

## DESIGNED OLIGOMERS OF CYANOVIRIN-N SHOW ENHANCED HIV NEUTRALIZATION

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*S.K.G conducted the experiments and analyzed the data showing that CVN<sub>2</sub>L0 is domain swapped in solution. S.K.G. created the original figure 4 (showing that the anti-HIV activity of CVN<sub>2</sub>L0 correlates with the number of functional binding sites), as well as supplemental figure S3 (showing that the partial binding site knockout mutants do not significantly affect the neutralization potency). S.K.G. wrote a draft to describe the experiments and results that led to figure 4 and S3. This draft was later rewritten and improved by J.R.K. J.R.K. also improved S.K.G.'s original figures (changed the original circles to squares) for publication.*

### 4.1 Abstract

Cyanovirin-N (CV-N) is a small, cyanobacterial lectin that neutralizes many enveloped viruses, including human immunodeficiency virus type I (HIV-1). This antiviral activity is attributed to two homologous carbohydrate binding sites that specifically bind high mannose glycosylation present on envelope glycoproteins such as HIV-1 gp120. We created obligate CV-N oligomers to determine whether increasing the number of binding sites has an effect on viral neutralization. A tandem repeat of two CV-N molecules (CVN<sub>2</sub>) increased HIV-1 neutralization activity by up to 18-fold compared to wild-type

CV-N. In addition, the CVN<sub>2</sub> variants showed extensive cross-clade reactivity and were often more potent than broadly neutralizing anti-HIV antibodies. The improvement in activity and broad cross-strain HIV neutralization exhibited by these molecules holds promise for the future therapeutic utility of these and other engineered CV-N variants.

## 4.2 Introduction

Cyanovirin-N (CV-N), a cyanobacterial lectin, is uniquely positioned to become a novel therapeutic and prophylactic for diseases caused by enveloped viruses. CV-N is a small, two-domain protein that neutralizes HIV by specifically binding to high mannose glycans on the envelope glycoprotein gp120, thereby preventing interaction of the virus with a host cell (1, 2). In addition to its potent activity against HIV, CV-N is also active against a number of other enveloped viruses including influenza (3, 4), Ebola (5, 6), hepatitis C (7), and herpesvirus 6 (2).

The two domains of CV-N are homologous in both their sequence (32% sequence identity and 58% sequence similarity (8)) and their three-dimensional structure (9, 10). Wild-type (WT) CV-N exists mainly as a monomer in solution and a domain-swapped dimer in crystals (Fig. S1). NMR structures of the monomer show that the protein is an ellipsoid with ten  $\beta$ -strands and four  $3_{10}$ -helical turns, approximately 55 Å in length and 25 Å wide (Fig. S1A). Each CV-N monomer contains two symmetrically-related structural domains (A and B), with each domain containing a carbohydrate binding site that specifically interacts with  $\alpha$  (1-2) linked oligomannose moieties within Man-8 or Man-9 glycans (9, 11-13). Domain A contains the N- and C-termini and includes residues 1–39 and 90–101, and domain B contains residues 40–89. Although purified as a monomer, a trapped, metastable domain-swapped dimer can be formed during folding and crystallization, and WT CV-N crystallizes exclusively as a domain-swapped dimer (10, 14, 15). In the domain-swapped dimer structure, domain A interacts with B' to form a “monomer-like unit,” whereas domain A' and B interact to form the second “monomer-like unit” (Fig. S1B and C). The domain swapping does not result in additional intramolecular interactions, but instead results from the extension of residues 50 to 53

across the interface. Carbohydrate binding is similar in the domain-swapped crystal structures and monomeric NMR structures, although binding in the B and B' domains are not seen in crystal structures due to potential steric constraints or crystal packing artifacts (15) (Fig. S1A and B).

The two binding sites in monomeric CV-N exhibit distinct affinities for carbohydrate in solution: the binding site in domain B, located distal from the N- and C-termini, has an equilibrium dissociation constant ( $K_D$ ) of approximately 140 nM for Man $\alpha$ 1  $\rightarrow$  2Man disaccharide, which is about 10-fold higher affinity than the binding site in domain A, located near the termini, which binds to Man $\alpha$ 1  $\rightarrow$  2Man disaccharide with a  $K_D$  of about 1.5  $\mu$ M (9). Numerous studies have shown, however, that both sites are necessary for viral neutralization and that destruction of either site renders the CV-N variant inactive (16, 17). However, a recent study showed that in the context of a CV-N dimer that was covalently crosslinked using disulfide bonds, two out of the four possible binding sites are sufficient to maintain neutralization activity, indicating that it is the number and not the identity of sites that is important for neutralization (18, 19). These results point toward a key role for avidity in the viral neutralization activity of CV-N.

A number of groups have attempted to study the oligomerization of CV-N to determine whether the domain swapping is a crystallization artifact or a biologically relevant state. However, because the domain-swapped dimer of WT CV-N is not stable at physiological temperatures, a significant amount of purified dimer may revert to monomer during the course of a viral neutralization assay (14). Therefore, mutations have been used to stabilize either the monomer (14) or the domain-swapped dimer (14, 20, 21). However, the effect of dimerization remains unclear, as some groups have concluded that the dimeric state is more active than monomeric WT CV-N (21), whereas others find that monomeric and dimeric variants have similar antiviral activities (20).

In this study, we show that by linking two CV-N molecules together in a head-to-tail fashion, we can stabilize the domain-swapped dimeric form of the protein in solution.

These linked dimers show enhanced HIV neutralization compared to WT CV-N against 33 strains from 3 clades. In addition, we show that although two carbohydrate binding sites are sufficient for activity as previously reported (18, 19), variants with more binding sites (three or four) have increased neutralization activity.

### 4.3 Results

**Design and Construction of CV-N Oligomers.** To directly assay the effects of multimerization on the activity of CV-N, we generated CV-N dimers (CVN<sub>2</sub>s) containing tandem repeats of CV-N in which the C-terminus of one copy of CV-N was linked to the N-terminus of the next copy through a flexible polypeptide linker. Because WT CV-N has the ability to domain swap, we hypothesized that the oligomeric molecules would adopt either a monomeric-like linked structure in which the two CV-N repeats are folded as monomers and connected through the linker (Fig. 1*A* and *B*) or a domain-swapped linked structure that takes a form similar to the domain-swapped dimeric crystal structures of WT CV-N but contains a direct linkage between the two CV-N repeats (Fig. 1*C* and *D*). We generated different versions of CVN<sub>2</sub> proteins using 10 different linker lengths ranging from 0 to 20 amino acids (Table S1). We also constructed trimers (CVN<sub>3</sub>) with linkers containing 0, 5, or 10 amino acids and a tetramer (CVN<sub>4</sub>) in which two CVN<sub>2</sub>L0 molecules were linked through a 20 amino acid linker.

**CV-N Oligomers Show Enhanced HIV Neutralization.** CV-N and CV-N oligomers were assayed for their ability to neutralize HIV in an in vitro luminescence-based neutralization assay (Fig. 2*A* and Table S2) (22). Initial characterizations of the CV-N proteins were conducted by assaying activity against the clade B HIV strain SC422661.8. WT CV-N showed half-maximal neutralization at concentrations (IC<sub>50</sub>s) between 1.0 and 9.4 nM (0.012 to 0.12 µg/mL) over 30 independent trials, with an average of  $4.4 \pm 2.6$  nM ( $0.054 \pm 0.032$  µg/mL), consistent with published values (20, 23-25).

Most dimeric variants (CVN<sub>2</sub>s) exhibited more potent HIV neutralization than WT CV-N, with enhancements of approximately 3- to 6-fold when correcting for the

increased molecular weight of the dimers (Fig. 2B). The single exception was CVN<sub>2</sub>L20, which displayed an IC<sub>50</sub> similar to that seen for WT CV-N. In this case, the long linker allows a different domain-swapping state relative to variants with shorter linkers. Additionally, CVN<sub>2</sub>L20 was only assayed on a single occasion (in triplicate) and therefore the true behavior of the molecule may not be accurately represented. Although there were significant increases in potency for most of the dimers, the addition of the third CV-N repeat to make the CVN<sub>3</sub> proteins did not improve HIV neutralization compared to the dimeric variants; in fact the trimers showed decreased potency compared to the lower molecular weight dimeric molecules (Fig. 2C). A tetrameric CV-N protein (CVN<sub>4</sub>) showed similar activity to the CVN<sub>3</sub> molecules (data not shown).

**CV-N Dimers Exhibit Broad Cross-Clade Reactivity and Are Comparable or More Potent Than Anti-HIV Antibodies.** We then assayed WT CV-N and two of our more potent dimers (CVN<sub>2</sub>L0 and CVN<sub>2</sub>L10) for their ability to neutralize other strains of HIV across different clades. The proteins were tested against a total of 33 strains from three clades. WT CV-N and the dimeric mutants effectively neutralized all 33 HIV pseudoviruses (IC<sub>50</sub>s less than 300 nM) (Table S3). Consistent with earlier results, the dimeric variants were more potent than WT CV-N against all strains tested (Fig. 3A). In 32 out of 33 cases, CVN<sub>2</sub>L0 neutralized virus with greater potency than CVN<sub>2</sub>L10, whereas CVN<sub>2</sub>L10 was the most potent against one clade C strain.

We were also interested in the overall potency of the CV-N proteins compared to known broadly neutralizing anti-HIV antibodies (NAbs): 4E10 (26), 2G12 (26, 27), 2F5 (26, 28), b12 (29), PG9 (30), PG16 (30), and VRC01 (31). IC<sub>50</sub> values for these NAbs were taken from the literature (22, 32, 33) or determined for this study (PG9, PG16, and VRC01) and converted to molarity for comparisons with the smaller CV-N proteins. When compared to each of the seven broadly neutralizing anti-HIV antibodies, CVN<sub>2</sub>L0 showed similar or greater potency against most of the strains tested (Fig. 3B). Since 2-fold differences in IC<sub>50</sub> values are generally not significant, we consider IC<sub>50</sub>s within 2-fold of the IC<sub>50</sub> of the antibody to be similar. CVN<sub>2</sub>L0 has similar or greater potency against 100% of the

viruses compared to 4E10, 2G12, and 2F5. Our molecule also fairs very well when compared to the newly discovered broadly neutralizing antibodies PG9 (72%), PG16 (66%), and VRC01 (77%).

**CV-N Dimers Are Domain-Swapped in Crystal Structures.** To elucidate a mechanism for the enhanced neutralization activity of the dimeric proteins, we solved crystal structures of CVN<sub>2</sub>L0 and CVN<sub>2</sub>L10 (*SI text*, Table S4, and Fig. S2). Both structures were very similar to each other and were intramolecularly domain-swapped with no major deviations relative to WT CV-N domain-swapped structures. Small differences between the CVN<sub>2</sub> structures and previously published WT CV-N structures were noted near the termini, as expected from the linkage, and also in the region of the domain swap. These differences, however, are minor and are unlikely to be responsible for the observed differences in antiviral activity.

**Binding Site Mutants of CVN<sub>2</sub>L0 Show That It Is Domain-Swapped in Solution.**

Although crystallographic studies definitively showed that the CVN<sub>2</sub> molecules are domain-swapped in crystals, it was still possible that the dimeric proteins were folded as two linked monomers (Fig. 1A) rather than as a domain-swapped dimer (Fig. 1B) in solution. To address this issue, we generated variants of CVN<sub>2</sub>L0 that contained previously described CV-N carbohydrate binding site knockouts (16, 34) (Fig. 4A). Each of the binding sites in a domain-swapped dimer (A, B, A', and B') are formed from residues in both CV-N repeats, so we created variants in which a binding site in either the A or B domain was knocked out completely in the context of the linked monomer dimeric model (CVN<sub>2</sub>L0<sub>ΔAmm</sub>, CVN<sub>2</sub>L0<sub>ΔBmm</sub>) or the domain-swapped dimeric model (CVN<sub>2</sub>L0<sub>ΔA</sub>, CVN<sub>2</sub>L0<sub>ΔB</sub>) (Table S5). In each case, if the model that the mutations were based on is correct, the mutations would form a single full binding site knockout (black squares in Fig. 4A). However, if the model is incorrect, the mutations would form two half-site knockouts (black triangles in Fig. 4A). We hypothesized that CVN<sub>2</sub>L0 mutants with a complete binding site knockout in solution would show decreased ability to neutralize HIV, whereas mutants with two half-site knockouts would be less severely

affected (34). Control variants with only a single half-site knockout showed only modest decreases in potency, verifying that half-site knockouts could be distinguished from complete binding site knockouts (Table S5, Fig. S3).

Variants with binding site knockouts made in the context of the domain-swapped dimeric model were significantly less active against HIV than those designed based on the monomeric model (Fig. 4B), indicating that the linked dimers are domain-swapped in solution as well as in crystals.

**The Number of Functional Binding Sites in CVN<sub>2</sub>L0 Is Directly Proportional to Its Anti-HIV Activity.** To determine whether CVN<sub>2</sub>L0 has enhanced neutralization activity relative to WT CV-N due to its increased number of binding sites, we created a series of binding site knockout mutants in which 1, 2, or 3 of the sites were fully knocked out (Fig. 4C). Variants with only a single binding site knockout showed approximately 20- to 35-fold decreases in potency relative to CVN<sub>2</sub>L0, whereas those with two binding site knockouts exhibited decreases of 80- to almost 2000-fold (Fig. 4D). Variants with three binding sites knocked out were unable to neutralize HIV at the concentrations tested (data not shown). These data are consistent with previously published accounts, which showed that at least two functional binding sites are required for activity (16, 19), and are also consistent with the hypothesis that avidity is an important factor for viral neutralization by CV-N. Interestingly, CVN<sub>2</sub>L0 variants that contain one functional A and one functional B binding site did not neutralize with the same potency as WT CV-N. CVN<sub>2</sub>L0<sub>ΔA||B</sub> and CVN<sub>2</sub>L0<sub>ΔAxB</sub> in which the active A and B binding sites are on the same pseudo-monomer or opposite pseudo-monomer (Fig. 4C), respectively, were 15- to 75-fold less potent than monomeric WT CV-N, which also contains only a single A and single B binding site. This observation indicates that factors in addition to avidity, including possible steric occlusion of binding sites and/or the relative orientation of binding sites, contribute to the potency of an oligomeric CV-N molecule.

#### 4.4 Discussion

By covalently linking two or more copies of CV-N together, we generated CV-N oligomers with significantly enhanced HIV neutralization activity compared to WT CV-N. CVN<sub>2</sub>L0 not only exhibited broadly neutralizing activity across three clades of HIV-1, but was also able to neutralize many HIV strains with potency similar to that of seven well-studied broadly neutralizing antibodies (4E10, 2G12, 2F5, b12, PG9, PG16, and VRC01). In addition, our crystallographic and mutagenesis studies revealed that the CVN<sub>2</sub>s form domain-swapped dimers in solution as well as in crystal form. By increasing the local concentration of CV-N through linking two molecules together, we have stabilized the domain-swapped dimeric form of the protein, allowing it to be stable under physiological conditions.

Previous studies were divided about whether the domain-swapped dimer of WT CV-N is more active than the monomeric form (20, 21). However, because the CV-N domain-swapped dimer is meta-stable under physiological temperatures and significant amounts can revert to monomer over the course of an assay (14), the domain-swapped form has been difficult to evaluate with current HIV neutralization assays. In contrast, our variants are covalently linked at their termini and are thereby effectively forced into the domain-swapped dimeric form by the effective increase in local concentration. Because there were no major structural differences between the linked dimers and WT CV-N, our results suggest that the dimeric species of WT CV-N would also be a more potent neutralization agent if it were stable during the assay. Therefore, other methods in which the dimer is stabilized may also result in increased neutralization activity.

In addition to the potential benefit of domain swapping, the simple increase in avidity in the CVN<sub>2</sub>s significantly improves the neutralization activity. WT CV-N itself has a high affinity for gp120 (1, 23), but by doubling the number of carbohydrate binding sites in the CVN<sub>2</sub> variants, the increase in avidity may prevent possible dissociation and escape of the virus. As shown by our knockout studies, CVN<sub>2</sub>L0 variants with more functional binding sites were significantly more potent at neutralizing HIV, indicating that higher



avidity translates to greater potency. Interestingly, we also found that deletion of a binding site in the B domain had a greater effect on the ability to neutralize HIV than deletion of a binding site in the A domain. CVN<sub>2</sub>L0<sub>ΔBB</sub>, which contains only two functional A sites, is approximately 15-fold worse at neutralizing HIV than CVN<sub>2</sub>L0<sub>ΔAA</sub>, which contains only two functional B sites. This is consistent with earlier studies that showed that binding site B has an approximately 10-fold lower K<sub>D</sub> for Man $\alpha$ 1-2Man than binding site A (9) and may indicate that the overall activity of CV-N could be improved by improving the affinity of site A.

An alternate mechanism for increased neutralization could result from the fact that the binding sites in CVN<sub>2</sub>s can potentially sample distances farther apart than the binding sites in monomeric WT CV-N. The wider spacing could allow CVN<sub>2</sub>s to crosslink glycosylation sites within a single gp120, across multiple gp120 subunits on an envelope spike or, less likely, across multiple spikes. This crosslinking would prevent a larger number of gp120 subunits from binding to CD4, the primary receptor for HIV, than would be blocked by WT CV-N, thus decreasing the IC<sub>50</sub>. An interesting note is that in one conformation of the domain-swapped structure of WT CV-N (Fig. S1B), every pair of carbohydrate binding sites is approximately 30 to 35 Å apart (Fig. 5A). The neutralizing antibody 2G12, which is also domain-swapped and binds carbohydrates on gp120, has carbohydrate binding sites that are also approximately 30 to 35 Å apart (35) (Fig. 5B). Perhaps by stabilizing the domain-swapped structure of CV-N, the carbohydrate binding sites of the CVN<sub>2</sub> variants are optimally positioned to interact with gp120.

While addition of a second CV-N molecule increases the potency of HIV neutralization significantly, the addition of a third or fourth CV-N repeat (CVN<sub>3</sub>, CVN<sub>4</sub>) does not increase it further. Although the mechanism for enhanced activity is not fully understood, perhaps non-domain-swapped CV-N repeats do not have a significant impact on the activity; for example, one of the three repeats in trimeric CVN<sub>3</sub> molecules may have little effect on activity. Alternatively, due to the close proximity of the N- and C-termini in the

WT structure and their proximity to the lower affinity carbohydrate binding site (site A), the additional CV-N molecule(s) may sterically occlude access to some of the carbohydrate binding sites in the molecule, rendering those sites nonfunctional and therefore inhibiting any additional effect. Longer linkers, including structured linkers, may be necessary to prevent steric occlusion of the binding sites.

WT CV-N and the CVN<sub>2</sub> molecules show excellent cross-clade and cross-strain reactivity. This property is promising for the development of these or other variants for therapeutic use, as they can potentially be used throughout the world. Because of the increase in potency relative to WT CV-N, CVN<sub>2</sub>L0 could be more effective in any prophylactic treatment protocol that WT CV-N is currently being investigated for, including gels, suppositories, and in vivo *Lactobacillus* delivery (36, 37). In addition to the increase in potency of CVN<sub>2</sub>L0, the lack of a proteolytically sensitive linker between the CV-N repeats suggests that this variant will probably have similar stability in vivo as WT CV-N. CVN<sub>2</sub>L0 shows similar potency to many of the broadly neutralizing antibodies that have recently been reported but is easier to express than intact antibodies and therefore could be used for a range of therapeutics that are intractable for antibodies. CV-N variants could also theoretically be used in combination therapy with anti-gp120 antibodies to direct gp120 evolution toward decreased glycosylation. Glycosylation itself has been shown to be important in the folding and function of viral glycoproteins (38), and in the case of HIV, deglycosylation of gp120 diminishes its binding to CD4, making the virus less infective (39, 40). Alternatively, deglycosylation of gp120 could reveal protein epitopes that can be recognized by the adaptive immune system, allowing the immune system to fight off infection more effectively.

#### 4.5 Materials and Methods

**Construct Generation.** The gene for WT CV-N was constructed using a recursive PCR method with 40-mer synthesized oligos (41), then sub-cloned into the NdeI and BamHI sites of pET11a. The protein contained an N-terminal 6-histidine purification tag followed by a Factor Xa protease cleavage site. CVN<sub>2</sub>L5 and CVN<sub>2</sub>L10 were constructed

using PCR-based cloning to insert a tandem repeat of the WT CV-N gene and sequence encoding the flexible polypeptide linker into the WT plasmid. The CVN<sub>3</sub>L5 gene was created by inserting an *Escherichia coli*-optimized WT CV-N DNA sequence between the two existing copies of the WT gene in CVN<sub>2</sub>L5. Other dimeric and trimeric genes of varying linker lengths were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) to insert or delete codons corresponding to linker amino acids. All constructs were verified through DNA sequencing and restriction analysis to ensure the correct sequence and number of CV-N repeats.

Binding site knockout mutant constructs were generated in the background of a CVN<sub>2</sub>L0 template gene containing two distinct DNA sequences for each CV-N repeat. Mutations were made using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene).

**Protein Expression and Purification.** The expression of WT CV-N and all oligomeric variants was induced with IPTG in BL21(DE3) *E. coli* cells in LB including ampicillin. The harvested cells were lysed using an EmulsiFlex-C5 (Avestin, Inc.), and the insoluble fraction was resuspended in buffer containing 6 M GnHCl and 10 mM imidazole and centrifuged to remove debris. The solubilized CV-N was then purified under denaturing conditions using a Ni-NTA gravity column (Qiagen) and refolded by dialyzing the Ni-NTA eluate against native buffer overnight at room temperature (42). Following refolding, proteins were additionally purified on a Superdex-75 column and eluted in 25 mM sodium phosphate pH 7.4, 150 mM NaCl. The N-terminal 6-histadine purification tag was not removed prior to functional or structural assays. Pure protein was concentrated or stored as eluted at 4°C.

Amino acid analysis was performed on WT CV-N, CVN<sub>2</sub>L5, CVN<sub>2</sub>L10, CVN<sub>3</sub>L5, and CVN<sub>3</sub>L10 to determine extinction coefficients at 280 nm (Texas A&M University). These experimentally determined extinction coefficients (WT: 10471 M<sup>-1</sup>cm<sup>-1</sup>; CVN<sub>2</sub>s: 20800 M<sup>-1</sup>cm<sup>-1</sup>; CVN<sub>3</sub>s: 32000 M<sup>-1</sup>cm<sup>-1</sup>) were used to calculate the protein concentration.

**HIV Neutralization Assays.** HIV-1 pseudovirus particles from pseudotyped primary virus strains were prepared as described (22, 43). The SC422661.8 strain (clade B) was used for all assays unless otherwise noted. HIV neutralization assays were performed either in-house (Fig. 2 and Table S2) or by the Collaboration for AIDS Vaccine Discovery (CAVD) core neutralization facility (Fig. 3 and Table S3) as previously described (22). Briefly, 250 infectious viral units of virus per well were incubated with three-fold dilutions of CV-N or a CV-N variant in triplicate (our assays) or duplicate (CAVD assays) for one hour at 37°C after which approximately 10,000 Tzm-B1 cells were added to each well and incubated for 48 h. The cells were then lysed using Bright Glo Luciferase Assay Buffer (Promega), and luciferase expression was assayed using a Victor<sup>3</sup> Multilabel Counter (PerkinElmer).

To determine the  $IC_{50}$  of neutralization, the luminescence was first averaged across the replicates, then the percent neutralization (*%Neutralization*) was calculated using Equation 1, where  $RLU$  is the average relative luminescence for a given concentration,  $CC$  is the average luminescence from the cell control wells, and  $VC$  is the average luminescence from the viral control wells. The percent of virus neutralized was then plotted as a function of neutralizing protein in Kaleidagraph (Synergy Software) and fitted to Equation 2, where  $IC_{50}$  is the concentration of CV-N at which half of the virus is neutralized,  $C$  is the concentration of CV-N, and  $m$  is a Hill coefficient.  $IC_{50}$ s are reported as the average of a minimum of four independent trials and the error reported is the standard deviation of the  $IC_{50}$ s from those trials. CVN<sub>2</sub>L20, CVN<sub>3</sub>L5, CVN<sub>3</sub>L10, and the binding site mutants were tested on only one occasion and therefore a standard deviation is not reported.

$$\%Neutralization = \left(1 - \frac{RLU - CC}{VC - CC}\right) * 100 \quad [1]$$

$$\%Neutralization = \frac{100}{1 + \left( \frac{IC_{50}}{C} \right)^m} \quad [2]$$

**Crystallization, Crystallographic Data Collection, and Refinement.** See *SI text*.

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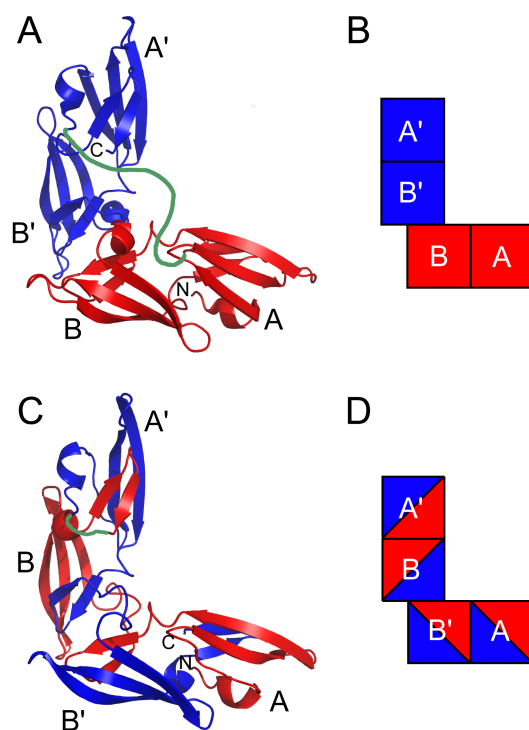
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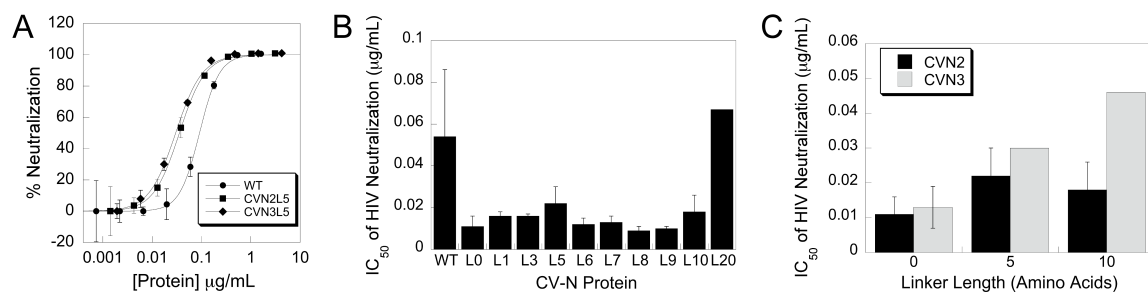
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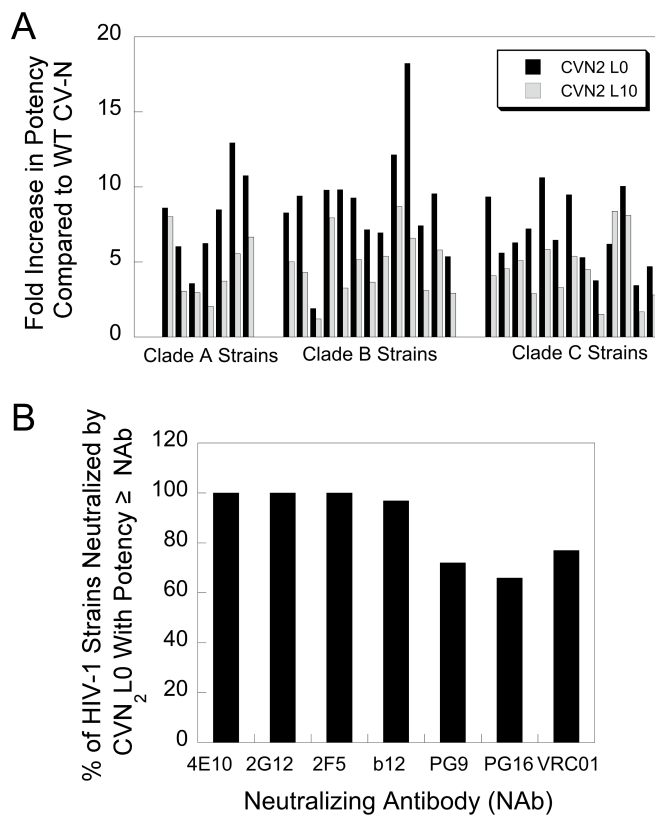
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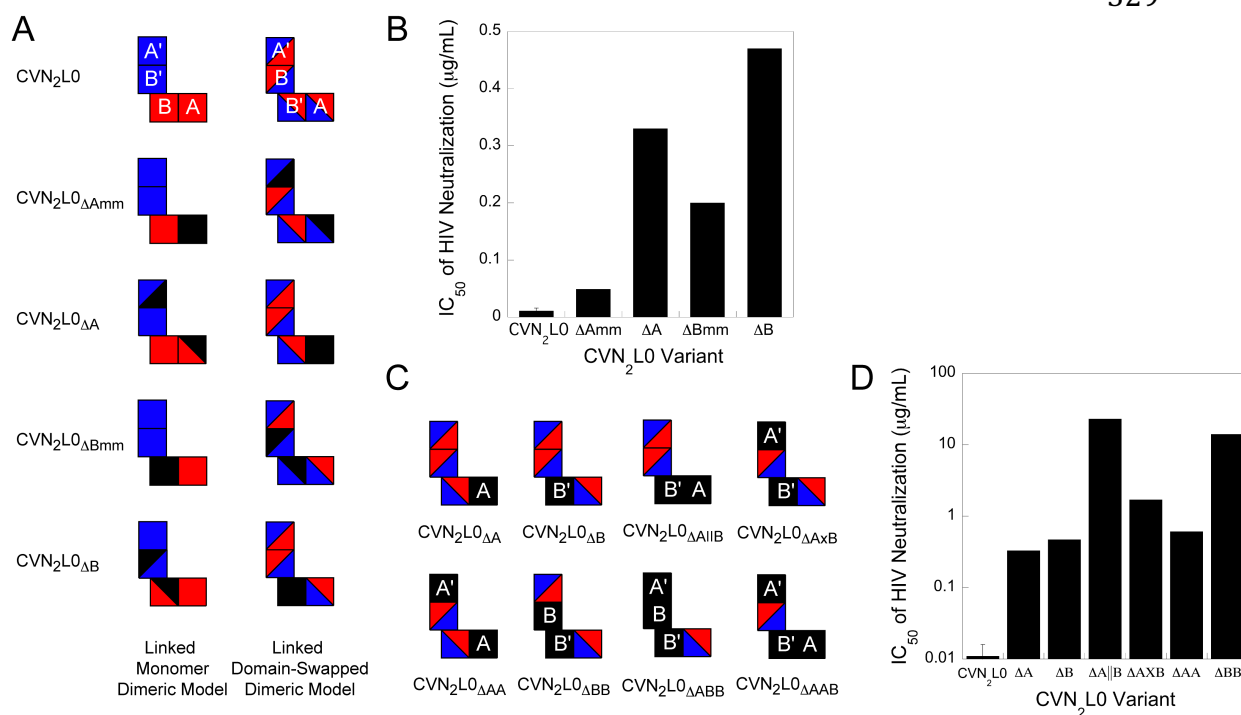
**Fig. 1.** Two models of CVN<sub>2</sub> proteins. (*A, B*) The linked monomer dimeric model for CVN<sub>2s</sub>. (*C, D*) The linked domain-swapped dimeric model for CVN<sub>2s</sub>. The CV-N repeats are shown in red and blue, and the flexible polypeptide linker is modeled in green. The N- and C-termini are labeled N and C. Each of the four carbohydrate binding domains are labeled A, B, A', or B' in each model. Models (*A*) and (*C*) are based on solved WT CV-N structures (10, 51) and (*B*) and (*D*) are block representations of the models in (*A*) and (*C*), respectively.



**Fig. 2.** CV-N oligomers exhibit enhanced HIV neutralization. (A) Typical neutralization data and curve fits for WT CV-N and two variants run in triplicate on the same 96-well plate. (B) The IC<sub>50</sub>s of HIV neutralization for WT CV-N and CVN<sub>2</sub> dimers containing varying linker lengths. All linked dimers show significant enhancements in their HIV neutralization compared to WT CV-N, and dimers containing 0 to 10 amino acid linkers are more potent than CVN<sub>2</sub>L20.  $N \geq 4$ ; CVN<sub>2</sub>L20,  $N = 1$ . (C) IC<sub>50</sub>s of HIV neutralization for CVN<sub>2</sub>s and CVN<sub>3</sub>s of the same linker length.  $N \geq 3$ ; CVN<sub>3</sub>L5 and CVN<sub>3</sub>L10,  $N = 1$ . (A-C) Error bars = SD.



**Fig. 3.** CV-N dimers neutralize HIV broadly and potency is similar to broadly neutralizing anti-HIV antibodies. (A) The designed dimers show enhanced neutralization activity relative to WT CV-N across all 33 strains tested from 3 clades. CVN<sub>2</sub>L0 is more potent than CVN<sub>2</sub>L10 in 32 of 33 cases. (B) When the IC<sub>50</sub>s of CVN<sub>2</sub>L0 neutralization against a panel of HIV-1 strains were compared to the IC<sub>50</sub>s of seven broadly neutralizing antibodies (Table S3), we saw that most strains were as sensitive to CVN<sub>2</sub>L0 as they were to the broadly neutralizing antibodies. Since 2-fold differences in potency are generally not significant, similar potency ( $\geq$ ) is defined as a potency for CVN<sub>2</sub>L0 that is within 2-fold of the potency of the antibody or higher.

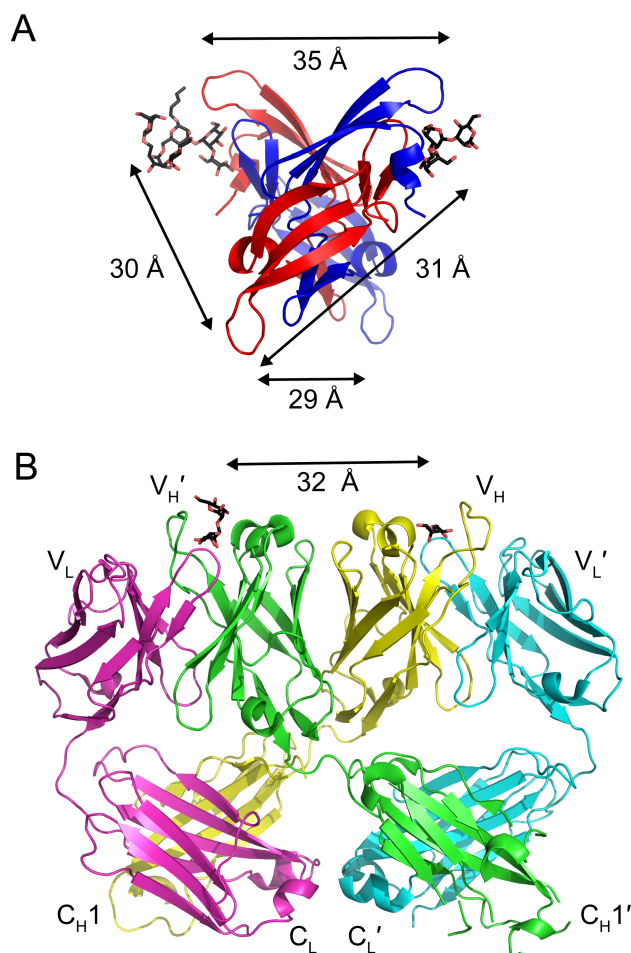


**Fig. 4.** Anti-HIV activity of CVN<sub>2</sub>L0 correlates with number of functional binding sites.

(A) Schematic representation of variants to determine whether CVN<sub>2</sub>L0 is in a linked monomer dimeric structure (mm) or domain-swapped dimeric structure. The two CV-N repeats are represented in red and blue, as in Fig. 1B and D. Black triangles represent partial carbohydrate binding site knockouts and red squares represent complete binding site knockouts. (B) HIV neutralization results for mutants in panel A. Mutants with full binding site deletions in the context of the domain-swapped dimer model have more significant increases in their HIV neutralization IC<sub>50</sub>s compared to mutants with full binding site deletions in the context of the monomer model. (C) Schematic representations of multiple binding site mutants. All variants contain one or more complete binding site knockouts according to the CVN<sub>2</sub>L0 domain-swapped dimer model. Black squares represent binding sites that have been knocked out, and squares

containing red and blue triangles represent WT (functional) binding sites. (*D*) HIV neutralization results for mutants in panel *C*. The number of functional binding sites in CVN<sub>2</sub>L0 is proportional to its ability to neutralize HIV. Mutants with two functional binding sites are less active than those with three sites. Additionally, the deletion of a B binding site has a greater effect on activity than the deletion of an A binding site.





**Fig. 5.** Similarity in carbohydrate binding site spacing in CV-N and the 2G12 anti-HIV (Fab)<sub>2</sub>. (A) Each of the four carbohydrate binding sites in one WT CV-N crystal structure (15) (P4<sub>1</sub>2<sub>1</sub>2 space group) is approximately 30 to 35 Å from the other sites (structure is viewed from the bottom with respect to Fig. 1). Carbohydrates (shown as sticks with black carbons and red oxygens) were only resolved in the A binding sites in the crystal structure. (B) Ribbon diagram of the domain-swapped (Fab)<sub>2</sub> from IgG 2G12, a broadly neutralizing antibody specific for carbohydrates on gp120 (34). The domain swapping creates a rigid (Fab)<sub>2</sub> dimer in which the carbohydrate binding sites at the antigen

combining sites are spaced approximately 30 to 35 Å apart. Carbohydrates are shown as sticks with black carbons and red oxygens and antibody domains are labeled.