per group. (**C**) Plasma viral loads in rhesus macaques (n=8 per group) challenged with a mixture of SHIV BaLP4 and SHIV 325c, 5 days after intravenous administration of either VRC01, PGDM1400 or VRC01/PGDM1400-10E8v4.

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Supplementary Materials for

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MATERIALS AND METHODS

Development of the trispecific antibodies. The format for the trispecific Ab was developed by integrating the previously described CODV-Ig bispecific antibody prototype (*23*) with a conventional antibody arm by heterodimerization using knob-in-hole (*24*) mutations in the CH3 domain of IgG1 Fc region. For CODV bispecific antibody design, two binding domains from any given antibodies can be linked together through various linkers in following order: light chain: 

, heavy chain: 

. More specifically, anti-HIV-1 neutralizing antibodies targeting CD4bs (VRC01, VRC07, N6), MPER (10E8), V1V2 glycan (PGT128, PGDM1400, VRC26.25), and V3 glycan (PGT121, 10-1074) were tested for possible combinations including each antibody position and linker type. The combinations with the best ability retaining the neutralizing breadth/potency and good manufacturability were used for trispecific antibody development. The following "Knob" (S354C/T366W) and "Hole" (Y349C /T366S/L368A/Y407V) mutations were engineered into CH3 domain of the monospecific or bispecific Fc region. Co-transfection of expression vectors expressing one bispecific light chain, one bispecific heavy chain, one conventional light chain, and one conventional heavy chain allows the heterodimerization and trispecific antibody formation. Homodimer of the monospecific antibody and homodimer of the bispecific antibody could also be produced, which were reduced to minimal level by using different ratios of the expression plasmids. Heavy and light chain mismatch can also happen, but generally at low level due to significant difference between monospecific and bispecific antibodies. Cationic exchange and size exclusion chromatography (SEC) protocols were developed to isolate the antibody of interest.

Construction of expression plasmids. Individual trispecific Abs were designed based on 5 parameters: 1) Selection of antibody binding sites; 2) Consideration of the position of each binding site; 3) Choice of linkers for the bispecific binding arm (i.e., heavy chain/light chain B in FIG. 1C); 4). "Knob" and "Hole" mutation integration into respective halves of the antibody; 5.) Choice of Fc isotype (IgG1 or IgG4). After assembly of the amino acid sequences for each trispecific molecule, four genes for each trispecific Ab were synthesized using human preferred codons (CambrY Applied Biosciences, Cambridge, MA, USA), and cloned into a eukaryotic expression vector.

Production and purification of trispecific antibodies. Trispecific antibodies were produced by transient transfection of 4 expression plasmids into Expi293 cells using ExpiFectamine™ 293 Transfection Kit (Thermo Fisher Scientific) according to manufacturer's protocol. Briefly, 25% (w/w) of each plasmid was diluted into Opti-MEM, mixed with pre-diluted ExpiFectamine reagent for 20-30 minutes at room temperature (RT), and added into Expi293 cells (2.5x106 cells/ml). An optimization of transfection to determine the best ratio of plasmids was often used to produce the trispecific antibody with good yield and purity. 4-5 days after transfection, the supernatant from transfected cells was collected and filtered through 0.45 μm filter unit (Nalgene). The trispecific antibody in the supernatant was purified using a 3-step procedure. First, protein A affinity purification was used, and the bound Ab was eluted using IgG elution buffer (Thermo Fisher Scientific). Second, the eluted product was dialyzed against PBS (pH7.4) overnight with 2 changes of PBS buffer. Any precipitate was cleared by filtration through 0.45 μm filter unit (Nalgene) before next step. Third, SEC purification (Hiload 16/600 Superdex 200pg, or Hiload 26/600 Superdex 200pg, GE Healthcare) was used to remove aggregates and different species in the preparation. The fractions were analyzed on reduced and non-reduced

SDS-PAGE to identify the fractions that contained the monomeric trispecific antibody before combining them. The purified antibody was then aliquoted and kept at -80°C for long term storage.

Validation of trispecific antibody binding activities using ELISA. The binding properties of the purified antibodies were analyzed either using ELISA or surface plasmon resonance (SPR) methods. For ELISA, corresponding antigens for each binding site in the trispecific antibody were used to coat a 96-well Immuno Plate (Thermo Fisher Scientific) overnight at 4°C using 2 µg/ml each antigen in PBS (pH7.4). The coated plate was blocked using 5% skim milk+2% BSA in PBS for one hour at RT, followed by washing with PBS+0.25% Tween 20 three times (Aqua Max 400, Molecular Devices). Serial dilution of antibodies (trispecific and control Abs) were prepared and added onto the ELISA plates (100 µl/well in duplicate), incubated at RT for one hour, followed by washing 5 times with PBS+0.25% Tween 20. After washing, the HRP conjugated secondary anti-human Fab (1:5000, Cat. No. 109-035-097, Jackson ImmunoResearch Inc) was added to each well and incubated at RT for 30 minutes. After washing 5 times with PBS+0.25% Tween 20, 100 µl of TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD, USA) was added to each well. The reaction was terminated by adding 50 µl 1M H_2SO_4, and OD450 was measured using SpectraMax M5 (Molecular Devices) and analyzed using SoftMax Pro6.3 software (Molecular Devices). The final data was transferred to GraphPad Prism software (GraphPad Software, CA, USA), and plotted as shown. EC50 was calculated using the same software.

Analysis of trispecific antibody binding using SPR. Three antigens were chosen for these studies. RSC3 (*45*) was used to evaluate binding of VRC01 and N6. Trimeric gp140ΔN6 (BG505) probed PGDM1400 interactions, and MPER peptide was used as a target for 10E8v4

binding (*7*). These antigens were used to measure the interaction of each determinant within the trispecific antibodies VRC01/PGDM1400-10E8v4 and N6/PGDM1400-10E8V4 and compared to the cognate parental Fab. Binding kinetics was determined using SPR on a BIACORE S200 (GE Healthcare). Abs were captured with anti-human IgG1 Fc specific antibody (Abcam. catalog no. ab1927) immobilized to CM5 sensor chip at pH 5 to ~11000 RU using standard amine coupling chemistry. An IgG1 isotope control (BioLegend; catalog no. 403102) was captured to the anti-IgG1 Fc specific antibody immobilized on the reference surface. The amount of antibody captured was controlled so that the maximal binding of each antigen was no more than 30 RU. All analyses were performed at a 30 µL/min flow rate with 1X PBS-P (GE Healthcare. catalog no. 28-9950-84) as running buffer. Increasing concentrations of each antigen were injected for 180 sec followed by 300 sec dissociation. For sequential binding of the three antigens to each trispecific Ab, saturating concentration (> 10 K_D) of each antigen (740 nM RSC3, 1000 nM 10E8 peptide, 2000 nM BG505) was injected for 8 min followed by 5 min dissociation. Surface regenerate was conducted by injecting 10 mM Glycine-HCl pH 2.5 for 60 sec at 30 µl/min. Data were fitted with 1:1 kinetic binding model and analyzed using Biacore S200 Evaluation Software v 1.0. Equilibrium dissociation constant (K_D) was calculated using association rate constant (k_{on}) and dissociation rate constant (k_{off}).

HIV-1 neutralization assays. Neutralization of replication-competent SHIV challenge stocks or single–round-of-entry Env-pseudoviruses (cross-clade) was evaluated in vitro by using Tzm-bl target cells and a luciferase reporter assay as described (1*9,46,47*). Briefly, HIV-1 Env pseudoviruses were generated by transfection in 293T cells of Env expression plasmids with full-length, Env-defective HIV genome SG3dEnv. To assess neutralization sensitivity of replication competent SHIVs, we used the same SHIV challenge stocks that were used for in vivo infection.

The SHIV challenge stocks were produced by transfection of 293T cells with infectious molecular clone plasmids, followed by propagation in human (SHIV325c) or rhesus (SHIV BaLP4) PBMCs. SHIV stocks or HIV-1 pseudoviruses were incubated with the antibody for 30 min at 37°C before Tzm-bl cells were added. The protease inhibitor indinavir was added to assays with SHIV stocks to a final concentration of 1 μM to limit infection of target cells to a single round of viral replication. Luciferase expression was quantified 48 h after infection upon cell lysis and the addition of luciferin substrate (Promega). For the neutralization assays done with a mixture of two SHIVs, each individual SHIV contributed ~50% of total infectivity of target cells, as measured by luciferase activity.

CODV Fab crystallization. Recombinant CODV-Fab containing PGDM1400-10E8v4 Fvs was expressed in ExpiHEK293 cells, purified and buffer exchanged into 20mM HEPES pH 7.0, 150mM sodium chloride, 30mM arginine. The protein was concentrated to 21mg/ml and crystallized in 1.68M ammonium sulfate, 0.1M sodium cacodylate pH 6.1 at 4°C (previous crystals in a similar condition were used as a nucleation seed). These crystals were cryoprotected in 20% glycerol and mother liquor. X-ray diffraction data were collected at Advanced Photon Source LS-CAT 21-ID-D with the Eiger 9M detector and processed using XDS. Molecular replacement was performed using Phaser and 10E8v4 Fab (PDB: 5IQ9, variable domain and constant domain searched separately) and the PGDM1400 variable domain (PDB: 4RQQ). Model rebuilding was performed in Coot and refinement was completed using Phenix. Data collection and refinement statistics are listed (Table S2). The coordinates and structure factors were deposited in the PDB (code 5WHZ)**.**

NHP PK study and antibody half-life quantitation and dual SHIV Challenge. Twenty-eight male and female rhesus macaques (M. mulatta) of Indian genetic origin were housed and cared

for in accordance with Guide for Care and Use of Laboratory Animals Report number NIH 82-53 (Department of Health and Human Services, Bethesda, Maryland, USA, 1985) in a biosafety level 2 National Institute of Allergy and Infectious Diseases (NIAID) facility. All animal procedures and experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases (NIH). For PK studies, four Indian-origin Rhesus macaques were administered low-endotoxin antibody preparations (<1 EU/mg) intravenously at 10 mg of Ab/kg of body weight. Whole blood samples were collected prior to injection and at multiple time points until week 4 post-administration. Antibody concentrations in serum were measured by a quantitative ELISA using HIV-1 resurfaced core envelope (RSC3)-coated microtiter plates to capture the trispecific antibodies followed by detection using a HRP-conjugated anti-human IgG antibody. Pharmacokinetic parameters were calculated in WinNonlin Software using the non-compartment model.

For the dual challenge study, twenty four Indian-origin Rhesus macaques were administered low-endotoxin antibody preparations (<1 EU/mg) intravenously at 5 mg of Ab/kg of body weight. Five days later, the animals were intra rectally challenged with a mixture of two SHIVs – SHIVBaLP4 and SHIV325c. SHIVs were grown in rhesus macaque peripheral blood mononuclear cells (PBMC). Blood samples were obtained before and after challenge at periodic intervals to quantitate antibody levels and plasma viremia. Antibody levels were measured using quantitative ELISA based methods in which either a resurface core gp120 (RSC3) (*45*) (for VRC01 and VRC01\PGDM-10E8v4 with Fc mutations) or a chimeric CNE58-strandC-CAP256.SU SOSIP trimer (for PGDM1400 with Fc mutations) coated microtiter plates were used to capture the administered antibodies followed by detection using a HRP-conjugated anti-

human IgG antibody. Plasma viremia was quantitated using a PCR-based method to quantify SIV gag RNA levels with a detection limit of 15 copies/ml as described previously (*48*).

SUPPLEMENTARY FIGURES

A

Name	Epitope	Breadth (<50 µg/ml)	Potency (IC_{50}) (µg/ml)
10E8	MPER	98%	0.437
VRC01	CD4BS	90%	0.329
PGT121	N332 glycan–V3 loop	63%	0.579
PGDM1400	glycan–V1V2 loop	80%	0.025

B

Virus	Clade	Neutralization				
		VRC01	N6	PGT121	PGDM1400	10E8v4
KER2008.12	A	S	S	S	S	R
620345.c1	AE	R	S	R	S	S
DJ263.8	AG	S	S	S	S	S
T266-60	AG	S	S	S	S	R
T278-50	AG	R	R	R	S	S
BL01.DG	B	R	R	R	R	S
BR07.DG	B	S	S	S	R	S
CNE57	B	S	S	S	R	S
H086.8	B	R	S	R	S	S
QH0692.42	B	S	S	S	R	S
SS1196.01	B	S	S	S	S	S
CNE21	BC	S	S	S	S	S
6471.V1.C16	C	R	R	R	R	S
CAP210.E8	C	R	S	R	S	S
DU156.12	C	S	S	S	S	S
DU422.01	C	R	S	S	R	S
TV1.29	C	R	R	S	S	S
ZM106.9	C	S	S	S	S	R
3817.v2.c59	CD	R	S	R	R	S
X2088.c9	G	R	S	S	R	R

Fig. S1. Neutralization activity of bnAbs used to develop multi-specific Abs. (A) Parental bnAbs were tested against a panel of 208viral strains from diverse clades. **(B)** a panel of 20 selected viral isolates used to test neutralization of bispecific Abs. Viruses resistant (R) or sensitive (S) to the indicated bnAb are highlighted in blue and red respectively.

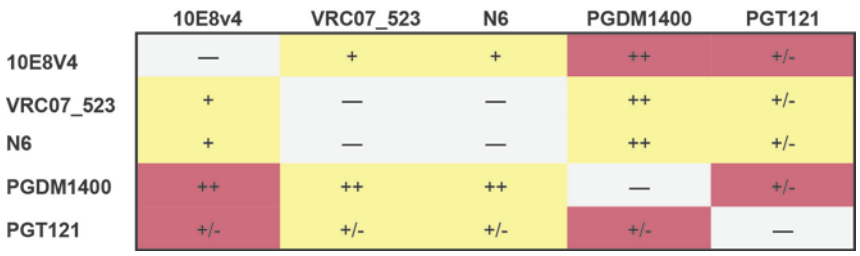


Fig. S2. Design of expanded set of bispecific Abs and virus screening panel. Combinations of mAb for bispecific Ab generation with high (++), medium (+), low (+/-) expression yield/solubility and relative good (red) or average (yellow) neutralization potency. – indicates that the Ab was not developed.

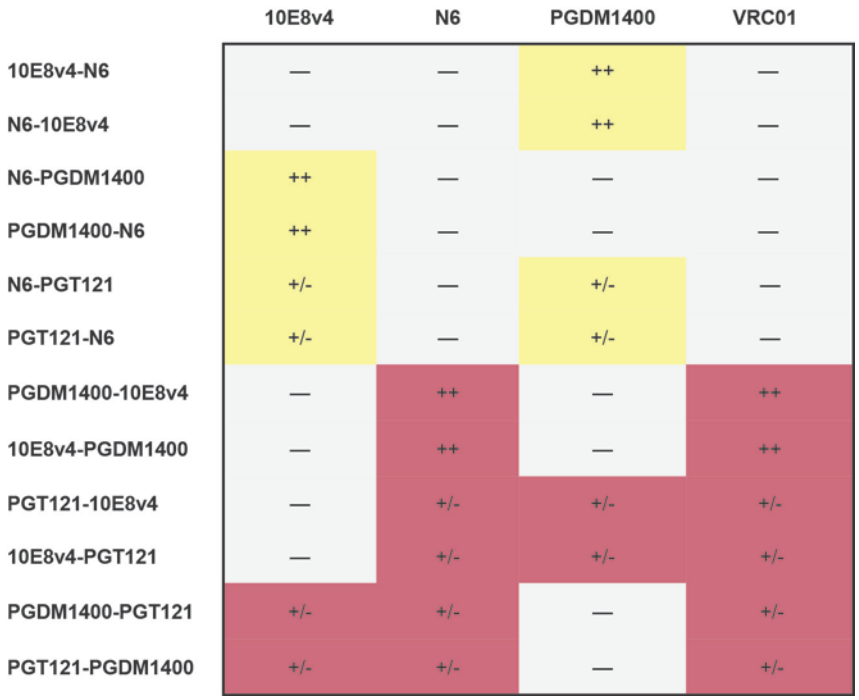
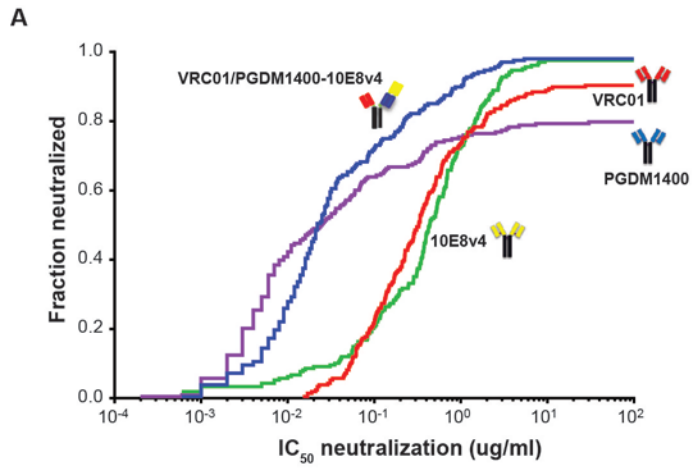


Fig. S3. Design of combinations of antibody binding sites into the trispecific format. The indicated combination of trispecific antibodies were produced with the antibody fragment making up the single arm of the trispecific shown horizontally along top and the double arms shown vertically along left. Solubility/yield of each trispecific is presented in ++ (highest), + (medium) or +/- (low). Relative potency against the virus panel in Fig. S1 is indicated as highest (red), medium/low (yellow). ─ indicates that the Ab was not developed.



B

	VRC01	N6	eCD4	10E8v4	PGDM 1400	PGT121	VRC01 10E8v4 PGDM 1400	N6 10E8v4 PGDM 1400	PGDM 1400 10E8v4 PGT121	N6 10E8v4 PGDM 1400	PGT121 PGDM 1400 N6	PGT121 PGDM 1400
%IC50 <50ug/ml	90	98	100	98	80	63	98	99	95	99	99	99
%IC50 <1.0ug/ml	74	96	85	73	75	52	90	99	87	98	85	98
Median IC50 (sensitive)	0.271	0.046	0.244	0.422	0.009	0.013	0.022	0.015	0.013	0.024	0.041	0.018
Geometric mean	0.280	0.046	0.129	0.309	0.017	0.024	0.034	0.021	0.025	0.028	0.075	0.020
Median IC50 (all)	0.329	0.046	0.244	0.437	0.025	0.579	0.022	0.015	0.015	0.026	0.041	0.018
Geometric mean	0.462	0.053	0.164	0.349	0.086	0.403	0.039	0.021	0.036	0.030	0.080	0.022

Fig. S4. Breadth and potency of trispecific Abs compared to parental bnAbs and eCD4-Ig against a representative panel of 208 HIV-1 strains. (A) The breadth and potency of the trispecific Ab VRC01/PGDM1400-10E8v4 compared to parental Abs. **(B)** Summary of neutralization results for the indicated monoclonal and trispecific antibodies presented as the percentage of viruses neutralized at IC_{50} of <50 µg/ml and IC_{50} of <1 µg/ml, the median IC_{50} and geometric mean for each Ab against all viruses, and the median IC_{50} and geometric mean for each Ab against viruses sensitive at IC_{50}<50 µg/ml.

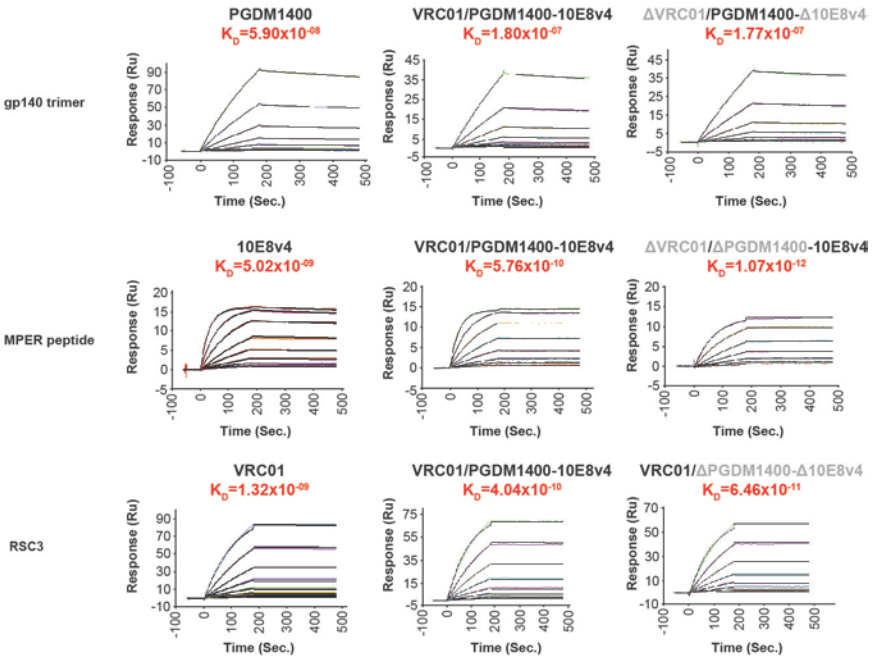


Fig. S5. Binding affinity of individual components in the trispecific Ab in comparison to parental bnAbs. Binding constants were determined by SPR as described in Materials and Methods. Dose dependent binding to the indicated antigens was performed and K_D for each Fab from the parental mAb, the trispecific Ab or its double-KO mutant was calculated using association rate constant (k_{on}) and dissociation rate constant (k_{off}).

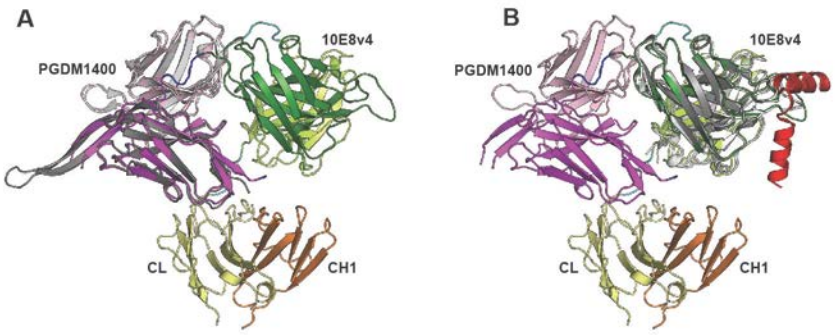


Fig. S6. Superposition of the PGDM1400-10E8v4 CODV Fab with parental antibody crystal structures. PGDM1400-10E8v4 CODV Fab structure is colored the same as in Figure 6. **(A)** PGDM1400 Fv structure from PDB 4RQQ (colored in grey) is overlaid on the same Fv in the CODV Fab. **(B)** 10E8 Fv structure from PDB 4G6F and 10E8v4 Fv structure from PDB 5IQ9 (colored in two different shades of grey) are superposed on the same Fv in the CODV Fab. The gp41 MPER peptide from the two PDBs are colored in two shades of red.

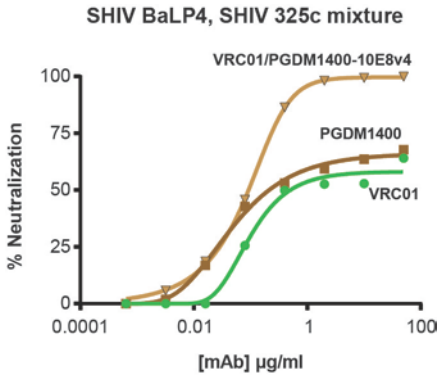


Fig. S7. Neutralization activity of VRC01, PGDM1400 and trispecific Ab against a dual SHIV mix. The neutralizing activity of VRC01, PGDM1400, and VRC01/PGDM1400-10E8v4 against an equal mixture of replication competent PBMC-derived SHIVs BaLP4 and SHIV 325c. As expected, individual parental mAbs could block only about 50% of the infection *in vitro*, while the trispecfic conferred complete neutralization.

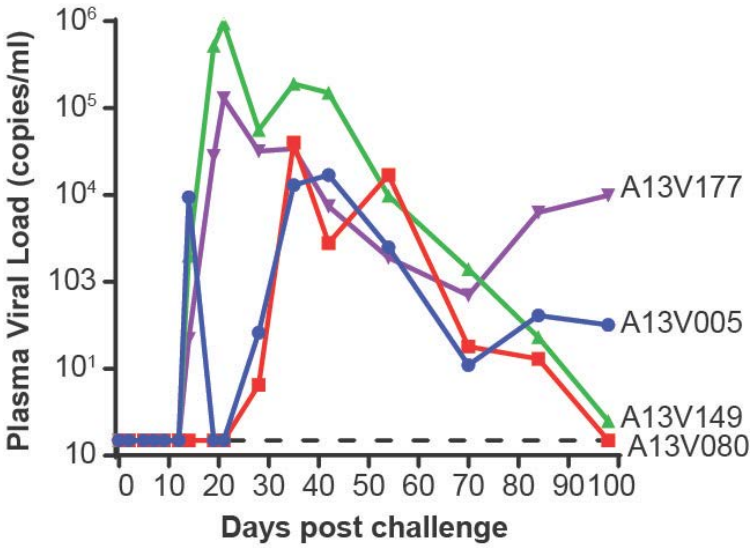


Fig. S8. SHIV 325c NHP infectivity study. Plasma viral loads in four naïve rhesus macaques challenged via intra rectal route with a single dose of SHIV 325c as described in Materials and Methods. The dashed line indicated the limit of detection for the assay (15 copies/ml).

SUPPLEMENTARY TABLES

		Trispecific Ab	Bispecific Abs		
Virus	Clade	N6/PGDM1400-10E8v4	10E8v4-PGDM1400	N6-PGDM1400	10E8v4-N6
KER2008.12	A	0.009	0.002	0.095	52.6
620345.c1	AE	1.11	1.1	>63	8.7
DJ263.8	AG	0.011	0.033	3.2	0.014
T266-60	AG	0.822	62.2	>63	4.89
T278-50	AG	3.94	0.291	42.3	14.2
BL01.DG	B	>50	19.4	>63	16.3
BR07.DG	B	1.91	9.87	>63	3.71
CNE57	B	0.744	3.94	>63	1.64
H086.8	B	0.113	0.693	>63	3.65
QH0692.42	B	3.96	>63	>63	14
SS1196.01	B	0.153	0.989	>63	0.605
CNE21	BC	0.011	0.002	0.169	5.9
6471.V1.C16	C	>50	>63	>63	>63
CAP210.E8	C	0.148	0.125	2.56	22
DU156.12	C	0.019	0.011	0.268	0.682
DU422.01	C	0.526	2.91	>63	7.77
TV1.29	C	0.022	0.002	0.153	4.14
ZM106.9	C	0.062	0.057	4.05	9.2
3817.v2.c59	CD	0.282	21.5	>63	37.8
X2088.c9	G	0.882	>63	>63	>63
% Neutralized (IC80 <1ug/ml)		70	50	20	15
Median IC80		0.218	0.693	1.414	6.835
Geometric Mean		0.197	0.344	0.990	4.720

Table S1. Neutralizing breadth and potency of bispecific and trispecific antibodies against a panel of 20 viruses selected for resistance to bnAbs. Values shown are neutralization IC80 concentrations (μg/ml). The percent of viruses neutralized is also shown along bottom, along with the median geometric mean IC80 for each A.

Table S2. Data collection and refinement statistics.

	4018 CODV-Fab
Wavelength (Å)	1.07812
Resolution range	54.83 - 3.55 (3.68 - 3.55)[1]
Space group	$P6_522$
Unit cell a, b, c (Å)/ α, β, γ (º)	151.226 151.226 200.595/90 90 120
Total reflections	327,933 (44,021)
Unique reflections	17,034 (2423)
Multiplicity	19.3 (18.2)
Completeness (%)	99.85 (99.88)
Mean I/sigma(I)	14.7 (1.0)
Wilson B-factor (Å2)	173
[2]R_{merge}	0.15 (3.24)
R_{pim}	0.04 (0.77)
Reflections used in refinement	16,982 (1660)
Reflections used for R-free	838 (98)
[3]R_{work}	0.239 (0.360)
[4]R_{free}	0.288 (0.408)
[5]$CC_{1/2}$	0.999 (0.510)
Number of non-hydrogen atoms	5169
Macromolecules	5169
Protein residues	677
RMS(bonds)	0.003
RMS(angles)	0.60
Ramachandran favored (%)	85.61
Ramachandran allowed (%)	12.89
Ramachandran outliers (%)	1.50
Rotamer outliers (%)	0.00
Clashscore	8.42
Average B-factor macromolecules	227
Number of TLS groups	6

[1]The numbers in parentheses refer to the highest resolution shell.
[2]$R_{merge} = \sum|Ij - < I > |/\sum Ij$, where Ij is the intensity of an individual reflection, and $< I >$ is the average intensity of that reflection
[3]$R_{work} = \sum||Fo| - |Fc||/\sum|Fc|$, where Fo denotes the observed structure factor amplitude,and Fc the structure factor amplitude calculated from the model.
[4]R_{free} is as for Rwork but calculated with 5% of randomly chosen reflections omitted from the refinement
[5]$CC_{1/2}$ is the correlation coefficient of the mean intensities between two random half-sets of data

Statistics for the highest-resolution shell are shown in parentheses.

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