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Evaluation of CD4-CD4i Antibody Architectures Yields Potent, Broadly Cross-Reactive Anti-Human Immunodeficiency Virus Reagents▽†

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The envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) has several adaptations that allow the virus to evade antibody neutralization. Nevertheless, a few broadly cross-reactive neutralizing antibodies as well as reagents containing portions of CD4, the HIV receptor, have demonstrated partial efficacy in suppressing viral replication. One type of reagent designed for improved HIV neutralization fuses the CD4 D1-D2 domains to the variable regions of an antibody recognizing the CD4-induced (CD4i) coreceptor binding site on the gp120 portion of the HIV envelope spike. We designed, expressed, purified, and tested the neutralization potencies of CD4-CD4i antibody reagents with different architectures, antibody combining sites, and linkers. We found that fusing CD4 to the heavy chain of the CD4i antibody E51 yields a bivalent reagent including an antibody Fc region that expresses well, is expected to have a long serum half-life, and has comparable or greater neutralization activity than well-known broadly neutralizing anti-HIV antibodies. A CD4 fusion with the anti-HIV carbohydrate antibody 2G12 also results in a potent neutralizing reagent with more broadly neutralizing activity than 2G12 alone.

The envelope spike of human immunodeficiency virus type 1 (HIV-1), a trimer of gp120/gp41 heterodimers, utilizes a number of strategies to avoid antibodies (Abs) elicited by the humoral immune response. These include variable loops, heavy glycosylation (36), conformational masking of key functional sites (19), and an architecture and surface density that reduce bivalent Ab engagement (18). Nevertheless, a small number of broadly cross-reactive neutralizing Abs have been found and extensively characterized (5, 32, 41). The targets of these Abs include the membrane proximal region of gp41 (24, 42), a cluster of high-mannose carbohydrates on gp120 (29), and the HIV receptor (CD4)-binding site (3, 28). A combination of several of these Abs has been evaluated in clinical trials as a passive immunotherapy to reduce viral rebound during an interruption of antiretroviral therapy (34).

Several CD4-containing proteins have also been explored clinically as possible therapeutics for treating HIV-1: soluble CD4 (13, 16), a CD4-Fc fusion protein (7), and the tetravalent CD4-immunoglobulin G2 (CD4-IgG2; PRO 542) reagent (1, 17). In patients with advanced disease, CD4-IgG2 treatment led to a ~0.5 $\log_{10}$ mean reduction in viral load (17). In addition, D1D2-Igαtp, an approximately dodecameric CD4 reagent created as a chimeric IgG1/IgA fusion protein (2), exhibited very potent HIV neutralization activity and targeted HIV-infected cells for lysis by natural killer cells (14).

Another approach to targeting gp120 is a fusion protein composed of CD4 linked to the variable regions of a CD4-induced (CD4i) Ab (11). CD4i Abs represent a potentially promising class of Abs because they bind to the conserved HIV-1 coreceptor binding site on gp120, which is exposed after a conformational change resulting from binding to CD4 (25, 27, 38). Examples of CD4i Abs include 17b (33), E51 (39), m9 (40), 412d (8), and 21c (38). These Abs are often broadly cross-reactive but generally show little neutralization potency in vivo due to limited steric accessibility when gp120 on the viral membrane is bound to CD4 on the surface of the target cell (20). Fusing CD4 to the combining site of a CD4i Ab solves the accessibility problem since the Ab epitope would be exposed by CD4 binding when the virion is not bound to the target cell. This class of reagent has two other favorable features: bivalent binding and targeting of functionally critical epitopes on gp120, the CD4 and coreceptor binding sites. One such reagent, sCD4-17b (referred to here as CD4-scFv$_{17b}$), contains the first two domains of CD4 linked to the single-chain fragment variable (scFv) form of the CD4i Ab 17b (Fig. 1) (11). This reagent was shown to potently neutralize multiple primary strains of HIV-1 (11), suggesting that CD4-CD4i Ab fusion proteins are promising candidates for passive immunization or gene therapy trials.

Critical properties for CD4-containing reagents include their breadth of neutralization activity, half-life, and, for reagents used in a gene therapy context, their expression level. We have undertaken a systematic effort to develop the optimal architecture for a CD4-CD4i Ab reagent by designing, constructing, and testing reagents with different CD4i Ab combining sites and including an Ab Fc region to increase valency and serum half-life (7). We varied the arrangements of the Ab

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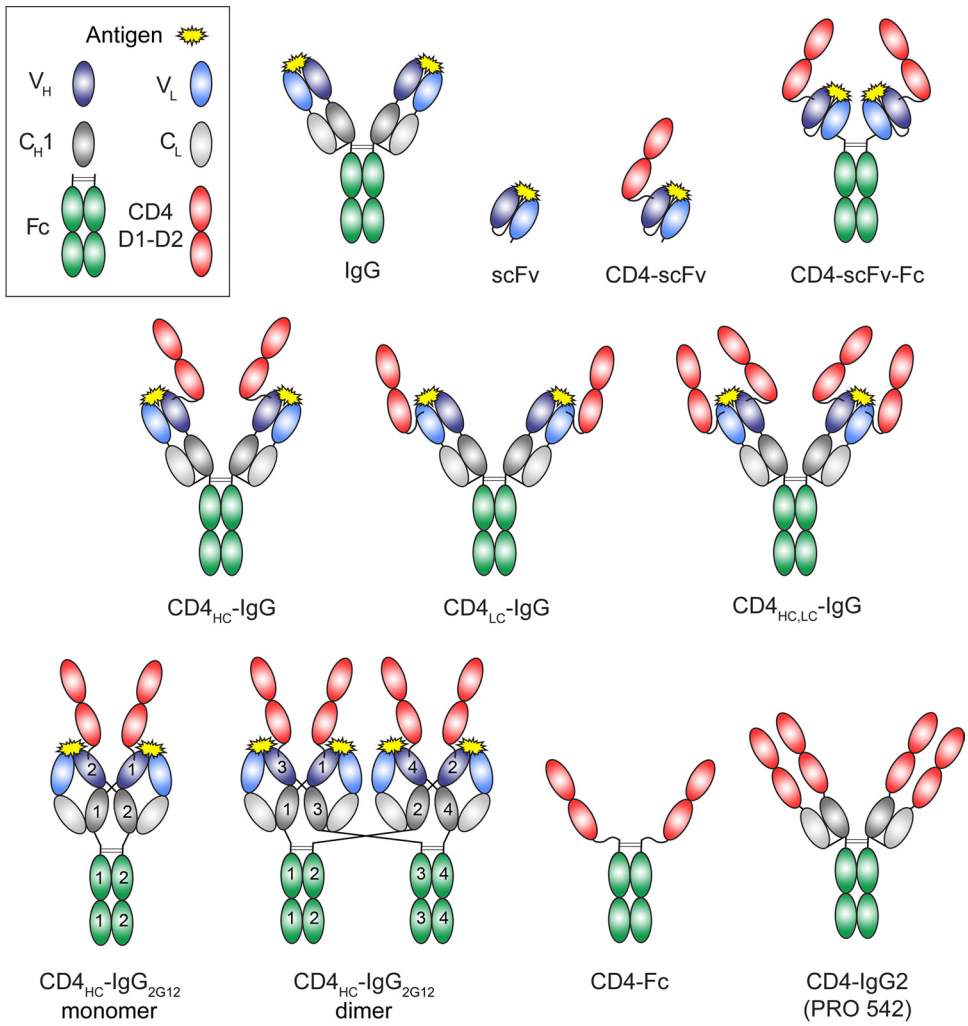

FIG. 1. Schematic depiction of CD4-CD4i reagents and related molecules. V_H, variable domain of the IgG heavy chain (HC); V_L, variable domain of the IgG light chain (LC), C_H1, constant region 1 of the HC; C_L, constant region of the LC; Fc, C_H2 and C_H3 domains of dimerized HCs; CD4 D1-D2, N-terminal two domains of CD4; scFv, single-chain fragment variable (V_H and V_L domains of an IgG); $CD4_{HC}$, CD4 linked to the V_H domain of an IgG; $CD4_{LC}$, CD4 linked to the V_L domain of an IgG; $CD4_{HC,LC}$, CD4 linked to the V_H and V_L domains of an IgG.

combining sites; the lengths, attachments, and forms of the linking regions; and the ways in which CD4 was fused to the CD4i Ab (Fig. 1). CD4-CD4i Ab reagents were evaluated using in vitro neutralization assays across a broad range of clade A, B, and C HIV-1 strains. One promising reagent, a fusion of CD4 domains 1 and 2 (D1-D2) to the heavy chain of the E51 CD4i Ab, was expressed at high levels in mammalian cells and exhibited neutralization potencies that compared favorably with or exceeded those of known broadly neutralizing Abs such as 4E10, b12, 2G12, and 2F5.

Since much of the activity of our CD4-CD4i reagents resulted from the CD4 component, we also explored the effects of attaching CD4 to an Ab with a different quaternary structure. The anticarbohydrate Ab 2G12 is unusual in that its heavy chains are involved in a domain swap creating a rigid $(Fab)_2$ unit in which the combining sites are separated by ~35Å (6). This domain swapping tendency also leads to the formation of 2G12 dimers containing two $(Fab)_2$ units and two Fc regions, which form when the domain swapping occurs intermolecularly between two IgGs rather than intramolecularly between the two Fab arms of a single IgG (37). The 2G12 dimer is 50- to 80-fold more potent than monomeric 2G12 in neutralizing clade B 2G12-sensitive strains; however, neither form of 2G12 neutralizes clade C strains of HIV-1 (37). In order to assess the effects of adding CD4 to the 2G12 monomer and dimer architectures and to explore whether addition of CD4 would broaden the range of HIV-1 strains that are sensitive to 2G12, we constructed CD4-2G12 fusion proteins and tested their neutralization potencies (Fig. 1). We found that these hybrid reagents had potent neutralizing activities with both the CD4 and Ab combining site components apparently contributing to this behavior.

MATERIALS AND METHODS

Isolation of genes encoding the 21c Ab. 21c IgG was purified from an Epstein-Barr virus-transformed human B-cell line obtained from James Robinson (Tulane University). N-terminal amino acid sequencing was performed after treatment with *Pfu* pyroglutamate aminopeptidase to remove blocking pyroglutamate,

TABLE 1. IC_{50}s for CD4-scFv reagents

Env	Clade	IC_{50} (nM)[a]					
		CD4-scFv$_{17b}$	CD4-scFv$_{m9}$	CD4-scFv$_{21c}$	CD4-scFv$_{E51}$	CD4	scFv$_{E51}$
SF162.LS	B	3.5	1.7	1.40	0.75	16	15
SC422661.8	B	91	ND	1200	24	480	61
TRJO4551.58	B	1000	720	1500	500	940*	320
QH0692.42	B	59	ND	ND	31	110	450
RHPA4259.7	B	110	ND	370	48	170	76
ZM53M.PB12	C	200	150	880	60	390*	66
ZM214M.PL15	C	91	110	330	23	480	120
Du172.17	C	24	66	89	26	150	190
Geometric mean		70	66	220	29	210	110
Arithmetic mean		200	210	620	89	340	160

[a] Results are from in-house neutralization assays except for values marked with an asterisk, which are from CAVD core neutralization facility assays. ND, not determined.

and 5′ primers were designed based on these sequences. Heavy and light chain genes were obtained by cDNA amplification using a SMART RACE kit (Clontech). The 3′ primers were the human C_H1 (constant region 1 of the heavy chain) domain primer CAGCTCCACCCTCTTGTCCACCTTGGTGTTGCTGGG and the human λ constant domain primer CTAAGAACATTCTGCATGGGC CATTGTCTTCTCC. Bridge PCR was used to generate the full-length heavy chain.

Materials. Genes encoding the variable regions (variable heavy and variable light, V_H and V_L, or the intact light chain V_L-C_L, where C_L refers to the constant light domain) of the E51, m9, and 412d Abs were synthesized (BlueHeron Biotechnologies or Integrated DNA Technologies). Intact IgG genes were constructed by subcloning the relevant variable sequences onto a human IgG1 sequence. The 2G12 and anti-glycoprotein D (gD) IgG genes were obtained from Dennis Burton (The Scripps Research Institute). The CD4-scFv$_{17b}$ gene (11) was obtained from Ed Berger (Laboratory of Viral Diseases, NIAID, NIH). The CD4-IgG2 (PRO 542) protein (1) was obtained from Progenics.

Sequences for all of the constructs are in Fig. S1 in the supplemental material; schematic structures are shown in Fig. S1 and Table S1 in the supplemental material. Genes encoding scFv versions of Abs E51, m9, and 21c were synthesized as the V_H domain followed by a $(Gly_4Ser)_3$ linker, the V_L domain, and a C-terminal six-His tag. CD4-scFv genes were constructed to be similar to the CD4-scFv$_{17b}$ gene (11) by fusing the DNA encoding the CD4 hydrophobic leader sequence and first two domains (D1-D2; residues 1 to 182 of the mature CD4 protein) to a $(Gly_4Ser)_7$ linker sequence followed by the His-tagged scFv construct. The CD4-scFv$_{17b}$ gene was fused to the human IgG1 Fc domain sequence by bridge PCR to create CD4-scFv$_{17b}$-Fc.

CD4-IgG heavy chain constructs (denoted by the prefix $CD4_{HC}$-) were made by fusing the CD4-V_H region of the 21c and E51 CD4-scFv constructs to the remaining domains (C_H1, C_H2, and C_H3) of a human IgG1 heavy chain gene using bridge PCR. Analogous constructs for 412d and m9 were synthesized. Bridge PCR was used to construct CD4-Fc [CD4 D1-D2 domains linked with $(Gly_4Ser)_7$, denoted GS7 in the construct names, to the Fc region of IgG1]. $CD4_{HC}$-(GS7)-IgG_{2G12} and $CD4_{HC}$-(GS7)-$IgG_{anti\text{-}gD}$ were constructed by inserting sequences encoding the relevant V_H and C_H1 domains into the CD4-IgG scaffold. The light chain (denoted LC in the constructs) of $CD4_{LC}$-(GS7)-IgG_{E51} was constructed by bridge PCR.

A gene encoding a flexible linker [$(Gly_4Ser)_9$, denoted GS9] was synthesized with NheI and BamHI restriction enzyme sites. The linker region sequence was inserted between the CD4 and V_H sequences of the $CD4_{HC}$-(GS7)-IgG_{E51} construct by subcloning, replacing the $(Gly_4Ser)_7$ sequence with the new linker sequence.

Protein expression and purification. The CD4-scFv$_{17b}$ gene was subcloned into baculovirus expression vector pBacPAK8 (BD Biosciences), and CD4-scFv$_{17b}$ protein was purified from insect cell supernatants by Ni-nitrilotriacetic acid (NTA) chromatography. All other constructs were subcloned into the mammalian expression vector pTT5 (NRC Biotechnology Research Institute), and the corresponding proteins were expressed transiently in suspension HEK 293-6E cells (NRC Biotechnology Research Institute) using 25-kDa linear polyethylenimine (PEI) (Polysciences) as described previously (12). When the heterodimeric constructs were expressed, the heavy chain and light chain vectors were mixed at a 1:1 ratio by mass. Cell culture supernatants were collected 6 days posttransfection. For Fc-containing constructs, supernatants were passed over protein A resin (Thermo Fisher Scientific), eluted using pH 3.0 citrate buffer, and then immediately neutralized. The scFv and CD4-scFv reagents were purified using Ni-NTA chromatography and eluted using 300 mM imidazole. All reagents were then subjected to size exclusion chromatography in 20 mM Tris (pH 8.0)–150 mM NaCl using a Superdex 200 16/60 or 10/30 column (GE Healthcare). Final yields of purified reagents are given in Table S1 in the supplemental material.

In vitro neutralization assays. A previously described pseudovirus neutralization assay, which measures the reduction in luciferase reporter gene expression in the presence of a potential inhibitor following a single round of pseudovirus infection in TZM-bl cells (21, 23), was used to evaluate the neutralization potencies of the reagents. Pseudoviruses were generated by cotransfection of HEK 293T cells with an Env expression plasmid and a replication-defective backbone plasmid. Neutralization assays were performed either by our laboratory (see Tables 1, 2, 4, and 5) or by the Collaboration for AIDS Vaccine Discovery (CAVD) core neutralization facility (see Table 3) using the same protocol (21, 23). Briefly, each sample was tested in triplicate (our assays) or duplicate (CAVD assays), with 200 infectious viral units per well incubated with a threefold dilution series and with 75 μg/ml DEAE-dextran. For experiments testing inhibition of 2G12 binding by fructose, the 1-h incubation at 37°C of reagent plus pseudovirus was done in the presence of 3% (wt/vol) fructose. For all assays, after a 1-h incubation at 37°C, 10,000 TZM-bl cells were added to each well and incubated for 2 days. Cells were then lysed and assayed for luciferase expression using BriteLite plus (PerkinElmer) and a Victor3 luminometer (PerkinElmer) (our assays) or equivalent equipment (CAVD assays). Percentage neutralization was determined by calculating the difference in luminescence between test wells (cells plus virus plus reagent) and cell control wells (cells only), dividing this value by the difference between the virus control wells (cells plus virus) and cell control wells, subtracting from 1, and multiplying by 100.

Nonlinear regression analysis was used to calculate concentrations at which half-maximal inhibition was observed (IC_{50}s). Average IC_{50}s across multiple HIV-1 strains are reported as both arithmetic and geometric means. Calculation of geometric means is suitable for data sets covering multiple orders of magnitude (31), as is the case for neutralization data across multiple viral strains. IC_{50}s are reported in Tables 1 to 5 as molar concentrations to compensate for different molecular weights of the reagents. IC_{50}s in μg/ml are presented for the Table 3 data in Table S2 in the supplemental material.

Calculation of the IC_{50eff} of a bivalent reagent. We calculated the effective IC_{50} (IC_{50eff}) for a reagent containing independently acting components with individual IC_{50}s of IC_{50a} and IC_{50b}. The fraction of Env binding sites occupied by component *a* is $[c]/(IC_{50a} + [c])$, where $[c]$ is the concentration of the reagent. Likewise, the fraction of sites occupied by component *b* is $[c]/(IC_{50b} + [c])$. The fraction occupied by components *a* and/or *b* is calculated as follows: $[c]/(IC_{50a} + [c]) + [c]/(IC_{50b} + [c]) - [c]^2/((IC_{50a} + [c]) \times (IC_{50b} + [c]))$. Solving for $[c]$ when the fraction occupied by *a* and/or *b* equals 0.5 gives the following IC_{50eff}: $IC_{50eff} = 0.5 \times (-IC_{50a} - IC_{50b} + \sqrt{(IC_{50a}^2 + IC_{50b}^2 + 6 \times IC_{50a} \times IC_{50b})})$. From Table 1, the geometric mean IC_{50}s for CD4-scFv$_{E51}$, CD4, and scFv$_{E51}$ are 29, 210, and 110 nM, respectively. For IC_{50a} of 210 nM (CD4) and IC_{50b} of 110 nM (scFv$_{E51}$), we calculate that the IC_{50eff} is 61 nM. Hence, the average IC_{50} for CD4-scFv$_{E51}$, 29 nM, is about twofold better than the independent model would predict. Using the IC_{50} of scFv$_{E51}$ for the activity of the CD4i component of CD4-scFv$_{E51}$ might underestimate the synergy of the components since larger CD4i reagents are thought to be significantly less potent than smaller ones (20).

Nucleotide sequence accession numbers. The 21c sequences have been deposited in GenBank under accession numbers GU179344 and GU179345.

RESULTS

To determine which CD4i Ab combining sites formed effective CD4-scFv CD4i reagents, we expressed fusions of 17b, E51, and m9 and evaluated these using an in vitro neutralization assay (Table 1). In addition, we made a CD4-scFv reagent using the CD4i Ab 21c, which was reported to be cross-reactive and highly potent against HIV-2 (10), after isolating the heavy and light chain genes encoding the Ab. All CD4-scFv reagents consisted of the first two domains of CD4 (D1 and D2) fused to an scFv with a $(Gly_4Ser)_7$ linker sequence (Fig. 1). Of these, 17b, E51, and m9 gave reagents with comparable potencies. The geometric mean molar IC_{50} for CD4-$scFv_{E51}$ (29 nM) is significantly lower than that of CD4 alone (210 nM) or $scFv_{E51}$ alone (110 nM) (Table 1), implying that both components are contributing to the neutralization potency of this reagent. The more weakly neutralizing 21c reagent was less effective (IC_{50} of 220 nM) than the other CD4-scFv reagents (IC_{50}s of 29 to 70 nM). The lower efficacy of this reagent might result if the linker in the CD4-$scFv_{21c}$ construct did not permit simultaneous binding by the CD4 and 21c components, perhaps due to a difference in the relative orientation of 21c on gp120 compared to other CD4i antibodies.

Next, we evaluated the effects of including an Ab Fc domain, which would convert a CD4-CD4i reagent into a dimer and increase the serum half-life through binding of the Fc to the protection receptor FcRn (7). Our initial Fc construct consisted of the CD4-$scFv_{17b}$ sequence (11) fused to the N terminus of the human IgG1 Fc domain to make CD4-$scFv_{17b}$-Fc (Fig. 1). In addition, we tried to express a two-chain reagent in which CD4 was fused to the N terminus of the constant domain of the light chain (C_L), and the 17b scFv was fused to the N terminus of the Fc domain. This two-chain form gave only trace quantities of the desired protein product (data not shown), whereas the single chain form, CD4-$scFv_{17b}$-Fc, yielded a small but testable quantity for neutralization assays (see Table S1 in the supplemental material). These assays demonstrated that CD4-$scFv_{17b}$-Fc had a molar neutralization potency about twofold better than CD4-$scFv_{17b}$ (for strain QH-0692, the IC_{50}s were 30 nM versus 59 nM, and for strain SF162.LS, the IC_{50}s were 2.5 nM versus 3.5 nM), as would be expected if each half of the dimeric reagent functioned independently. Hence, no enhancement due to avidity was observed for this reagent.

Although the CD4-$scFv_{17b}$-Fc reagent maintained its antiviral activity and likely would have an increased serum half-life due to the Fc domain, it had a relatively poor expression yield (see Table S1 in the supplemental material), and its neutralization properties suggested no evidence for an avidity effect. Since we had observed that two-chain IgG reagents were often expressed well, we designed a CD4-CD4i reagent with a more IgG-like architecture by attaching CD4 to the N terminus of a complete Ab heavy chain, which would be expressed together with a conventional Ab light chain. The $CD4_{HC}$-(GS7)-IgG_{E51} construct was expressed well (~10 mg/liter), and we compared the neutralization potencies of this reagent with CD4-$scFv_{E51}$ (Table 2). $CD4_{HC}$-(GS7)-IgG_{E51} had a molar neutralization potency (mean IC_{50} of 3.1 nM) higher than what would be expected for monovalent binding by only one arm of the reagent at a time (expected IC_{50} of ~7.5 nM), suggesting that this geometry allowed an avidity enhancement resulting from simultaneous binding of both arms. Simultaneous binding might have been facilitated by the greater distance between the CD4 arms in this reagent compared to the CD4-$scFv_{17b}$-Fc construct.

TABLE 2. IC_{50}s for CD4-IgG_{E51} constructs compared to CD4-$scFv_{E51}$

Env	Clade	IC_{50} (nM)[a]			
		CD4-$scFv_{E51}$	$CD4_{HC}$-(GS7)-IgG_{E51}	$CD4_{LC}$-(GS7)-IgG_{E51}	$CD4_{HC,LC}$-(GS7)-IgG_{E51}
SF162.LS	B	0.75	0.47	1.3	1.7
SC422661.8	B	24	16	30	47
QH0692.42	B	31	2.1	ND	ND
RHPA4259.7	B	48	3.5	ND	ND
Du172.17	C	26	4.9	5.5	32
Geometric mean		15	3.1	6.0	14
Arithmetic mean		26	5.4	12	27

[a] ND, not determined.

To fully explore the potential of the IgG-like architecture, we made additional reagents in which CD4 was fused to the N terminus of the Ab light chain ($CD4_{LC}$-(GS7)-IgG_{E51}) or to both the heavy chain and the light chain [$CD4_{HC,LC}$-(GS7)-IgG_{E51}]. These reagents were expressed at yields of ~30% and ~10% of the heavy chain $CD4_{HC}$-(GS7)-IgG_{E51} form, and the average neutralization potencies of these reagents were modestly reduced compared to the original reagent (Table 2).

Using the $CD4_{HC}$-(GS7)-IgG_{E51} reagent as a benchmark, we next varied the linker between CD4 and the variable domain of the heavy chain (V_H). We constructed a longer flexible glycine-serine linker $(Gly_4Ser)_9$ to make $CD4_{HC}$-(GS9)-IgG_{E51}. To more thoroughly evaluate these reagents, as well as reagents containing 17b, m9, and 412d Ab combining sites in place of E51, we compared neutralization potencies over a panel of clade A, B, and C HIV-1 strains (Table 3). The neutralization potency of the longer $CD4_{HC}$-(GS9)-IgG_{E51} construct was slightly, but consistently, ~30% better than $CD4_{HC}$-(GS7)-IgG_{E51} ($P < 0.001$ [two-tailed paired t test on $\log_{10}$-transformed values]).

To determine the degree to which the combining site of the CD4i Ab contributed to the activity of $CD4_{HC}$-(GS7)-IgG_{E51}, we constructed a similar reagent using an Ab against an irrelevant antigen (the herpes simplex virus gD protein) (4) to create $CD4_{HC}$-(GS7)-$IgG_{anti\text{-}gD}$. Control experiments demonstrated that the parental anti-gD Ab was inactive in HIV-1 neutralization assays (data not shown); thus, any potential neutralization activity of the $CD4_{HC}$-(GS7)-$IgG_{anti\text{-}gD}$ reagent would be derived solely from its CD4 component. We also evaluated the neutralization potencies of other reagents whose neutralization activities would be solely due to CD4: the tetravalent CD4-IgG2 reagent (also known as PRO 542) (1) and a bivalent CD4-Fc construct similar to a previously described CD4-immunoadhesin reagent (7) (Fig. 1). $CD4_{HC}$-(GS7)-IgG_{E51} had a geometric mean IC_{50} of 5 nM and was about twofold more potent than the corresponding reagent lacking CD4i Ab activity, $CD4_{HC}$-(GS7)-$IgG_{anti\text{-}gD}$, which had a geo-

TABLE 3. Comparison of IC_{50}s for CD4 reagents and commonly studied broadly neutralizing anti-HIV-1 Abs

Clade and Env	IC_{50} (nM)[a]															
	$CD4_{HC}$-(GS7)-IgG_{E51}	$CD4_{HC}$-(GS9)-IgG_{E51}	$CD4_{HC,LC}$-(GS7)-IgG_{E51}	$CD4_{HC}$-(GS7)-IgG_{17b}	$CD4_{HC}$-(GS7)-IgG_{m9}	$CD4_{HC}$-(GS7)-IgG_{412d}	$CD4_{HC}$-(GS7)-$IgG_{anti\text{-}gD}$	CD4-IgG2	CD4-Fc	CD4	4E10	b12	2G12	2F5	PG9	PG16
Clade B																
SF162.LS	0.37	<0.26	ND	<0.26	ND	ND	ND	ND	ND	2.3	0.07	0.06	4.0	0.68	>330	>330
PVO.4	12	7.1	9.8	7.0	27	14	5.2	27	300	310	45	>330	8.0	>330	27	36
CAAN5342.A2	14	6.8	ND	7.5	110	90	25	41	870	750	19	>330	>330	24	38	59
WITO4160.33	3.2	1.6	5.5	1.4	17	8.2	4.6	8.0	120	250	2.1	20	7.3	4.1		
AC10.2.29	7.3	2.8	>420	3.5	51	ND	14	11	230	400	2.1	12	>330	8.8		
SC422661.8	7.0	5.7	10	7.4	28	14	6.7	14	240	220	6.2	1.3	14	4.8	5.3	7.5
6535.30	1.7	1.1	9.8	0.8	6.8	ND	1.5	13	29	37	1.4	9.1	13	13	1.5	250
THRO4156.18	2.8	1.8	ND	ND	4.7	5.1	2.6	2.3	12	14	2.1	3.2	>330	>330	83	8.9
REJO4541.67	2.3	1.4	30	ND	8.4	ND	3.1	2.9	17	23	4.9	4.5	>330	4.1		
TRJO4551.58	20	11	ND	ND	66	ND	35	19	500	940	31	>330	>330	>330	2.9	7.7
QH0692.42	3.1	1.8	16	ND	10	9.7	1.0	2.3	14	23	9.7	1.9	19	6.8	>330	>330
TRO.11	86	52	4.7	ND	31	ND	9.3	45	770	540	2.1	>330	2.7	>330	36	1.5
RHPA4259.7	3.2	1.5	370	ND	34	20	5.2	5.2	82	84	48	0.6	>330	82		
Clade C																
MW965.26	<0.26	<0.26	ND	<0.26	ND	ND	ND	ND	ND							
ZM197M.PB7	22	12	>420	7.2	61	63	35	91	>1,000	180	3.5	130	>330	84		
ZM249.PL1	13	4.6	ND	4.1	83	42	19	30	1000	450	15	21	>330	>330		
ZM53M.PB12	2.8	1.1	ND	1.5	28	31	4.6	12	350	390	49	170	>330	>330		
ZM214M.PL15	7.0	6.0	>420	5.2	22	16	13	16	470	370	28	19	>330	>330		
Du156.12	51	35	>420	35	120	53	87	40	>1,000	630	1.4	5.2	>330	>330		
Du422.1	1.6	0.8	>420	1.3	150	85	68	40	630	420	4.9	1.3	>330	>330		
Du172.17	5.2	2.9	160	ND	21	28	8.8	8.6	56	79	2.1	6.5	>330	>330		
CAP45.2.00.G3	3.6	2.0	ND	ND	46	11	20	6.3	>1,000	1200	18	4.5	>330	>330		
CAP210.2.00.E8	6.3	2.9	ND	ND	18	20	7.7	3.4	50	160	8.3	130	>330	>330		
ZM233M.PB6	6.6	2.3	ND	ND	42	40	11	33	460	140	8.3	>330	>330	>330		
ZM109F.PB4	0.52	<0.26	ND	ND	2.1	2.0	0.52	0.29	3.1	9	4.2	>330	>330	>330		
ZM135M.PL10a	13	9.3	ND	ND	71	43	22	8.0	420	280	4.2	>330	>330	>330		
Clade A																
DJ263.8	<0.26	<0.26	ND	<0.26	ND	ND	ND	ND	ND							
Q23.17	3.6	2.1	>420	1.8	69	53	15	18	530		120	>330	>330	50		
Q842.d12	45	43	>420	33	200	86	110	90	>1,000		97	>330	>330	59		
Q259.d2.17	2.7	1.8	>420	1.8	43	38	12	13	93		99	>330	>330	72		
3718.v3.c11	19	9.4	>420	19	ND	94	55	41	>1,000		80	>330	>330	23		
0330.v4.c3	2.4	1.3	9.3	1.0	ND	12	ND	13	130		40	>330	4.7	65		
3415.00	16	8.1	12	10	ND	18	ND	90	640		160	160	14	250		
Geometric mean	5.0	2.9	75	2.9	32	25	11	14	210	150	10	33	110	78	27	33
Arithmetic mean	12	7.2	220	7.1	51	36	22	25	440	320	30	150	240	190	96	110

[a] IC_{50}s for the 4E10, b12, 2F5 Abs and CD4 were taken from previously reported results (21, 22) or were provided by the CAVD neutralization core. IC_{50}s for 2G12 were taken from references 21 and 22 and converted to molar concentrations, assuming the monomeric form. PG9 and PG16 were tested on a larger panel of viruses (35), but only those that overlapped with our panel are reported in this table. Over a 162-pseudovirus panel, PG9 and PG16 had geometric mean IC_{50}s of 5.7 and 5.5 nM, respectively. In calculating average IC_{50}s, measurements outside of the range of the assay (indicated by > or < symbol) were assigned to that limiting value. ND, not determined.

TABLE 4. IC_{50}s for CD4-2G12 constructs compared with 2G12-only and CD4-only reagents

Env	Clade	IC_{50} (nM)[a]				
		Monomer		Dimer		CD4-IgG2
		2G12	$CD4_{HC}$-IgG_{2G12}	2G12	$CD4_{HC}$-IgG_{2G12}	
PVO.4	B	33*	4.2*	<0.17*	0.30*	27*
SC422661.8	B	140	4.4	0.38	0.46	7.3
QH0692.42	B	72	1.8	0.71	0.60	0.83
TRO.11	B	13	6.8	0.11	0.63	92
Du156.12	C	>670*	>530*	>330*	>260*	40*
Du172.17	C	>670	6.3	>330	1.1	2.5
Geometric mean		110	9.5	2.9	1.6	11
Arithmetic mean		270	92	110	44	28

[a] Results are from in-house neutralization assays except for values marked with an asterisk, which are from CAVD core neutralization facility assays. In calculating average IC_{50}s, measurements outside of the range of the assay (indicated by a > symbol) were assigned to that limiting value.

metric mean IC_{50} of 11 nM ($P < 0.02$, by a two-tailed paired t test on $\log_{10}$-transformed values). We also found that the $CD4_{HC}$-(GS9)-IgG_{E51} and $CD4_{HC}$-(GS7)-IgG_{17b} reagents were about threefold more potent than the $CD4_{HC}$-(GS7)-$IgG_{anti\text{-}gD}$ and CD4-IgG2 reagents lacking CD4i Ab combining sites (all pairwise comparisons have P values of <0.0001) (Table 3). However, the m9 and 412d reagents ($CD4_{HC}$-(GS7)-IgG_{m9} and $CD4_{HC}$-(GS7)-IgG_{412d}) were two– to threefold less potent than either CD4-IgG2 or $CD4_{HC}$-(GS7)-$IgG_{anti\text{-}gD}$, suggesting that the m9 and 412d combining sites were not paired with CD4 to achieve optimal binding of the CD4 or Ab components to gp120. All of these reagents were considerably more potent than CD4-Fc, which had a geometric mean IC_{50} of 210 nM, compared with mean values of <10 nM for the E51 and 17b $CD4_{HC}$ reagents (Table 3). A direct comparison between the two bivalent CD4-containing proteins with no CD4i components [$CD4_{HC}$-(GS7)-$IgG_{anti\text{-}gD}$ and CD4-Fc] demonstrates that increasing the potential distance between the CD4 components yields a far more effective reagent (mean IC_{50} of 11 nM versus 210 nM). The fact that the bivalent $CD4_{HC}$-(GS7)-$IgG_{anti\text{-}gD}$ has a potentially longer CD4 separation distance than the tetravalent CD4-IgG2 reagent (Fig. 1) may explain their similar neutralization potencies (mean IC_{50}s of 11 nM and 14 nM) despite the greater number of CD4 moieties in CD4-IgG2. Similarly, a longer CD4 separation distance combined with the inclusion of CD4i components is likely to account for the increased neutralization potencies of $CD4_{HC}$-(GS7)-IgG_{17b} (mean IC_{50} of 2.9 nM) and $CD4_{HC}$-(GS9)-IgG_{E51} (mean IC_{50} of 2.9 nM) compared with CD4-IgG2 (mean IC_{50} of 14 nM) (Table 3).

In order to test a multivalent CD4 architecture with a different geometry, we constructed a fusion of CD4 D1-D2 with the non-CD4i Ab 2G12, which recognizes a cluster of carbohydrates on gp120 via a rigid $(Fab)_2$ created by three-dimensional domain swapping (6). Expression and purification of a CD4 fusion to the 2G12 heavy chain yielded monomeric and dimeric fractions similar to those observed with unmodified 2G12 IgG (Fig. 1) (37). The neutralization potencies of these reagents (Table 4) suggested that both the CD4 components and the antibody combining sites of these reagents contribute to neutralization since (i) $CD4_{HC}$-(GS7)-IgG_{2G12} is active on a clade C stain (Du172.17) that is not neutralized by unmodified 2G12 monomers or dimers and since (ii) $CD4_{HC}$-(GS7)-IgG_{2G12} neutralizes the clade B TRO.11 strain 13-fold (2G12 monomer) and 150-fold (2G12 dimer) better than the pure CD4-reagent CD4-IgG2 (PRO 542). The second observation implies that either the 2G12 anticarbohydrate binding activity is functional in $CD4_{HC}$-(GS7)-IgG_{2G12} and/or that the geometry of the CD4 components is more favorable than in the CD4-IgG2 reagent. The more similar neutralization potencies observed between $CD4_{HC}$-(GS7)-IgG_{2G12} and CD4-IgG2 on other strains suggest that differences in the CD4 geometries are not likely to explain the TRO.11 results, suggesting that carbohydrate recognition contributes to the neutralization potencies of the $CD4_{HC}$-(GS7)-IgG_{2G12} reagents against 2G12-sensitive viral strains.

To confirm that antigen binding by 2G12 contributes to the activity of $CD4_{HC}$-(GS7)-IgG_{2G12}, we conducted additional neutralization assays in the presence of fructose, which has been reported to inhibit 2G12 binding to its carbohydrate epitope on gp120 (6). In order to observe a potential inhibitory effect of fructose addition, we chose clade B strains that were sensitive to 2G12. In the presence of 3% fructose, we observed 3- to 11-fold weaker neutralization of 2G12 IgG and $CD4_{HC}$-(GS7)-IgG_{2G12}, whereas neutralization by $CD4_{HC}$-(GS7)-IgG_{E51}, a CD4-CD4i reagent, was unaffected by including fructose (0.7- to 1.2-fold differences) (Table 5).

DISCUSSION

CD4-CD4i reagents are designed to allow both the CD4 and CD4i components to bind gp120 simultaneously, in which case neutralization of HIV-1 should occur at a lower concentration

TABLE 5. IC_{50}s for $CD4_{HC}$-IgG_{2G12}, 2G12, and CD4-CD4i reagents in the presence and absence of 3% fructose

Env	Effect on IC_{50} of fructose[a]								
	$CD4_{HC}$-IgG_{2G12} monomer			2G12 monomer			$CD4_{HC}$-(GS7)-IgG_{E51}		
	IC_{50} (nM)		Fold change	IC_{50} (nM)		Fold change	IC_{50} (nM)		Fold change
	+	−		+	−		+	−	
WITO4160.33	200	18	11	650	65	10	110	91	1.2
SC422661.8	430	46	9.3	260	64	4.1	310	300	1.0
QH0692.42	16	6	2.7	130	44	3.0	18	27	0.7
TRO.11	28	5.7	4.9	33	5.3	6.2	ND	ND	ND

[a] +, treatment with 3% fructose; −, no fructose; ND, not determined.

because the effective affinity of this type of bivalent reagent would be increased compared to its components. In other words, if binding of the CD4 portion of the reagent to gp120 is in equilibrium, then binding of the CD4i portion to gp120 would reduce the rate at which the reagent dissociates from the virus by either stabilizing the CD4-bound conformation or by permitting rebinding of the CD4 component.

Our comparative study of the neutralization potencies of various CD4-CD4i reagents allows an estimate of the enhanced potencies of these reagents resulting from potential bivalent binding. Analysis of the neutralization activity of CD4-$scFv_{E51}$ compared to its CD4 and $scFv_{E51}$ components suggests about a twofold enhanced potency for the combined reagent (see Materials and Methods), similar to the increased potency of $CD4_{HC}$-(GS7)-IgG_{E51} compared to $CD4_{HC}$-(GS7)-$IgG_{anti\text{-}gD}$ (Table 3). The relatively modest enhancement we see for CD4-CD4i reagents compared to other bivalent reagents (neutralization potencies of IgG versus Fab can be up to 1,000-fold different for some viruses) (30) may be due to gp120 conformational changes associated with CD4 binding. First, the CD4i Ab component is unlikely to bind unless CD4 is already bound. Second, dissociation of CD4 may be quickly followed by a conformational change in gp120 that eliminates the CD4i binding site and thereby causes dissociation of the whole CD4-CD4i reagent. In theory, the CD4-CD4i Ab fusion protein can reduce the rate at which the reagent dissociates from the virus by either stabilizing the CD4-bound conformation or by permitting rebinding of the CD4 component. These mechanisms may be limited by the kinetics of gp120 conformational change.

Although it is not yet clear if it is possible to enhance the synergy between the components of a CD4-CD4i fusion protein, we were able to construct reagents with increased neutralization potencies compared with CD4-scFv reagents. By comparing the expression levels and efficacies of different architectures of CD4-CD4i Ab fusion proteins (Fig. 1; see also Table S1 in the supplemental material) in neutralization of a broad range of primary HIV-1 strains, we found that including an IgG Fc region improved the neutralization potency of a CD4-CD4i reagent. In addition to dimerizing a CD4-CD4i reagent, addition of Fc would also be expected to improve a reagent's serum half-life due to rescue from degradation via binding to the neonatal Fc receptor, which serves as a protection receptor for IgGs and other Fc-containing proteins (26). After testing several architectures for CD4-IgG constructs, we found that reagents in which CD4 was linked to the heavy chain of a CD4i Ab [i.e., $CD4_{HC}$-(linker)-IgG] showed favorable expression characteristics and a low nanomolar geometric mean IC_{50} across a broad range of primary HIV strains (Table 3). The polymeric reagent D1D2-Igαtp has also been reported to neutralize HIV strains very efficiently at low-nanomolar concentrations (2), and even without binding to gp120 or virus, this reagent exhibited a functionally irreversible association with CD16 on natural killer cells (14). Although D1D2-Igαtp should be a powerful reagent for elimination of virally infected cells, our monomeric reagents may have advantages for providing passive immunity (e.g., as smaller molecules there should be more complete distribution to potential sites of exposure).

We also compared our reagents to an existing set of broadly neutralizing Abs (Table 3). We were unable to fully compare our reagents with recently described highly potent broadly neutralizing Abs (35) because the new Abs were tested on a different panel of primary HIV-1 strains than used in our study; however, IC_{50}s are presented for the common strains that were tested (Table 3). Three reagents, $CD4_{HC}$-(GS7)-IgG_{E51}, $CD4_{HC}$-(GS9)-IgG_{E51}, and $CD4_{HC}$-(GS7)-IgG_{17b}, neutralized a range of clade A, B, and C HIV-1 strains with comparable if not higher potencies than the broadly neutralizing Abs and CD4-IgG2. Of the three new CD4-CD4i reagents, we achieved the highest expression levels for $CD4_{HC}$-(GS7)-IgG_{E51}, suggesting that this reagent would be the best suited for applications involving expression by gene therapy.

In addition to the CD4-CD4i fusion proteins, we tried fusing CD4 to 2G12, an Ab that binds to a constellation of carbohydrates on gp120 using a domain-swapped $(Fab)_2$ unit (6). We previously showed that 2G12 forms stable dimers that exhibit a 50- to 80-fold increase in neutralization potency compared with monomeric 2G12 against a collection of clade B HIV-1 strains, but that 2G12 dimers, like their monomeric counterparts, were unable to neutralize clade C HIV-1 strains (37). We found that the CD4-2G12 fusion protein, similar to unmodified 2G12, was expressed as a mixture of monomers and dimers, with two and four antibody combining sites, respectively (Fig. 1). The $CD4_{HC}$-(GS7)-IgG_{2G12} proteins showed nearly comparable (in the case of the dimer) or enhanced (in the case of the monomer) neutralization potencies against clade B HIV-1 strains, but most promisingly, fusion of CD4 to 2G12 conferred the ability to neutralize some clade C HIV-1 strains (Table 4).

The IgG-based CD4-CD4i and CD4-2G12 reagents reported here represent a new class of anti-HIV-1 protein therapeutics of potential use for passive immunization or gene therapy efforts. The addition of CD4 conferred a broader range of neutralization activity and/or facilitated binding of a CD4i Ab. Comparison of CD4-CD4i reagents with CD4-only reagents suggested that the addition of a CD4i Ab enhanced the potency of CD4 reagents two- to threefold. The moderate degree of synergy may be related to a nonoptimal geometry between the CD4 and CD4i Ab components; thus, alternative linkers may be more efficient for promoting simultaneous binding. Also, since we sometimes observed partial degradation of the CD4-CD4i reagents on storage (A. P. West, Jr., R. P. Galimidi, and P. J. Bjorkman, unpublished observations), improvement of the linker design may yield more stable and potent reagents.

The maximal potential potency of CD4-CD4i reagents may be inherently limited by kinetic factors or other properties; for example, in the trimeric envelope spike, engagement of multiple CD4 molecules might be required to elicit the conformational change that exposes the coreceptor binding sites. Furthermore, binding studies involving trimeric Env proteins and CD4 and/or a CD4i Ab have revealed unexpected stoichiometries. For example, only a single CD4i Fab X5 bound to an HIV-1 Env trimer complexed with three CD4 molecules (9), apparently reflecting steric constraints on CD4i Ab binding to Env trimers. Recently, a specific mechanism of action for soluble CD4 and CD4-mimetic compounds was discovered; engagement with these reagents causes a short-lived activated state of Env trimers that is followed by irreversible decay into

a nonfunctional form (15). A greater understanding of steric and other factors that limit the activity of CD4-CD4i reagents would facilitate design of bispecific reagents with increased potency relative to the individual components.

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