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Vaccine Focusing to Cross-Subtype HIV-1 gp120 Variable Loop Epitopes

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Abstract

We designed synthetic, epitope-focused immunogens that preferentially display individual neutralization epitopes targeted by cross-subtype anti-HIV V3 loop neutralizing monoclonal antibodies (mAbs). Vaccination of rabbits with these immunogens resulted in the elicitation of distinct polyclonal serum Abs that exhibit cross-subtype neutralization specificities mimicking the mAbs that guided the design. Our results prove the principle that a predictable range of epitope-specific polyclonal cross-subtype HIV-1 neutralizing Abs can be intentionally elicited in mammals by vaccination. The precise boundaries of the epitopes and conformational flexibility in the presentation of the epitopes in the immunogen appeared to be important for successful elicitation. This work may serve as a starting point for translating the activities of human broadly neutralizing anti-HIV-1 monoclonal antibodies (bNAbs) into matched immunogens that can contribute to an efficacious HIV-1 vaccine.

Keywords

immunofocusing; HIV-1 vaccine; V3 loop; antibodies

INTRODUCTION

The envelope spike of HIV (gp120, gp41) is highly antigenically variable and is the primary target of human antibody-mediated HIV neutralization (1-4). Clinical and preclinical studies

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have demonstrated that most HIV-specific antibodies (Abs) elicited via gp120 immunization are strain-specific or narrowly reactive and cannot protect against HIV acquisition(5-12). Nevertheless, a few Abs raised by immunization can protect against infection by HIV or related viruses in animal models(13-25) and in humans (26-29). In addition, some monoclonal antibodies isolated from HIV-infected subjects cross-react broadly with HIV-1 viruses(30-35). Focused presentation of the epitopes targeted by these Abs on non-HIV scaffolds has been proposed as a way to specifically elicit desirable Abs in mammalian hosts by vaccination, while avoiding elicitation of the type-specific or narrowly reactive Abs that dominate the response to immunization with whole gp120. “Epitope-focusing”, which can be viewed as a form of “reverse vaccinology”, has long been proposed as a method to achieve focused presentation of epitopes on synthetic immunogens(36, 37). These methods start from a high-resolution crystal structure of the complex of a neutralizing monoclonal antibody (mAb) isolated from HIV-infected human subjects and seek to mimic, by protein design, the 3D structure of the epitope within a non-HIV scaffold protein. To this point, immunogens designed to elicit specific anti-HIV bNabs in mammalian serum via vaccination using epitope-focused immunogens have uniformly failed to elicit cross-strain HIV neutralizing serum Abs(38-41).

We sought to design immunogens to specifically and preferentially elicit polyclonal cross-subtype HIV neutralizing Abs targeting cross-strain neutralizing epitopes also targeted by mAbs 3074 and 2219, which occur in the third sequence variable (V3) loop of gp120 in up to 80% of circulating HIV viruses (42-47). We designed these immunogens using the novel epitope-focusing strategy described in this report, and tested them for immunogenicity along with native constructs in a parent study (48). Here, we analyze in detail the functional and structural properties of these constructs with regard to whether we preferentially elicited polyclonal serum antibodies mimicking the specificity of the mAb, based on which the immunogen used for elicitation was designed.

METHODS

Immunogen Design and Production

Antigens, consisting of sequence-modified V3 loops, for both the protein immunogens and for their mirror chimeric SF162-V3 loop pseudoviruses (psVs) were designed as described in Figure 1. V3 loop sequences designed in this manner were inserted into a cholera toxin subunit B (CTB) scaffold at an appropriate fusion point, and tested by ELISA for reactivity with specific mAbs as previously described (49). Genes for CTB with the indicated inserts consisting of full-length V3 loops with the V3 crown sequence modified as in Figure 1 were chemically synthesized and cloned into pSUMO plasmids, followed by expression in *E. coli* and purification by affinity chromatography. The final sequences for the designed immunogens were: 2219- MTPQNITDLC AEYHNTQIHT LNDKIFSYTE SLAGKREMAI **ITFC**TRPSNN **TRKSINFGPG QTFYATGEII GDIRQAHC**AT FQVEVPGSQH IDSQKKAIER MKDTRLRIAYL TEAKVEKLCV WNNKTPRAIA AISMAN 3074- MTPQNITDLC AEYHNTQIHT LNDKIFSYTE SLAGKREMAI **ITFC**TRPSNN **TTESINIGPG QTFYATGEII GDIRQAHC**AT FQVEVPGSQH IDSQKKAIER

MKDTLRIAYL TEAKVEKLCV WNNKTPRAIA AISMAN The inserted V3 sequence is in bold and the mutations are red; Figure 1.

Binding of the immunogen constructs to anti-V3 mAbs

Immunogen-Ab binding was evaluated by ELISA as previously described (49).

Rabbit immunizations

The DNA-prime, protein-boost immunizations and serum harvesting were performed as previously described (50). A codon-optimized *env* gene from HIV clade C primary isolate 92BR025.9 for the DNA prime was prepared where the V3 sequence is: CTRPNNNTRKSIRIGPGQAFYATGEIIGDIRQAHC. Five animals of each group received the DNA prime 3 times via Gene Gun followed by two boosts with either V3₂₂₁₉-CTB, V3₃₀₇₄-CTB or V3₄₄₇-CTB (V3 sequence is identical with clade B consensus) at weeks 10 and 14. A total of 100µg/per injection of each V3-CTB was administered intramuscularly with incomplete Freund's adjuvant (IFA). Blood samples were collected prior to immunization and two weeks after each immunization.

Virus construction

Chimeric pseudoviruses (psVs) were constructed and produced by standard methods that have been previously described (51). SF162 Env variants containing modified V3 sequences were generated by sequentially introducing the necessary modifications by site-directed mutagenesis using the QuikChange kit, as described by the manufacturer (Stratagene, Inc.). The sequences of all mutant Envs were confirmed by sequencing the complete gene (Genewiz, Inc.).

The sequences of the V3 loops of the chimeric psVs used in neutralization experiments in Figure 2 were: psV-SF162-V3²²¹⁹: CTRPSNNTRKSINFGPGQAFYATGDIIGDIRQAHC
psV-SF162-V3³⁰⁷⁴: CTRPSNNTRESIRIGPGQTFYATGDIIGDIRQAHC

These have the same distribution of 2219, 3074 and 447 epitopes as the V3 loop sequences inserted into the scaffold to produce V3₂₂₁₉-CTB or V3₃₀₇₄-CTB, but they differ in the minor underlined positions from the immunogen V3 loop sequences (as they were constructed for testing before the immunogen designs were finalized). These minor non-epitope amino acid differences are believed not to have a significant structural influence on the V3 loop crown due to the Ab specific behavior of similarly altered psV. The sequences of the chimeric psV's bearing consensus subtype V3 loop sequence shown in Figure 3 were previously published (50). The sequences of the V3 loops of the chimeric psVs with specific epitopes perturbed (Figure 5B) are: Consensus B:

CTRPNNNTRKSIHIGPGRAFYTTEIIGDIRQAHC “-447, +2219, +3074:

CTRPNNNTRKSIHIGPGQAFYTTEIIGDIRQAHC “-3074, +2219”:

CTRPNNNTRKSIHMGPGRAFYTTEIIGDIRQAHC “-2219, +3074”:

CTRPNNNTRRESIHIGPGRAFYTTEIIGDIRQAHC “-3074, -2219”:

CTRPNNNTRRESIHMGPGRAFYTTEIIGDIRQAHC where the bolded underlined residues are the mutations perturbing the respective epitopes.

Neutralization assays

Neutralization assays using chimeric psVs were performed as described previously (47,51). Standard NAb and serum neutralization assays performed by the Vaccine Immune Monitoring Center of the Center for AIDS Vaccine Collaboration were also performed as previously described(52, 53). Briefly, 8 pseudoviruses from the clade B and 6 from the clade C standard Tier 2 psV panels(54, 55) were used along with an additional four Tier 1A and Tier 1B psVs from clades AG, B and C. Two-fold serial dilutions of heat-inactivated sera were prepared starting at a dilution of 1:10. The serum/psV mixtures were then incubated with the TZM.bl target cells and luciferase activity was measured at 48 hr. Pools of pre-bleed sera were tested as negative controls against each psV, and all sera were also tested against a negative control psV carrying the envelope of murine leukemia virus. The percent neutralization was calculated relative to the effect of the pre-immune serum from the same rabbit at the same dilution. All sera were assayed in duplicate in at least two experiments against each virus. The 50% neutralizing titers (NT₅₀) were determined using the method of Least Squares.

RESULTS

Three structural classes of V3 loop-targeted cross-subtype neutralizing mAbs have been defined: those similar to mAb 3074, those similar to mAb 2219 and those similar to mAb 447-52D(44, 57-59). We designed antigenic sequences to mimic the flexible structure of the crown of the V3 loop with amino acid mutations incorporated to disrupt the neutralization epitopes targeted by 447-52D but preserve the neutralization epitopes targeted by 3074 or 2219 respectively (Figure 1). The same approach was applied to the other two V3 mAbs (3074 and 2219). The designed sequences consisted of the consensus subtype C V3 loop sequence with point mutations introduced to “knock out” the neutralization epitopes targeted by 447-52D and either 2219 or 3074 (see Methods). Briefly, we reasoned that the peptide segment bearing the desired epitope needed to be present in a flexible loop and that this could be achieved by starting with a known V3 loop (consensus subtype C sequence) and introducing point mutations to “knock out” all but one desired neutralization epitope. The identity of the specific amino acid mutated was determined by our previously derived highly specific sequence motifs for the neutralization epitopes targeted by mAbs 2219, 3074, and 447-52D (46). In addition, we hypothesized that charged amino acids attract antibody responses, so we “knocked out” charged amino acids in the consensus V3 loop sequence that do not contribute to the target epitope. The designed antigens were inserted into an optimal location on the 3D-structure of the cholera toxin subunit B (CTB) protein as previously described (49) to create chimeric, epitope-focused HIV-1 V3-CTB immunogens. The immunogen designed to preferentially present the 2219-targeted neutralization epitope specifically binds mAb 2219, indicating a successful design (Figure 2A). The second immunogen intended to preferentially mimic the neutralization epitope targeted by 3074 was more broad in its binding, confirming the lesser clade and sequence dependence of mAb 3074.

The neutralization selectivity of the designed V3 loop antigens was tested by chimeric HIV-1-derived pseudovirus (psV) bearing an exposed V3 loop crown whose sequence

exhibited the identical epitope repertoire to that of the designed 3074- and 2219-mimicking antigenic loop in the protein immunogens. Both of these psV constructs were neutralized extremely strongly (IC₅₀ at single nanograms/ml) only by the 3074 antibody and not by the other two mAbs 2219 and 447-52D. As expected, a similarly designed chimeric HIV-1-derived psV preferentially presenting the 2219 epitope in its V3 loop crown also showed 2219-specific behavior (Figure 2B). Thus, the two immunogens preferentially display 3074(broadest) and 2219 (broad) Ab specific binding and neutralization epitopes, as well as the relatively strong clade B (R at position 18) dependence of mAb 447-52D.

Groups of five rabbits were immunized with the immunogens using a previously described protocol consisting of a DNA gp120 prime followed by a protein boost consisting of one of the two designed chimeric V3-CTB proteins (49). The resulting sera from the rabbits potentially neutralized 7 chimeric psVs modified from the SF162 strain and bearing the consensus V3 loop sequence of a different HIV subtypes(51) (Figure 3). The SF162 strain of HIV exhibits a perpetually exposed V3 loop, thus all synthetic psVs derived from this strain exhibit a V3 loop that is fully accessible to anti-V3 loop Abs (i.e. unmasked V3 loops). Specifically, the results show that the Abs raised in the rabbit serum by immunization with V3₃₀₇₄-CTB neutralize psVs bearing the unmasked V3 loops of all seven major HIV group M subtypes at a relatively high titers (46). Psedoviruses with non-subtype-B V3 loops appear to be more strongly neutralized by the V3₃₀₇₄-CTB elicited Abs. These results are consistent with the broad occurrence of the epitope targeted by mAb 3074(46). Abs raised in rabbit serum by immunization with the V3₂₂₁₉-CTB immunogen also neutralize 6 of the 7 diverse consensus V3 loops in SF162 psV, with maximal activity against subtype B but at much higher titers in contrast to the profile observed for immunization with V3₃₀₇₄-CTB. The results show that the Abs raised in the rabbit serum by immunization with V3₃₀₇₄-CTB (blue bars) or V3₂₂₁₉-CTB (orange bars) neutralize unmasked V3 loops from multiple subtypes, which suggests that the 3074-like polyclonal antibody response and the 2219-like polyclonal antibody response raised in the serum was potent and crosses subtypes. The Abs raised by a CTB fusion protein bearing the consensus subtype B V3 loop insert (CTB-V3/B) show a profile consistent with that seen in prior studies and distinct from that observed with V3₃₀₇₄-CTB or V3₂₂₁₉-CTB (48) (50). Indeed, sera from both groups of rabbit experiments neutralized multiple strains from different subtypes in a standard panel of psVs (Table I, Figure 4), as well as primary isolates BX08, BZ167 and DJ263 from different subtypes B and AG (data not shown). This is a similar pattern of neutralization to that observed for the 3074 and 2219 monoclonal antibodies (30). Thus, cross-subtype serum neutralizing Abs similar in breadth of viral reactivity to mAbs 3074 and 2219 were raised by these immunogens via vaccination.

The variability of response between the immunized animals was significant in the previous parent study (48), posing difficulties in drawing a conclusion about the fine specificity of the neutralizing response on average. Here we attempted to determine the neutralization specificity of the rabbit serum response to our immunogens. Six subtype C Tier 2 psVs were tested both for neutralization by mAb 3074 and for neutralization by the serum elicited in a rabbit by V3₃₀₇₄-CTB. Interestingly, the same three psVs are neutralized by both mAb 3074(30) and by the most potent individual rabbit serum elicited by V3₃₀₇₄-CTB (Table II). It is well established that Tier 2 HIV-1 viruses are difficult to neutralize by most antibodies

or sera (53). In this study, the three viruses not neutralized by the mAb were also not neutralized significantly by the V3₃₀₇₄-CTB-elicited serum. Notably, of the three viruses that were neutralized by both: serum and mAb, the order and proportional change of ID₅₀ of the serum from weakest to strongest is nearly the same as the order and proportional change of the IC₅₀ of mAb 3074 against those same viruses (Table II). The serum elicited by V3₂₂₁₉-CTB did not significantly neutralize any subtype C Tier 2 viruses. Overall, 6 of 18 of the psV tested from published standard panels were neutralized to some extent at greater than 1:10 titer by at least one of the vaccinated rabbits: all of the Tier 1 psVs, 2 out of the 7 Tier 2, subtype B psVs and 3 out of the 7 Tier 2, subtype C psVs (Figure 4).

To further characterize the neutralization specificity, chimeric psV were designed to perturb one or both of the epitopes targeted by mAbs 3074 and 2219. For each designed psV, mutations were introduced into the consensus B V3 loop that rendered the psV resistant only to the intended mAb and not to other mAbs (Figure 5A). These psVs represent specific probes for the neutralization specificity of the immune sera of the rabbits. For example, if most of the Abs elicited by vaccination with V3₃₀₇₄-CTB are 3074-like, the serum would show little neutralization potential against the psV lacking the 3074-targeted epitope. The neutralization of the psV lacking only the 3074 epitope by pooled sera from the animals immunized with V3₃₀₇₄-CTB was indeed dramatically reduced as compared to a psV containing the unmodified epitope (Figure 5B). Similarly, the neutralization of the psV lacking only the 2219 epitope by pooled sera from animals immunized with V3₂₂₁₉-CTB was significantly reduced as compared to a psV containing the unmodified epitope. The neutralization of a psV lacking both the 3074 and 2219 epitopes was reduced radically for both immune sera tested (Figure 5B). This shows that specific point mutations in V3 were able to selectively eliminate the 2219 and 3074 epitopes. Thus, in both cases, the serum antibodies elicited were similar in neutralization specificity to 3074 and 2219 respectively, based on each of the respective immunogens was used

DISCUSSION

Undesirable, narrowly-reactive or strain specific Abs responses dominate upon gp120-based immunizations in mammals, yet epitopes associated with protective or broadly neutralizing Abs are present in most gp120 proteins. We pursuing an epitope-focused approach in a previously published parent study (48), in which only desirable V3 loop epitopes were presented for elicitation. The results indicated that the isolated V3 loop could potentially elicit cross-subtype neutralizing Abs of equivalent activity to whole recombinant gp120 immunogens (48). The more detailed analytic data presented in this report suggest further that this response was successfully rationally engineered to preferentially present the neutralization epitopes targeted by mAbs 3074 – V3₃₀₇₄-CTB and V3₂₂₁₉-CTB, which elicited, via vaccination, a corresponding HIV neutralizing antibody response in rabbits. The response was almost entirely composed of and recapitulated, to a measurable degree, the specificity of mAbs 3074, 2219 and 447-52D. This conclusion is supported by the observations that: 1) the responses to the two different immunogens trended towards different specificities against different psVs (Figure 3); 2) perturbation of the epitope in question knocked out most of the neutralizing response observed in the serum (Figure 5);

and 3) the same three Tier 2 panel C viruses neutralized by mAb 3074 were also neutralized by the Abs elicited to mimic 3074 in the most responsive animal (Table II).

The response to V3₃₀₇₄-CTB immunogen appears to have been distinct from that for V3₂₂₁₉-CTB, and both resulted in neutralizing animal sera that recapitulated the cross-subtype breadth of their parent cross-subtype neutralizing Abs. A degree of cross-elicitation appears to have occurred indicating that perturbation of just one of the epitopes does not completely eliminate the neutralization activity in either of the sera, while perturbation of both nearly does. This suggests that the formation of these particular epitopes are linked structurally to some degree, which is not surprising since they overlap in the same peptide segment of the V3 loop. Although significant specific cross-subtype neutralizing Ab mimicry was achieved, the approach can be improved. Most importantly, the inter-animal variability was high and most animals did not respond to the maximal degree.

Despite the extremely high neutralization titers achieved by V3₃₀₇₄-CTB immunization against unmasked/accessible epitopes (Figure 3), these sera have much lower neutralizing activity for Tier 2 and primary isolates (Table II, Figure 4). Nevertheless, the goal of the present study was to engineer an elicited Ab response with the same specificity as mAbs, and this neutralization pattern against Tier 2 viruses and primary isolates matches that observed for the targeted mAbs. On the other hand, close to half of the Tier 1 and Tier 2 pseudoviruses from a published standard panel were weakly neutralized by at least one of the polyclonal sera resulting from one or the other immunogen (Figure 4), which is among the best elicited neutralization reported to date for any gp120-based immunogen (48). This performance mirrors the pattern seen with the mAbs on which the immunogens were designed to elicit the Ab responses in the sera (30). For example, comparing the potency of a mAb (in $\mu\text{g/ml}$) to a serum titer is not possible without knowing the exact amount of the relevant Ab in the serum. However, we found that the order and proportional change of ID₅₀ of the serum elicited by V3₃₀₇₄-CTB from weakest to strongest is nearly the same as the order and proportional change of the IC₅₀ of mAb 3074 against those same viruses (Table II). Interestingly, this suggests that the elicited serum might also mirror the potency of mAb 3074 as well as its breadth of reactivity across strains.

Our elicitation of specific serum neutralizing antibodies by preliminary design reveals several factors that may be important in improving the specificity of the desired response and in reproducing this success for other bNAbs. First, the molecular design was based on our hypothesis that the antigen needs to be structurally flexible and not rigid(48). We intentionally added and subtracted elements in a flexible antigenic loop to engineer a flexible 3D shape mimicking the Ab combining site. This requirement probably derives from the fact that the target epitope across HIV-1 viruses in immunologic reality is slightly distorted for each strain, so an antibody with too rigid specificity is not useful for neutralization across multiple strains. Instead, a flexible design may elicit a polyclonal, but narrow, envelope of specificities for a range of distortions of the epitope. This concept may underlie the failure to elicit neutralizing serum Abs when high affinity binding Abs are elicited by a rigid design (38, 39, 49, 60). These Abs cannot accommodate slight distortions in the target epitope needed for neutralization across multiple strains.

Second, this novel approach was enabled by precise computational delineation of the boundaries of neutralization epitopes (43, 45, 46), essentially projecting 3D shapes to the single dimension of amino acid sequence which allowed us to perform epitope “knock-outs” in a flexible linear region without the need for a rigid 3D structural approach. The designed antigen via this approach is predicted to be both structurally specific and conformational flexible. It is constrained to oscillate between closely related conformations centered on the core 3D structural fingerprint of the epitope.

Finally, we used a DNA prime, protein boost approach to immunization(61). Interactions between the prime and the boost are not well understood, and new findings may offer opportunities to enhance the potency of the response without altering specificity(62).

Durability and reproducibility of the immunogenicity profile of an HIV antigen are major challenges currently in the field of HIV vaccine research. In a previous published study, our epitope-focused protocol resulted in retention of 75% of the initial Ab titers one year after immunization(48). The Ab responses to epitope focused Cholera toxin subunit B (CTB)/gp120 epitopes constructs in rabbits has proven reproducible across several studies (48,49) including this one. The approach we have described here may therefore have promise for engineering and rationally improving epitope-specific anti-HIV Ab responses in mammals.

The recent identification of anti-variable loop Abs targeting the V1V2 domain as the only known inverse correlate of risk for HIV infection suggests that antibodies targeted to specific epitopes within gp120 variable loops indeed play a significant role in the human protective response to protection from circulating HIV-1 strains (26). As mAbs 3074 and 2219 are functional, HIV-neutralizing anti-variable loop mAbs, the rules for their specific elicitation, as revealed by this study, represent a starting point for eliciting similar functional anti-variable loop HIV-protective polyclonal Abs by vaccination, including those targeting specific monoclonal epitopes in the V1V2 domain(63).

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Figure 1. Design of V3 loop crown sequences to preferentially present specific epitopes when grafted onto a non-HIV protein scaffold, cholera toxin subunit B (CTB)

The consensus subtype C V3 loop sequence was modified by point mutations to perturb the epitopes targeted by specific nAbs. **A)** The precise neutralization epitopes for bNAbs 447-52D, 2219 and 3074 are defined as the ensemble of individual amino acids in the V3 loop crown that abrogate neutralization by these Abs when any individual amino acid in the ensemble is mutated. A sequence motif, as indicated in different colors for each Ab on the left, therefore precisely represents the minimal determinants for each Ab-targeted epitope (43, 46). An example sequence bearing each minimal epitope determinant (colored

according to the motif color in the left panel) is shown on the right. Superscripts indicate the amino acid position in the V3 loop as numbered from 1 (first disulfide cysteine in V3 loop) to 35 (terminal disulfide cysteine in V3 loop). **B)** The designed sequence specific for the neutralization epitope targeted by 2219. All mutations were made in the consensus subtype C V3 loop crown representing positions 9 to 22 to encode the design. Color-coded text and arrows indicate the point mutations that were made to disrupt the 447-52D (grey) and 3074 (light blue) epitopes, while leaving the 2219 epitope (green letters in the amino acid sequence) intact. We hypothesized that nearby non-epitope charged residues may attract antibody responses to less conserved (more narrowly reactive across HIV-1 strains) epitopes: these amino acids were modified from charged to polar residues to reduce this attraction (dark blue arrows and text). An illustration of the 3D structure of the resulting immunogen is shown at the bottom of the panel. The pentavalent cholera toxin subunit B (CTB) scaffold portion without inserted designed V3 loops is shown in green and the chimeric CTB-V3 loop immunogen with the inserted designed V3 loop is shown in purple with the V3 crown location corresponding to the designed sequence circled with a dashed line. In the actual immunogen, five purple structures form a pentamer like the purple one shown presenting 5 valencies of the designed V3 loop-based antigen. **C)** The designed sequence specific for the neutralization epitope targeted by 3074. All design changes were made in the same manner as for the 2219 design in B). Color-coded text and arrows indicate the point mutations that were made to disrupt the 447-52D (grey) and 2219 (green) epitopes, while leaving the 3074 epitope (light blue letters in the amino acid sequence) intact. Amino acids modified from charged to polar residues are indicated by dark blue arrows and text.

A	mAb	V3-CTB ²²¹⁹	V3-CTB ³⁰⁷⁴
	3074	4.2	3.9
	2219	4.2	0.5
	447-52D	4.1	0.6
	1418	0.1	0.1

B	mAb	psV-SF162-V3 ²²¹⁹	psV-SF162-V3 ³⁰⁷⁴
	3074	Sensitive (0.00098)	Sensitive (0.0011)
	2219	Sensitive (0.0036)	Resistant (>20)
	447-52D	Resistant (>20)	Resistant (1.0)

Figure 2. Binding and neutralization of engineered antigenic V3 loop sequences grafted into CTB immunogens and preferentially presenting epitopes targeted by 2219 [V3₂₂₁₉-CTB] or 3074 [V3₃₀₇₄-CTB]

A) ELISA binding of three different HIV neutralizing anti-V3 mAbs (2219, 3074 and 447-52D) with different epitope specificities and one anti-parvovirus negative control mAb (“1418”). The middle column shows the optical density (OD) values resulting from an ELISA assay measuring the binding of the respective antibody to the V3₂₂₁₉-CTB immunogen. The right-hand column shows the results of mAb binding to the V3₃₀₇₄-CTB immunogen. Strong binding/high OD values are colored red; low OD values indicative of lack of reactivity of the mAb with the protein are uncolored. **B)** Viral neutralization assay of three different anti-V3 mAbs. The middle column shows the sensitivity results of an *in vitro* neutralization assay measuring the antibody-mediated neutralization of a chimeric SF162-based pseudovirus bearing the antigen designed to preferentially present the neutralization epitope targeted by nAb 2219 (psV-SF162-V3²²¹⁹; see Methods and Figure 1 for details). The right column shows the sensitivity results from an *in vitro* neutralization assay measuring the antibody-mediated neutralization of a chimeric SF162-based psV bearing the antigen designed to preferentially present the neutralization epitope targeted by mAb 3074 [psV-SF162-V3³⁰⁷⁴; see Methods and Figure 1 for details]. If the IC₅₀ for the mAb against any psV is < 1 µg/ml, the psV is labeled as “sensitive” to the mAb, otherwise the cell is labeled as “resistant”. Actual IC₅₀ values in µg/ml are shown in parentheses.

Figure 3. Neutralization sensitivities of SF162 psVs with designed V3 loops to 3 anti-V3 mAbs Chimeric pseudoviruses (psV) of the SF162 HIV-1 strain in which the SF162 V3 loop has been replaced with the consensus V3 amino acid sequence of 8 clades (Y-axis) tested for sensitivity to neutralization (NT₅₀, X-axis) by the serum resulting from immunization of rabbits with the V3₃₀₇₄-CTB immunogen (blue bars), the V3₂₂₁₉-CTB immunogen (orange bars) and a previously published (50) “wild-type” immunogen consisting of the consensus subtype B V3 loop grafted onto CTB (V3_B-CTB). NT₅₀ (X-axis) is the geometric mean titer, or mean reciprocal serial dilution, of the indicated rabbit serum required to achieve 50% neutralization of the indicated psV by the serum. C* indicates the subtype C V3 loop with its N-terminal glycan removed.

Figure 4.

A) Neutralization titration curves for each rabbit immunized with V3₃₀₇₄-CTB. Each of the five panels represents the bleed from a different rabbit, labeled “NYU-7-R1xx”. The Y-axis indicates the percent inhibition of infectivity of each respective virus by the indicated serum. The X-axis indicates the Geomean titer, or serial dilution of the serum, associated with the inhibition. Each curve is for a different HIV-1 pseudovirus as indicated in the legend on the right. The non-HIV murine leukemia virus (MuLV) is a negative control. **B)** As in A, but the

curves show the neutralization activity of the serum resulting from rabbits immunized with V₃₂₂₁₉-CTB.

Figure 5. Dissection of epitope specificities of V3 specific mAbs and in sera raised by immunization with V3₂₂₁₉-CTB or V3₃₀₇₄-CTB using designed chimeric psV

A) Four different chimeric psVs (colors and legend) were designed to introduce perturbations in specific epitopes in their V3 loops targeted by three different anti-V3 mAbs (listed on Y-axis). Each psV consists of the SF162 HIV-1 strain with its V3 loop replaced by the consensus subtype B V3 loop sequence (yellow bars) or a designed sequence with the indicated epitope composition: BR18Q/-447D/green bars represents the psV with only its 447-52D targeted epitope perturbed and the epitopes targeted by 2219 and 3074 preserved; BI14M/3074-/blue bars represents the psV with only its 3074 targeted epitope perturbed; B-K10E/2219/orange bars represents the psV with only its 2219 epitope perturbed. The consensus B psV contains the epitopes for all three viruses. The IC₅₀ of the sensitivity of each psV to antibody-mediated neutralization by each of the three mAbs, measured by the µg/ml of mAb required to neutralize 50% of virus is plotted on the X-axis. The IC₅₀% numbers in µg/ml neutralization concentration of mAbs is given in the small table below the graph. **B)** Geometric mean 50% titers for the neutralization of psV with perturbed 3074 and 2219 epitopes from A (Y-axis) by rabbit sera pooled from 5 rabbits immunized with the immunogen V3₂₂₁₉-CTB (orange) or V3₃₀₇₄-CTB (blue). “+3074, +2219” refers to the psV consisting of the SF162 psV with the V3 loop of the SF162 strain replaced by the consensus subtype B V3 loop sequence, which contains the motifs targeted by both the 3074 NAb and

the 2219 NAb. “-2219, +3074” refers to the virus depicted in the orange bars in panel A, which is the same chimeric psV as “+3074, +2219”, but harboring a point mutation eliminating the motif for the epitope targeted by 2219 (K10E). “-3074, +2219” refers to the virus depicted in the blue bars in panel A, which is the same chimeric psV as “+3074, +2219”, but harboring a point mutation eliminating the motif for the epitope targeted by 3074 (I14M). “-2219, -3074” refers to the same chimeric psV as “+3074, +2219”, but with two point mutations eliminating the motifs for the epitopes targeted by both 3074 and 2219 incorporated. Data are the result of 3 experiments.

Table I

List view summary of the neutralization results shown in Figure 4: Pseudoviruses from the standard panel published in (53) that were 50% neutralized at a minimal titer of 10 (1:10 dilution) by the rabbit serum resulting from the immunization with the immunogen listed in the respective column heading. The neutralization curves summarized by this table are shown in Figure 4). Virus clade is indicated in parentheses. Neutralization assays were performed as described in (53).

<u>V3₂₂₁₉-CTB</u>	<u>V3₃₀₇₄-CTB</u>
MW965.26 (C)	MW965.26 (C)
BaL.26 (B)	BaL.26 (B)
Bx08.16 (B)	Bx08.16 (B)
QH0692.42 (B)	QH0692.42 (B)
SS1196.1 (B)	SS1196.1 (B)
6535.3 (B)	-
ZM109F.PB4 (C)	ZM109F.PB4 (C)
-	CAP210.2.00.E8 (C)
-	ZM233M.PB6 (C)

Table II

Neutralization of six Tier 2 Clade C viruses by mAb 3074 compared with neutralization of the same six viruses by polyclonal serum elicited in rabbits by immunization with V3₃₀₇₄-CTB. “-” in the “3074 IC₅₀” column indicates that infectivity of the respective virus was not inhibited by 50% by the addition of up to 50 µg/ml of the 3074 mAb in the neutralization assay. “+” in the “3074 IC₅₀” column indicates that infectivity of the respective virus was inhibited by 50% by addition of less than 50 µg/ml of mAb 3074 in the neutralization assay: the exact amount in µg/ml to achieve 50% neutralization is indicated in parentheses. [All data in the “3074 IC₅₀” column were adapted from ref (30)]. “-” in the “ID₅₀ V3₃₀₇₄-CTB Serum” column indicates that infectivity of the respective virus was not inhibited by 50% by a 1:10 dilution of the serum resulting from vaccination of the rabbit with V3₃₀₇₄-CTB in the DNA prime, protein boost protocol described in the methods in the neutralization assay. “+” in the “ID₅₀ V3₃₀₇₄-CTB Serum” column indicates that infectivity of the respective virus was inhibited by 50% by a 1:10 or greater dilution of the serum resulting from vaccination of the rabbit with V3₃₀₇₄-CTB in the neutralization assay: the exact dilution interpolated to achieve 50% neutralization is shown in parentheses.

<i>Virus</i>	<i>3074 /C₅₀</i>	<i>ID₅₀ V3₃₀₇₄-CTB Serum</i>
CAP210.2.00.E8	+ (41.48)	+ (19)
Du156.12	-	-
ZM109F.PB4	+ (7.33)	+ (52)
ZM135MPL10a	-	-
ZM197M.PB7	-	-
ZM233.M.PB6	+ (40.78)	+ (11)