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Affinity maturation by targeted diversification of the CDR-H2 loop of a monoclonal Fab derived from a synthetic naïve human antibody library and directed against the internal trimeric coiled-coil of gp41 yields a set of Fabs with improved HIV-1 neutralization potency and breadth

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ABSTRACT

Previously we reported a broadly HIV-1 neutralizing mini-antibody (Fab 3674) of modest potency that was derived from a human non-immune phage library by panning against the chimeric gp41-derived construct N_{CCG}-gp41. This construct presents the N-heptad repeat of the gp41 ectodomain as a stable, helical, disulfide-linked trimer that extends in helical phase from the six-helix bundle of gp41. In this paper, Fab 3674 was subjected to affinity maturation against the N_{CCG}-gp41 antigen by targeted diversification of the CDR-H2 loop to generate a panel of Fabs with diverse neutralization activity. Three affinity-matured Fabs selected for further study, Fabs 8060, 8066 and 8068, showed significant increases in both potency and breadth of neutralization against HIV-1 pseudotyped with envelopes of primary isolates from the standard subtype B and C HIV-1 reference panels. The parental Fab 3674 is 10–20-fold less potent in monovalent than bivalent format over the entire B and C panels of HIV-1 pseudotypes. Of note is that the improved neutralization activity of the affinity-matured Fabs relative to the parental Fab 3674 was, on average, significantly greater for the Fabs in monovalent than bivalent format. This suggests that the increased avidity of the Fabs for the target antigen in bivalent format can be partially offset by kinetic and/or steric advantages afforded by the smaller monovalent Fabs. Indeed, the best affinity-matured Fab (8066) in monovalent format (~50 kDa) was comparable in HIV-1 neutralization potency to the parental Fab 3674 in bivalent format (~120 kDa) across the subtype B and C reference panels.

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Introduction

Fusion of the HIV-1 virus and host cell membranes is mediated by the surface envelope (Env) glycoproteins gp120 and gp41 (Berger et al., 1999; Eckert and Kim, 2001a). The binding of gp120 to the primary receptor CD4 and the coreceptor CXCR4 triggers a series of conformational changes in both gp120 and gp41 that lead to the formation of a pre-hairpin intermediate (PHI) of the ectodomain of gp41 (Furuta et al., 1998). In the PHI the C-heptad repeat (C-HR; residues 623–663) and the helical coiled-coil trimer of the N-heptad repeat (N-HR, residues 542–591) do not interact with one another but bridge the viral and target cell membranes in an overall extended conformation through the C- and N-termini of gp41, respectively (Chan et al., 1998; Furuta et al., 1998; Eckert and Kim, 2001a; Gallo et al., 2003; Melikyan et al., 2006). The subsequent formation of a six-helix bundle (6-HB) with the N-HR trimer surrounded by three C-HR helices (Chan et al., 1997; Tan et al., 1997; Weissenhorn et al., 1997; Caffrey et al., 1998) brings the viral and target cell membranes into contact eventually leading to fusion. The N-HR and C-HR in the PHI are accessible and can therefore be targeted by gp41-directed fusion inhibitors (Wild et al., 1992; Jiang et al., 1993; Wild et al., 1994; Eckert et al., 1999; Louis et al., 2001; Root et al., 2001; Eckert and Kim, 2001b; Bewley et al., 2002; Louis et al., 2003; Matthews et al., 2004; Root and Steger, 2004; Eckert et al., 2008).

The conserved nature of the gp41 N-HR suggests that it may represent an attractive target for generating antibodies with broadly neutralizing activity. However, the majority of antibodies raised against both the N-HR and 6-HB of gp41 have been only weakly inhibitory or non-neutralizing (Jiang et al., 1998; Chen et al., 2000; Golding et al., 2002; Louis et al., 2003), presumably because of the

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transient exposure of the N-HR trimer during the fusion process and the large size of IgGs making access difficult (Hamburger et al., 2005; Steger and Root, 2006; Eckert et al., 2008). To date, six modestly neutralizing antibodies directed against the N-HR have been reported: Fab 3674 (Gustchina et al., 2007), D5 (Miller et al., 2005; Luftig et al., 2006), 8K8 (Nelson et al., 2008), DN9 (Nelson et al., 2008), m46 (Choudhry et al., 2007) and m44 (Zhang et al., 2008).

In recent work (Louis et al., 2005; Gustchina et al., 2007) we made use of a chimeric protein known as N_{CCG}-gp41 (Louis et al., 2001) which presents the N-HR as a stable, helical, disulfide-linked trimer that extends in helical phase from the 6-HB core of gp41, to select monoclonal antibodies by phage display from a synthetic human combinatorial antibody library (HuCAL GOLD) comprising more than 10^{10} human specificities (Knappik et al., 2000; Kretzschmar and von Ruden, 2002; Rothe et al., 2008). The HuCAL GOLD library includes diversification of all six complementarity determining regions (CDRs) according to the sequence and length variability found in naturally rearranged human antibodies (Rothe et al., 2008). Our initial attempts resulted in a set of Fabs that inhibited Env-mediated cell fusion in a vaccinia virus-based fusion assay but were non-neutralizing in an Env-pseudotyped virus neutralization assay (Louis et al., 2005). Subsequently, Fab 3674 with broad neutralizing activity against HIV-1 pseudotyped with Envs from diverse laboratory-adapted B strains of HIV-1 and primary isolates of subtypes A, B and C, were isolated (Gustchina et al., 2007). Fab 3674 recognizes both the internal timeric N-HR coiled-coil, and the 6-HB of gp41 (Gustchina et al., 2007).

The D5 monoclonal antibody was derived from a naive scFv library selected by panning against the gp41-derived construct 5-helix (Miller et al., 2005; Luftig et al., 2006). 5-helix is a single chain construct in which the N-HR trimer is surrounded by only 2 C-HR helices, thereby exposing two of the three N-HR helices (Root et al., 2001). There are a number of interesting differences between Fab 3674 and D5. While Fab 3674 and the C34 peptide (derived from the C-HR and comprising residues 628–661 of gp41; (Chan and Kim, 1998)) neutralize HIV-1 either additively (Fab in monovalent format) or synergistically (Fab in bivalent format) (Gustchina et al., 2007), binding of D5 and C34 appears to be mutually exclusive (Miller et al., 2005). 8K8 and DN9 (Nelson et al., 2008) monoclonal antibodies were selected from rabbit (b9 rabbit immunized against $N35_{CCG}$-N13) and human (infected HIV-1 individual) phage display libraries, respectively, by panning against the N-HR trimer mimetic $N35_{CCG}$-N13 (Louis et al., 2003). In contrast to Fab 3674, 8K8 and DN9 are targeted specifically to the N-HR trimeric coiled-coil and do not bind to the 6-HB of gp41. m46 (Choudhry et al., 2007) and m44 (Zhang et al., 2008) monoclonal antibodies were selected from a human immune phage library by panning against gp140, a soluble form of the Env ectodomain comprising uncleaved gp120 and gp41; m46 binds only to 5-HB but not to 6-HB or the N-HR trimer, while m44 binds to 5-helix and the 6-HB, but not to the N-HR trimer.

In this paper we set out to improve the potency of Fab 3674 (Gustchina et al., 2007) by affinity maturation against the N_{CCG}-gp41 antigen using targeted diversification of the CDR-H2 loop. Due to their unique modular structure HuCAL antibodies are ideal for the specific optimization of CDRs (Knappik et al., 2000). Recently, an affinity improvement of 5000-fold was achieved by parallel CDR-L3 and CDR-H2 diversification and subsequent combination of optimized CDR-L3 and CDR-H2 antibodies (Steidl et al., 2008).

Affinity maturation of the CDR-H2 of Fab 3674 resulted in the generation of a panel of Fabs that displayed significantly improved affinity towards the target antigen N_{CCG}-gp41 and exhibited diverse HIV-1 neutralizing activity. The three best Fabs displayed enhanced HIV-1 neutralization properties relative to the original Fab 3674, both in terms of IC_{50} values and neutralization breadth over a standard panel of Envs from primary isolates of HIV-1 subtypes B and C.

Results and discussion

Affinity maturation of Fab 3674

The parental neutralizing Fab 3674 (Gustchina et al., 2007) was derived from the synthetic human Fab library HuCAL Gold comprising more than 10^{10} antibody genes (Knappik et al., 2000) by panning against N_{CCG}-gp41 (Louis et al., 2001), a construct that presents the N-HR of gp41 as a stable helical, disulfide-linked trimer that extends in helical phase from the 6-HB core. Fab 3674 was subjected to affinity maturation by diversification of the heavy chain CDR-H2 loop. Specifically, the original CDR-H2 sequence of the Fab 3674 gene was replaced by a repertoire of CDR-H2 sequences designed according to the respective amino acid distribution at each position of naturally occurring, human rearranged antibody genes (Virnekas et al., 1994; Knappik et al., 2000; Rothe et al., 2008). A total of 1.3×10^6 Fab genes with different heavy chain CDR-H2 loops were generated in this manner. After two rounds of panning against N_{CCG}-gp41 coupled to magnetic beads, applying increased washing stringency relative to that used for the parental Fab 3674, the enriched pool of Fab genes was subcloned into an expression vector. 368 clones were tested for binding to N_{CCG}-gp41 coupled beads, the 10 clones with the best signal and an additional 10 clones selected at random were sequenced, and 12 unique Fabs were found. The 10 Fabs with the highest ELISA signal on N_{CCG}-gp41 were selected for further characterization and were expressed in both monovalent (mF) and bivalent (bF) formats. The latter is obtained by the addition of a helix–loop–helix dimerization domain to the C-terminus of the heavy chain (Pluckthun, 1992).

Table 1 summarizes the CDR-H2 sequences together with the equilibrium dissociation constants (K_D) for the binding of Fab 3674 and the 10 affinity-matured Fabs (in monovalent format) to N_{CCG}-gp41. The K_D values, determined by solution equilibrium titration using an electrochemiluminescence (ECL) based affinity measurement (Haenel et al., 2005; Steidl et al., 2008), range from 7 to 25 nM, compared to ~97 nM for the parental Fab 3674. Western blot analysis against a panel of gp41 ectodomain-derived constructs comprising N_{CCG}-gp41, $N35_{CCG}$-N13, $N35_{CCG}$, 6-HB and 5-HB revealed the same pattern of binding for all the Fabs, including the parental Fab 3674 (cf. Fig. 2 of Gustchina et al. (2007)). Thus all the Fabs recognize the fully exposed internal trimer of N-HR helices (cf. N_{CCG}-g41, $N34_{CCG}$, $N35_{CCG}$-N13), the $(N\text{-}HR)_3/C\text{-}HR_3$ six-helix bundle (cf. 6-HB and N_{CCG}-gp41), and a partially exposed trimer of N-HR helices (cf. 5-helix in which two of the three N-HR helices are exposed) (Fig. 1A). Likewise, the pattern of intensities observed for the single (Fig. 1B) and double (Fig. 1B) alanine mutants of 6-HB comprising surface exposed residues of the N-HR (Gustchina et al., 2007) is similar, indicating that the parental and affinity-matured Fabs bind to the

Table 1
Affinity of monovalent, affinity-matured Fabs for the antigen N_{CCG}-gp41 and corresponding CDR-H2 sequences.

Antibody	K_D (nM)	CDR-H2 sequence																	
3674	96.5	G	I	I	P	I	F	G	M	A	N	Y	A	Q	K	F	Q	G
8059	7.2	S	I	I	P	L	F	G	T	T	N	Y	A	Q	K	F	Q	G
8060	10.1	S	I	I	P	I	F	G	S	T	N	Y	A	Q	K	F	Q	G
8061	21.4	S	I	I	P	M	M	G	S	T	N	Y	A	Q	K	F	Q	G
8062	16.8	S	I	I	P	L	F	G	F	A	V	Y	A	Q	K	F	Q	G
8063	19.9	S	I	I	P	V	I	G	S	T	N	Y	A	Q	K	F	Q	G
8064	9.5	S	I	I	P	W	F	G	S	T	N	Y	A	Q	K	F	Q	G
8065	25.1	S	I	I	P	W	H	G	G	T	N	Y	A	Q	K	F	Q	G
8066	15.3	S	I	I	P	I	F	G	T	T	N	Y	A	Q	K	F	Q	G
8068	15.9	S	I	I	P	L	M	G	T	T	N	Y	A	Q	K	F	Q	G
8069	7.1	S	I	I	P	L	F	G	W	A	N	Y	A	Q	K	F	Q	G
		50	51	52	52a	53	54	55	56	57	58	59	60	61	62	63	64	65

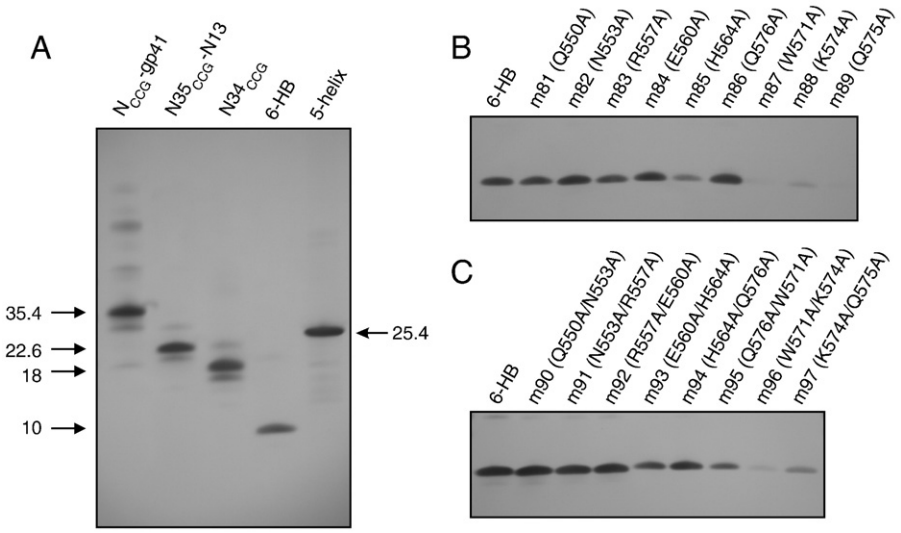

Fig. 1. Western blot analysis under non-reducing conditions of gp41-derived constructs reacting with the monovalent Fab mF-8066. (A) N_{CCG}-gp41, $N35_{CCG}$-N13, $N34_{CCG}$, 6-HB and 5-helix. (B) Single and (C) double alanine scanning mutants of 6-HB involving solvent exposed N-HR residues that lie in the shallow groove between the two C-HR helices (Gustchina et al., 2007). The molecular weights of trimeric N_{CCG}-gp41 (35.4 kDa), $N35_{CCG}$-N13 (22.6 kDa) and $N34_{CCG}$ (18 kDa) held together by disulfide bridges are indicated. 6-HB is a 10 kDa popypeptide chain spanning the N-HR and C-HR that adopts a trimeric fold (comprising the minimal thermostable ectodomain core of gp41) during Western blotting upon transfer of the protein from the gel to the nitrocellulose membrane. 5-helix is a single chain polypeptide of 25.4 kDa.

same gp41 epitope comprising the shallow groove of N-HR residues that is surface exposed and lies between two C-HR helices of the 6-HB.

Neutralization activity of affinity-matured Fabs against laboratory-adapted strains of subtype B HIV-1

The neutralization activity of the parental Fab 3674 in monovalent format and the affinity-matured monovalent Fabs against diverse laboratory-adapted HIV-1 subtype B strains using an Env-pseudotyped virus neutralization assay are reported in Table 2. Examples of dose–response curves against diverse laboratory-adapted HIV-1 subtype B strains for one of the Fabs, mF-8066, is shown in Fig. 2A. As expected no neutralization activity is observed for two negative control viruses pseudotyped with an amphotrophic Env from Murine leukemia virus (MuLV) and vesicular stomatitis virus (VSV) (Fig. 2B).

Surprisingly, not all of the Fabs with improved binding affinity towards the target N_{CCG}-gp41 antigen showed HIV-1 neutralization activity. This points to the importance of kinetic restriction and suggests that the association and/or dissociation rate constants also play a key role in determining neutralization activity (Steger and Root, 2006). Indeed, 4 Fabs (mF-8061, mF-8062, mF-8063 and mF-8065) with K_D values ranging from 17–25 nM showed no detectable neutralization activity. By way of contrast, the parental mF-3674 ($K_D \sim 97$ nM) neutralized 6 out of 7 the strains with IC_{50} values ranging from ~480 to ~2100 nM. Of the 6 affinity-matured Fabs that were neutralizing, the K_D values for binding to N_{CCG}-gp41 were ≤15 nM, but beyond that there was no apparent correlation between the actual K_D and IC_{50} values. For example, mF-8069 had the lowest K_D value (7 nM) of all the affinity-matured Fabs but was comparable to or slightly worse than the original mF-3674 in terms of HIV-1 neutralization activity. Three matured Fabs, 8060, 8066 and 8068, however, displayed significantly improved neutralization activity relative to mF-3674 and were selected for further study. The best, mF-8066, exhibited a 5–20-fold increase in HIV-1 neutralization potency relative to mF-3674 for 6 out of the 7 HIV-1 strains; for the seventh HIV-1 strain, 89.6, mF-8066 was neutralizing while mF-3674 was not.

A comparison of the CDR-H2 sequences shown in Table 1 in the light of the neutralization results is of interest. Three (8061, 8062 and 8065) of the four non-neutralizing Fabs, have a substitution of Phe54 to a less bulky amino acid (Met, Ile and His, respectively). The fourth non-neutralizing Fab (8063), as well as the poorly neutralizing Fab 8069 has a substitution to an aromatic residue (Phe and Trp, respectively) at position 56. An aromatic residue at position 53 (cf. Fabs 8064 and 8065) is also unfavorable. The three best Fabs, 8060, 8066 and 8068, all have an Ile or Leu at position 53, and a Ser or Thr at position 56. The original Fab, 3674 also has an Ile at position 53, but a

Table 2
Neutralization activity of affinity-matured Fabs against HIV-1 subtype B laboratory-adapted strains and primary isolates BaL01 and BaL26.

Antibody	HIV-1 Env strain, IC50 (nM)[a]						
	HXB2	NL4-3	SF162	JR CSF	89.6	BaL01	BaL26
3674	630 ± 160	480 ± 220	2500 ± 690	2100 ± 600	NA	1100 ± 270	1900 ± 960
8059	400 ± 89	98 ± 26	3200 ± 850	1500 ± 470	1100 ± 240	1100 ± 310	970 ± 420
8060	130 ± 30	36 ± 7	1000 ± 200	620 ± 380	500 ± 130	360 ± 120	360 ± 130
8061	NA	NA	NA	NA	NA	NA	NA
8062	NA	NA	NA	NA	NA	NA	NA
8063	NA	NA	NA	NA	NA	NA	NA
8064	790 ± 270	270 ± 140	5200 ± 1800	NA	1300 ± 560	2300 ± 1300	1100 ± 520
8065	NA	NA	NA	NA	NA	NA	NA
8066	82 ± 29	26 ± 9	510 ± 150	230 ± 160	380 ± 110	200 ± 73	280 ± 69
8068	160 ± 72	34 ± 11	1400 ± 380	110 ± 92	350 ± 110	450 ± 110	580 ± 220
8069	880 ± 300	360 ± 210	NA	NA	660 ± 400	NA	1900 ± 760

[a] NA, no activity. Neutralization activity was too weak to reliably determine an IC_{50}.

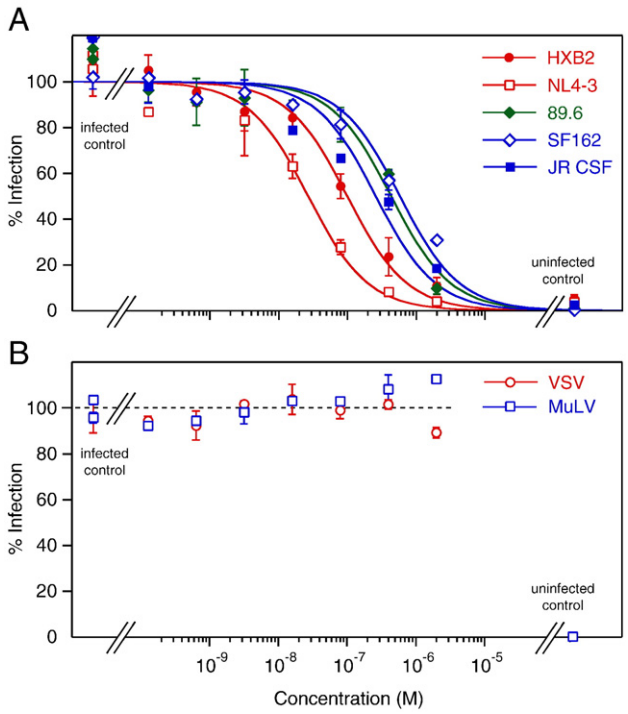

Fig. 2. Dose–response curves for antiviral activity of the monovalent Fab mF-8066 against (A) diverse laboratory-adapted HIV-1 subtype B strains and (B) MuLV and VSV (negative controls) in an Env-pseudotyped virus neutralization assay.

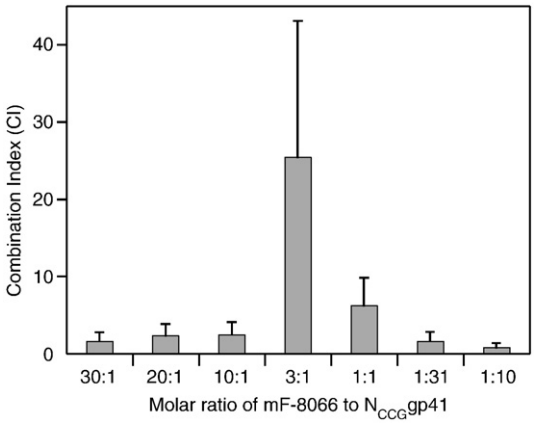

Fig. 3. Antagonism of Fab mF-8066 and the N_{CCG}-gp41 antigen observed in an HXB2 Env-pseudotyped HIV neutralization assay. IC_{50}s were obtained from dose–response curves measured for a range of fixed molar combination ratios of mF 8066 and N_{CCG}-gp41. The IC_{50}s for mF-8066 and N_{CCG}-gp41 alone are 82 ± 29 and 34 ± 10 nM, respectively. The K_D for the binding of mF-8066 to N_{CCG}-gp41 is 15 nM (Table 1). Combination index (CI) values greater than 1 indicate that two inhibitors are mutually antagonistic. Maximum antagonism is observed at a molar ratio of mF-8066 to N_{CCG}-gp41 of 3:1, as expected given that N_{CCG}-gp41 is a symmetric trimer with three symmetric binding sites for mF-8066.

longer amino acid, Met, at position 56. One can therefore conclude that residues at positions 53, 54 and 56, with an optimal sequence of Ile/Leu, Phe and Ser/Thr, respectively, likely make key contacts with gp41.

N_{CCG}-gp41 and Fab mF-8066 are mutually antagonistic

To rule out any potential non-specific effects inherent in the Env-pseudotyped HIV neutralization assay, we tested whether addition of the target antigen, N_{CCG}-gp41, in the assay could adsorb out the neutralization activity of the most potent monovalent Fab mF-8066. Since N_{CCG}-gp41 was designed to bind to the C-HR of the PHI and is itself a potent nanomolar inhibitor of HIV-1 fusion (Louis et al., 2001), we tested the neutralizing activity of mixtures of mF-8066 and N_{CCG}-gp41 in various fixed molar ratios ranging from 30:1 to 1:10, and analyzed the combination effects using the method of Chou and Talalay (1981, 1984) as described previously (Gustchina et al., 2006). Since the N_{CCG}-gp41 trimer has three symmetrically related binding sites for Fab 8066, one would expect that maximum antagonism between mF-8066 and N_{CCG}-gp41 would be observed at a molar ratio of 3:1. When an excess of either mF-8066 or N_{CCG}-gp41 is present in the mixture, one would predict that neutralization activity is dominated by the inhibitor in excess. The dose reduction index (DRI) of inhibitor x in combination with inhibitor y is given by $DRI_x = (IC_{50})_x / IC_{50})_{x,y}$, where $(IC_{50})_x$ and $IC_{50})_{x,y}$ are the IC_{50} of x alone and in combination with y, respectively. The combination index (CI) which describes the summation of the effects of the two inhibitors is given by $CI = (DRI_x)^{-1} + (DRI_y)^{-1} + (DRI_x DRI_y)^{-1}$ (Chou and Talalay, 1984; Gustchina et al., 2006). CI values equal to, greater than, or less than 1 are indicative of additive, antagonistic and synergistic effects, respectively. The results are displayed in Fig. 3 and are as predicted above. At high molar ratios (mF-8066 to N_{CCG}-gp41 ratios of 30:1 and 1:10), the effects are essentially additive with CI values close to 1. Maximum antagonism is observed at a molar ratio of mF-8066 to N_{CCG}-gp41 of 3:1 with a CI of ~25. At intermediate molar ratios, increasing antagonism (i.e. larger CI values) is observed as the molar ratio approaches 3:1 of mF-8066 to N_{CCG}-gp41.

Breadth of neutralizing activity of Fabs 8060, 8066 and 8068 against a Standard Reference Panel of subtype B and C strains

On the basis of the neutralization data obtained with laboratory-adapted HIV-1 strains, further studies were confined to the three most potent Fabs, 8060, 8066 and 8068. The breadth of HIV-1 neutralization activity of the Fabs in both mono- and bivalent formats was investigated using HIV pseudotyped with Envs from the Standard Reference Panels of HIV-1 subtypes B (Mascola et al., 2005) and C (Williamson et al., 2003) (Tables 3 and 4). A summary of the results in the form of a comparison of neutralization activity of the affinity-matured Fabs to the parental Fab 3674 is presented in Table 5.

For the parental Fab 3674, the bivalent format is 10–20-fold more potent than the monovalent format in terms of neutralization activity across the panel of HIV-1 B and C clades. The differential in neutralization potency between bivalent and monovalent formats is reduced to 2–5-fold for the affinity-matured Fabs (Tables 3–4). Thus, the affinity-matured monovalent Fabs showed markedly improved potency relative to the parental monovalent Fab, compared to their bivalent counterparts. On average the IC_{50} value for the most potent Fab, mF-8066, was reduced by over 5-fold, when compared to the IC_{50} of the parental mF-3674 (average $IC_{50}^{mF\text{-}8066}/IC_{50}^{mF\text{-}3674}$ ratio of 0.17 ± 0.1 and 0.16 ± 0.1 for HIV-1 subtype B and C panels, respectively), whereas the IC_{50} value for bF-8066 was only reduced by about 2-fold (average $IC_{50}^{bF\text{-}8066}/IC_{50}^{bF\text{-}3674}$ ratio of 0.44 ± 0.2 and 0.45 ± 0.3 for HIV-1 subtype B and C panels, respectively). All of the affinity-matured monovalent Fabs neutralized all pseudoviruses of the HIV-1 subtype B and C Env panels, which correspond to Tier2 contemporary primary isolates (Mascola et al., 2005). This is a significant improvement in breadth of neutralization, compared to the parental mF-3674, which neutralized 92% of pseudoviruses from the subtype B panel and only 67% of pseudoviruses from the subtype C panel.

With regard to the affinity-matured Fabs in bivalent format it is interesting to note that while the neutralization activity against many of the strains is only slightly better than that for the parental Fab bF-3674, there are four B strains (TRJO4551.58, THRO41456.18, WITO4160.33 and RHPA4259.7) and three C strains (ZM249M.PL1, CAP45.2.00.G3 and ZM197M.PB7) for which improvements in potency ranging from 5 to 50-fold are observed.

Table 3
Neutralization activity of monovalent and bivalent, affinity-matured Fabs against primary isolates of subtype B HIV-1.

Env	IC50 (nM)[a]							
	Monovalent Fabs				Bivalent Fabs			
	mF-3674	mF-8068	mF-8060	mF-8066	bF-3674	bF-8068	bF-8060	bF-8066
Ac10.0.29	2000 ± 680	520 ± 300	450 ± 290	340 ± 110	120 ± 47	130 ± 91	35 ± 25	85 ± 34
WITO4160.33	1800 ± 820	270 ± 110	310 ± 170	180 ± 73	200 ± 83	43 ± 26	31 ± 17	47 ± 27
THRO4156.18	6800 ± 2100	830 ± 270	550 ± 230	500 ± 110	2000 ± 470	340 ± 77	370 ± 96	210 ± 31
CAAN5342.A2	2700 ± 480	1000 ± 360	580 ± 170	450 ± 83	340 ± 51	290 ± 58	210 ± 67	220 ± 45
PVO.4	NA	1200 ± 690	2000 ± 1100	1100 ± 450	NA	450 ± 200	310 ± 160	210 ± 58
TRO.11	2000 ± 470	710 ± 310	940 ± 460	610 ± 180	200 ± 25	170 ± 79	150 ± 57	150 ± 35
RHPA4259.7	3000 ± 890	970 ± 430	710 ± 290	560 ± 180	940 ± 460	360 ± 160	270 ± 120	190 ± 77
TRJO4551.58	990 ± 410	540 ± 320	460 ± 260	300 ± 100	1100 ± 420	140 ± 66	96 ± 48	93 ± 23
6535.3	9000 ± 4000	430 ± 230	740 ± 220	540 ± 170	550 ± 190	280 ± 110	310 ± 89	280 ± 77
REJO4541.67	5900 ± 1500	3800 ± 1500	2600 ± 860	1400 ± 190	2300 ± 810	5200 ± 4200	1200 ± 400	1300 ± 180
SC422661.8	2300 ± 1100	1100 ± 380	860 ± 310	450 ± 120	160 ± 45	170 ± 62	94 ± 35	110 ± 32
QH0692.42	7100 ± 1900	770 ± 270	850 ± 380	700 ± 240	2000 ± 690	1800 ± 720	1400 ± 650	860 ± 230

[a] NA, no activity. Neutralization activity was too weak to reliably determine an IC_{50}.

Temporal window of inhibition of viral infectivity

Using HXB2 Env-pseudotyped HIV infections that are synchronized and temperature-arrested at the CD4-bound step, we measured viral infectivity as a function of time of addition of fully inhibitory concentrations of antibody (Gustchina et al., 2008). The results are summarized in Table 6. We have previously shown that the neutralizing activity of bF-3674 had a comparable half-life to that of the C34 peptide, indicating that the two fusion inhibitors act at approximately the same stage of the fusion process (Gustchina et al., 2008). Although we were able to demonstrate that mF-3674 is able to inhibit HIV infection at the same stage of the fusion process as C34, bF-3674 and the affinity-matured Fabs, a very high concentration (approximately 5.5 μM) of mF-3674 was required to achieve such inhibition. Within a reasonable concentration range (≤2 μM) mF-3674 was essentially inactive post-CD4 engagement due to its poor IC_{50} in the post-attachment neutralization assay (Table 6). At a concentration of 2 μM the affinity-matured Fabs neutralized HIV-1 infection post-CD4 engagement with essentially the same half-lives (23–26 min) as C34 (Table 6), which may provide an explanation for the marked increase in their neutralization potency.

Concluding remarks

Affinity maturation of the broadly neutralizing Fab 3674 resulted in the generation of a panel of Fabs with improved binding affinity (ranging from 4 to 12-fold) towards the target antigen, N_{CCG}-gp41 a chimeric construct that presents both the trimeric N-HR coiled-coil and the 6-HB (Table 1). However, not all of the Fabs selected on the basis of binding affinity possessed improved HIV-1 neutralization activity (Table 2). Thus higher binding affinity to the target antigen did not necessarily correlate with improved neutralization activity of the Fabs in the Env-pseudotyped HIV neutralization assays, providing evidence for the importance of kinetic restriction as a determinant of neutralization activity (Steger and Root, 2006). However, for the three Fabs (8060, 8066 and 8068) selected for further study on the basis of their improved neutralization activity (Table 2), a marked decrease in IC_{50} values against HIV pseudotyped with the Envs of Tier2 viruses was observed (Tables 3–5). The improved neutralization potency was on average more pronounced for the Fabs in monovalent than bivalent format. This observation raises the question as to whether the avidity advantage of the Fabs in bivalent format gives way to a potential kinetic and/or steric advantage for the monovalent Fabs once high affinity binding has been achieved. In addition, the breadth of neutralization activity of the monovalent Fabs was significantly enhanced (Tables 3–5). The monovalent Fabs mF-8060, mF-8066 and mF-8068 showed neutralization activity against 100% of HIVs pseudotyped with Tier2 Envs from the Standard Reference Panels of subtypes B and C, whereas the parental monovalent Fab, mF-3674, neutralized 92% of the panel B and only 67% of the panel C pseudoviruses.

Materials and methods

gp41-derived proteins

Expression, purification and folding of N_{CCG}-gp41, N-terminal His-tagged $N35_{CCG}$-N13, $N34_{CCG}$, and 6-HB were performed as described

Table 4
Neutralization activity of monovalent and bivalent, affinity-matured Fabs against primary isolates of subtype C HIV-1.

Env	IC50 (nM)[a]							
	Monovalent Fabs				Bivalent Fabs			
	mF-3674	mF-8068	mF-8060	mF-8066	bF-3674	bF-8068	bF-8060	bF-8066
DU172.17	4900 ± 2500	140 ± 62	180 ± 82	93 ± 22	77 ± 31	72 ± 41	40 ± 17	48 ± 14
ZM214M.PL15	6500 ± 1400	2200 ± 760	1200 ± 320	770 ± 130	550 ± 140	680 ± 270	230 ± 99	310 ± 48
DU422.1	1300 ± 450	240 ± 96	240 ± 69	170 ± 40	93 ± 22	85 ± 30	57 ± 23	60 ± 18
ZM197M.PB7	NA	890 ± 380	1700 ± 720	640 ± 160	1900 ± 660	410 ± 210	280 ± 110	300 ± 110
ZM135M.PL10a	NA	680 ± 220	840 ± 450	340 ± 110	340 ± 92	380 ± 120	200 ± 80	150 ± 45
CAP210.2.00.E8	NA	490 ± 190	560 ± 230	310 ± 88	450 ± 120	150 ± 55	110 ± 33	83 ± 25
CAP45.2.00.G3	NA	490 ± 140	610 ± 300	250 ± 110	500 ± 260	78 ± 53	53 ± 32	53 ± 33
ZM249M.PL1	2900 ± 1100	690 ± 250	510 ± 230	370 ± 76	3000 ± 190	170 ± 110	66 ± 44	56 ± 23
DU156.12	2500 ± 1100	290 ± 150	520 ± 240	190 ± 82	110 ± 46	60 ± 39	41 ± 23	46 ± 23
ZM109F.PB4	800 ± 120	490 ± 54	340 ± 47	330 ± 93	260 ± 74	510 ± 130	350 ± 88	250 ± 85
ZM53M.PB12	1000 ± 310	280 ± 91	450 ± 180	240 ± 51	150 ± 37	120 ± 48	97 ± 34	59 ± 20
ZM233M.PB6	430 ± 210	90 ± 42	120 ± 64	73 ± 27	29 ± 10	35 ± 15	31 ± 13	27 ± 13

[a] NA, no activity. Neutralization activity was too weak to reliably determine an IC_{50}.

Table 5
Improvement in potency and breadth of neutralizing activity of affinity-matured Fabs against diverse primary isolates from the standard subtype B and C reference panels.

Antibody	Panel B			Panel C		
	Average ratio of IC_{50}s relative to Fab 3674	Fold decrease in IC_{50}s relative to Fab 3674	Neutralization breadth	Average ratio of IC_{50}s relative to Fab 3674	Fold decrease in IC_{50}s relative to Fab 3674	Neutralization breadth
Monovalent						
mF-3674	1	1	92	1	1	67
mF-8068	0.31 ± 0.19	1.6–20.7	100	0.25 ± 0.18	1.6–35.3	100
mF-8060	0.26 ± 0.15	2.1–12.4	100	0.24 ± 0.14	2.3–27.1	100
mF-8066	0.17 ± 0.09	3.2–16.8	100	0.16 ± 0.12	2.4–53.0	100
Bivalent						
bF-3674	1	1	92	1	1	100
bF-8068	0.76 ± 0.60	0.5–7.7	100	0.79 ± 0.55	1.5–17.9	100
bF-8060	0.43 ± 0.24	1.3–11.3	100	0.51 ± 0.39	0.8–45.4	100
bF-8066	0.44 ± 0.24	1.4–11.7	100	0.45 ± 0.30	1.1–53.4	100

previously (Louis et al., 2001, 2003, 2005). (The His-tag comprises 20 residues.) N_{CCG}-gp41 is a chimeric protein comprising $N35_{CCG}$ (residues 546–580 of gp41 HIV-1 Env with Leu576, Gln577 and Ala578 substituted by Cys, Cys and Gly, respectively) fused onto the minimal thermostable ectodomain core of gp41 (Louis et al., 2001). Thus, each chain of N_{CCG}-gp41 comprises $N35_{CCG}$–N34-(L6)-C28, where N34 and C28 represent portions of the N-HR and C-HR regions of gp41 (residues 546–579 and 628–655, respectively) and L6 is a six-residue linker (SGGRGG). $N35_{CCG}$-N13 is a 48 residue polypeptide that comprises $N35_{CCG}$ immediately followed by N13 (residues 546–548) (Louis et al., 2003). Three chains of N_{CCG}-gp41, $N35_{CCG}$-N13 and $N34_{CCG}$ are linked covalently via three intermolecular disulfide bridges. 6-HB is the minimal thermostable ectodomain core of gp41 comprising N34-(L6)-C28 (Tan et al., 1997). Single (m81-89) and double (m90-m97) alanine mutants of His-tagged 6-HB involving surface exposed residues of the N-HR that lie in a shallow groove between two C-HR helices were as follows (Gustchina et al., 2007): m81, Q550A; m82, N553A; m83, R557A; m84, E560A; m85, H564A; m86, Q576A; m87, W571A; m88, K574A; m89, Q575A; m90, Q550A/N553A; m91, N553A/R557A; m92, R557A/E560A; m93, E560A/H564A; m94, H564A/Q567A; m95, Q567A/W571A; m96, W571A/K574A; and m97, K574A/Q575A. C-terminal His-tagged 5-helix (Root et al., 2001) was expressed and purified as described previously (Gustchina et al., 2007). All proteins underwent a final purification step using size exclusion chromatography and were characterized by gel electrophoresis and ES-MS.

Affinity maturation of Fab 3674 by exchange of the CDR-H2 loop

DNA encoding Fab 3674 was subcloned from an expression vector into a phagemid vector based on pMorph23 (US Patent 6,753,136). The heavy chain CDR-H2 sequence was removed by restriction digest and a repertoire of CDR-H2 sequences was cloned into this construct. The CDR-H2 repertoire was designed according to the respective amino acid distribution at each position of naturally occurring, human rearranged antibody genes using trinucleotides for gene synthesis (Virnekas et al., 1994; Knappik et al., 2000; Rothe et al., 2008). Electroporation into *Escherichia coli* Top10F' competent cells (Invitrogen) resulted in a library of about 1.3×10^6 antibodies with different heavy chain CDR-H2 loops.

Two rounds of panning were performed on N_{CCG}-gp41 coupled magnetic beads (M-450 Epoxy, Invitrogen) applying increased washing stringency compared to the standard panning conditions used for selection of the parental Fab 3674 (Gustchina et al., 2007). After the second round of panning, the enriched pool of Fab genes was subcloned into the expression vector pMORPHx9_Fab-MH for the expression of myc-his_6 tagged monovalent Fab fragments (Rauchenberger et al., 2003). *E. coli* TG1F$^-$ (TG1 without the F plasmid) was transformed with the ligation mixture and plated. 384 colonies were randomly picked and transferred to a 384-well microtiter plate. Then 368 clones were tested for binding to N_{CCG}-gp41 coupled beads by FLISA on a CDS8200 instrument (Applied Biosystems). Briefly, N_{CCG}-gp41 coupled beads, Fab containing *E. coli* lysates and fluorescence-labeled anti-human Fab secondary antibody were mixed and incubated for 1 h. Fluorescence on beads settled at the bottom of the wells indicated positive lysates. More than 200 hits were obtained in the primary screening. The 10 clones with the best signal and in addition 10 clones selected at random were sequenced and 12 unique antibodies were found. These were expressed and purified via the his_6 tag by affinity chromatography. Specific binding to N_{CCG}-gp41 was confirmed by ELISA and the 10 antibodies with the highest signal on N_{CCG}-gp41 were selected for further characterization. These Fabs were also subcloned and expressed in the bivalent Fab-dHLX-MH format (Jarutat et al., 2006; Gustchina et al., 2007). Composition and purity of antibodies were confirmed by SDS-PAGE, and Western blot analysis against the panel of gp41 ectodomain-derived constructs was carried out as described previously (Gustchina et al., 2007).

Determination of Fab affinities for N_{CCG}-gp41 using Solution Equilibrium Titration (SET)

Electrochemiluminescence (ECL) based affinity determination by solution equilibrium titration was performed essentially as described previously (Haenel et al., 2005; Steidl et al., 2008).

Cell lines and molecular clones

The HIV-1 expression plasmid SG3Δenv (catalog no. 11051), the Standard Reference Panel of subtype B HIV-1 Env clones (catalog no. 11227), the Standard Reference Panel of subtype C HIV-1 Env clones (catalog no. 11326), the HIV-1 Env molecular clone pCAGGS SF162 gp160 (catalog no. 10463), the Murine leukemia virus (MuLV) Env clone SV-A-MLV-env (catalog no. 1065), the vesicular stomatitis virus (VSV) G glycoprotein clone pHEF-VSVG (catalog no. 4693), and indicator cells TZM-b1 (or JC53BL-13, catalog no. 8129) were obtained

Table 6
Comparison of pre-attachment (regular assay) and post-attachment (following CD4 engagement) HIV-1 HXB2 neutralization activity together with the half-lives of the inhibitor-sensitive state obtained from assays synchronized at the CD4-bound step.

Fusion inhibitor	Pre-attachment IC_{50} (nM)	Post-attachment IC_{50} (nM)	Half-life of inhibitor-sensitive state (min)
bF-3674	42 ± 12	85 ± 11	24 ± 2
mF-3674	630 ± 160	1600 ± 320	24 ± 2
mF-8068	160 ± 72	820 ± 68	26 ± 3
mF-8060	130 ± 30	480 ± 110	24 ± 1
mF-8066	82 ± 29	400 ± 61	23 ± 2
C34[a]	6 ± 2	4 ± 0.4	23 ± 1

[a] C34 is a peptide fusion inhibitor derived from the C-HR of gp41 that targets the N-HR of gp41 (Chan et al., 1998).

from the National Institutes of Health AIDS Research and Reference Reagent Program. 293T cells were obtained from the American Type Culture Collection. gp160 expression plasmids pSVIII HBXc2, 89.6 and JR CSF were provided by Dr. J.Sodroski, Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute (Sullivan et al., 1995; Karlsson et al., 1996), and the NL4-3 gp160 expression plasmid pHenv was provided by Dr. E. Freed, HIV Drug Resistance Program, NCI (Freed et al., 1989). gp160 expression plasmids pSVIII BaL01 and BaL26 (Li et al., 2005, 2006) were provided by Dr. J. Mascola, BSL-3 Core Virology Laboratory, Vaccine Research Center, NIAID.

Env-pseudotyped HIV-1 preparation

Env-pseudotyped HIV-1 stocks were prepared essentially as described (Li et al., 2005, 2006; Gustchina et al., 2007). Exponentially dividing 293T cells were cotransfected using FUGENE6 transfection kit (Roche, Nutley, NJ) with the Env-deficient HIV-1 expression plasmid SG3Δenv and an Env-expressing plasmid in ratios proportional to their individual sizes (~16 μg total DNA per 50 to 80% confluent T-150 culture flask). Culture supernatants were collected 2 days post-transfection, filtered through 0.45 μm filter, and stored at −80 °C.

HIV-1 neutralization assay

Env-pseudotyped HIV neutralization assays were performed essentially as described (Li et al., 2005, 2006; Gustchina et al., 2007). Serial dilutions of Fabs in phosphate buffer saline (10 μl) were added to Env-pseudotyped virus (in 40 μl Dulbecco's modified Eagle medium plus 10% fetal calf serum), followed by the addition of freshly trypsinized TZM-bl indicator cells (JC53BL-13), a HeLa-derived cell line genetically modified to constitutively express CD4, CCR5, and CXCR4 (10,000 cells in 20 μL of the same medium). After incubation at 37 °C overnight, 150 μL of fresh growth medium was added. Approximately 48 h post-infection cells were lysed and luciferase activity was measured using the BrightGlo luciferase assay kit (Promega, Madison, WI) with a Synergy2 luminescence microplate reader (BioTek Instruments, Inc., Winooski, VT). Pseudovirus stocks were diluted to yield approximately 200 to 1000-fold increase of luminescence for an infected control over the uninfected control. IC_{50} values were obtained by a non-linear least-squares fit of a simple dose–activity relationship, given by % infection = 100 / (1 + [Fab] / IC_{50}), to the experimental data.

Analysis of neutralizing activity of mF-8066 in combination with N_{CCG}-gp41

The neutralizing activity of multiple constant-ratio combinations of mF-8066 antibody and N_{CCG}-gp41 protein was tested in serial dilutions in HXB2 Env-pseudotyped HIV neutralization assays. The data were analyzed as described previously (Gustchina et al., 2006).

Post-attachment HIV-1 neutralization assay

The neutralizing activity of Fabs after attachment of pseudovirus to the target cells following CD4 engagement was measured as described previously (Gustchina et al., 2008).

Synchronized and "time-of-addition" Env-pseudotyped HIV neutralization assay

Synchronized viral infection assays in the context of the HXB2 Env-pseudotyped virus neutralization assay were performed using the spinoculation technique (O'Doherty et al., 2000; Reeves et al., 2002) as described previously (Gustchina et al., 2008). The infectivity (y) as a function of the time post-infection (t) of addition of the Fabs was fit to a sigmoidal function given by $y = y_{max} / [1 + e^{(t_{1/2} - t)/k}]$ where $t_{1/2}$ is the half-life of the inhibitor-sensitive state of Env and k is a constant that determines the shape of the sigmoidal curve (Reeves et al., 2002).

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