Distinct conformations of the HIV-1 V3 loop crown are targetable for broad neutralization

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The V3 loop of the HIV-1 envelope (Env) protein elicits a vigorous, but largely non-neutralizing antibody response directed to the V3-crown, whereas rare broadly neutralizing antibodies (bnAbs) target the V3-base. Challenging this view, we present V3-crown directed broadly neutralizing Designed Ankyrin Repeat Proteins (bnDs) matching the breadth of V3-base bnAbs. While most bnAbs target prefusion Env, V3-crown bnDs bind open Env conformations triggered by CD4 engagement. BnDs achieve breadth by focusing on highly conserved residues that are accessible in two distinct V3 conformations, one of which resembles CCR5-bound V3. We further show that these V3-crown conformations can, in principle, be attacked by antibodies. Supporting this conclusion, analysis of antibody binding activity in the Swiss 4.5 K HIV-1 cohort (n = 4,281) revealed a co-evolution of V3-crown reactivities and neutralization breadth. Our results indicate a role of V3-crown responses and its conformational preferences in bnAb development to be considered in preventive and therapeutic approaches.
**HIV-1 entry depends on the interaction of the variable loop 3 (V3) of its envelope (Env) protein with an HIV co-receptor, commonly CCR5 or CXCR4**. In line with its critical function in entry, the three sections of the V3 - (i) the base (residues 296–299 and 327–331 within the HxB2 reference strain), (ii) the stem (residues 300–303 and 321–326), and (iii) the crown (residues 304–320) - are largely conserved, making the V3 loop a potential prime target for neutralizing antibodies (nAbs), inhibitors and vaccine approaches. Yet, V3 is effectively shielded from antibody recognition by interaction with the variable loops 1 and 2 (V1V2) [26-10]. Conformational changes in the Env trimer upon CD4 receptor binding trigger a displacement of V1V2, lifting its trimer stabilizing function and enabling V3 to interact with the HIV co-receptors. The ensuing trimer opening exposes highly neutralization sensitive sites within the V3-crown [59,11,12]. However, the window of accessibility is normally not sufficient to allow V3-crown specific antibodies to effectively block infection. Prototypically, a vigorous V3 antibody response is elicited in almost all HIV-1 infected individuals, but bears little to no neutralization activity as it is mostly constituted of V3-crown Abs [13,14].

Rare broadly neutralizing antibodies (bnAbs) targeting V3 overcome the access restriction on the trimer by binding to the conserved V3-base, involving the GDIR motif and surrounding glycans [5,15,16]. Considering the crucial function and conservation of the V3-crown, V3-crown inhibitors that can bypass V1V2 shielding could have immense therapeutic potential. If V1V2 shielding is artificially released, V3-crown Abs display extreme potency and breadth, thus confirming that accessibility, not specificity is the limiting factor [6]. Although a few cross-neutralizing V3-crown specific neutralizing Abs have been identified, overall they lack breadth compared to V3-base directed bnAbs [15–20]. The properties that distinguish cross-neutralizing V3-crown Abs from non-neutralizing V3 Abs are currently not fully understood. A capacity to recognize the V3-crown in distinct conformations has been proposed as a potential requirement [5,19,21–25].

Using the Designed Ankyrin Repeat Proteins (DARPin) technology [26], we previously developed the V3-crown specific DARPin 5m3_D12 that shares features with cross-neutralizing V3-crown Abs and has activity against difficult to neutralize (Tier-2) strains of HIV-1 subtype B [27]. Notably, 5m3_D12 partially by-passes V1V2 shielding, suggesting that generation of DARPin based broadly neutralizing V3-crown inhibitors may be possible. DARPins are based on a small rigid binding-protein scaffold, providing high target affinity and specificity for biomedicai applications [26,28–30]. Due to their rigid binding surface complementary to folded protein domains, DARPins often bind in a conformation-specific manner [26,27,31]. Exemplary for this, 5m3_D12 displays a strong binding preference for a specific V3-crown conformation [27]. Identification of novel broadly neutralizing V3 DARPins may thus reveal V3 conformations that are relevant for infection. Building on the discovery of 5m3_D12, we here exploit the DARPin technology in order to define conformational states of the V3-crown that are targetable for broad neutralization.

**Results**

**Broad neutralization via the V3-crown is possible.** Starting from highly diverse DARPin libraries we performed five independent DARPin selections (Selections A-E) by Ribosome Display [32,33]. In these selections we utilized different combinations of Env-derived panning targets with the aim to select V3-specific DARPins with high neutralization breadth (Fig. 1a and Supplementary Figs. 1, 2 and 3a, b). From each ribosome display selection pool, individual DARPin clones were subsequently screened for binding to their respective selection targets and tested for cross-neutralizing activity (Fig. 1a and Supplementary Fig. 2). The best neutralizers of each selection were retained, yielding a final set of six DARPins (Supplementary Fig. 2b). V3 specificity was confirmed by the binding to linear V3 peptide (Fig. 1a). Sequence analysis showed that the selected DARPins were distinct clones that were neither related to each other, nor to the previously identified cross-neutralizing DARPin 5m3_D12 [27] (Supplementary Fig. 4a). All six selected DARPins were mono-meric in solution (Supplementary Fig. 4b).

**Broadly neutralizing V3-crown DARPins access V3 on Env post CD4 engagement.** Since the V3-crown is well shielded in the closed prefusion conformation of the native Env trimer, inhibitors that interact with the V3 must either be able to bypass V1V2 shielding or access the V3 during the entry process, when V1V2 is displaced upon CD4 engagement. We therefore probed the capacity of bnDs to bind native Env of the Tier-2 strain JR-FL expressed on 293-T cells, in the presence or absence of sCD4 (Fig. 2a and Supplementary Fig. 7). All V3-crown DARPins lacked the ability to bind native Env, unless triggered by sCD4. DARPin binding increased with higher concentrations of sCD4, suggesting that the virus-neutralizing capacity of the V3-crown bnDs lies in their ability to access V3 post CD4 triggering.

To rate the dependency on an open Env conformation, we compared the capacity of bnDs and prototypic V3-crown directed mAbs (F425-B4e8, 3074, DH753, 2219, 10A37, 1Δ47-52D) to neutralize wild-type (wt) and corresponding V1V2-deleted viruses (Supplementary Figs. 5b, 8, Supplementary Data 4). The gain in neutralization potency in the absence of V1V2 was dramatic for mAbs (ranging from 922 to 9,708 fold difference in geometric mean IC50 ratio (wt-Env/ΔV1V2-Env), but comparatively modest for bnDs (geometric mean IC50 difference of 30-, 15-, and 184-fold for bnD.1, bnD.2 and bnD.3, respectively).

Thus, despite their dependence on trimer opening, the V3-crown bnDs appear to have a considerable capacity to bypass V1V2 shielding. Opening of the trimer upon CD4 engagement occurs gradually with V1V2 retaining some shielding activity [36]. In line with this model, the V3 antibodies gained substantially better neutralization activity when triggered by sCD4.
**Fig. 1 Selection of V3-crown reactive broadly neutralizing DARPinbs (bnDs).** a Scheme of the selection workflow to identify V3-crown-reactive neutralizing DARPinbs. For details on panning, ribosome display selections, and the screening procedure, see Supplementary Fig. 1 and 2 as well as the Materials and Methods section. The right panel depicts V3-crown binding and neutralizing activity of the top six neutralizing DARPinbs and the reference DARPin 5m3_D12 selected for follow-up experiments (magenta). V3-crown nAbs (black) and V3-base bnAbs (blue) are shown for comparison. V3-crown binding is depicted as 50% effective concentration (EC50) (nM) derived by ELISA. Bars indicate the geometric mean EC50 values from three independent replicates (circles). Error bars depict the geometric standard deviation (SD). The neutralization breadth-potency plot is based on results from a multi-clade 18-virus panel (maximum concentrations probed: DARPins (10,000 nM or 20,000 nM) and mAbs (167 nM (=25 μg/ml, commonly used for mAbs in this type of assay) Supplementary Data 1). The geometric mean of 50% inhibitory concentration (IC50) values in a and c were calculated over all sensitive strains. b Extended neutralization analysis of bnDs and nAbs on a 40-virus panel (Supplementary Data 1). IC50 values (geometric mean of 1-5 independent replicates) are shown. Minimum (5 nM: only for mAbs 3074, DH753 and F425-B4e8) and maximum concentrations of DARPins (10,000 nM) and mAbs (10,000 nM or 167 nM (=25 μg/ml) probed are indicated by dashed lines. Viruses not neutralized are shown as dots above the dashed lines. Colors of individual data points indicate virus clade. c Neutralization breadth-potency plot summarizing the data in b. d Neutralization breadth of V3-crown bnDs and nAbs depending on their concentration according to data in b. e Dendrogram based on Spearman correlation (Supplementary Data 2) and single linkage hierarchical clustering depicting the similarity of neutralization fingerprints on the 40-virus panel for bnDs and a range of bnAbs and nAbs with different epitope specificity.
more than bnDs from V1V2 deletion, suggesting that bnDs may already access the V3 at an earlier stage of the CD4 triggering when the trimer is not yet fully opened.

This capacity of bnDs is likely favored by their small molecular size. Indeed, Fab fragments of the probed V3 crown mAbs gained neutralizing potency in the absence of V1V2 to a similar extent as bnDs (ranging from 68- to 459-fold, Supplementary Fig. 8a, b, Supplementary Data 4). Overall, Fabs showed higher potency against V1V2-deleted viruses compared to bnDs but lacked in activity against wild-type viruses, where bnD.2 and bnD.3 reached higher breadth (Supplementary Fig. 8c). Together this suggests that a particular capacity to bypass V1V2 shielding, rather than binding affinity, shapes the neutralization capacity of bnDs.

Due to their rigid interaction surface, DARPinss often bind to a specific conformation of their target, as exemplified by the cross-neutralizing V3 DARPin 5m3_D12. We thus examined the structural preferences of the V3-crown bnDs by comparing their reactivity with a linear V3-crown peptide and four structurally constrained V3-crown mimetic peptides. The V3-crown mimetic peptides (named V3-IY, V3-RF, V3-RY, V3-RF) were designed to residues forming inter-strand hydrogen bonds and were previously been identified in complexes with V3-crown mAbs.

**Fig. 2** V3 DARPinss depend on CD4 triggering to bind native HIV-1 Env. **a** Binding of biotinylated DARPinss to cells expressing Env (JR-FL) was assessed by flow cytometry. Histograms of normalized fluorescence intensities and corresponding plots showing mean fluorescence intensities (MFI) as a function of concentration are depicted. **b** Competition ELISA to probe for V3-crown conformational preferences of DARPinss. DARPin binding to immobilized recombinant Env-derived proteins was measured at a concentration just before reaching saturation. Env proteins were chosen to maximize the dynamic binding range for the individual DARPin (bnD1: BG505gp120ΔV1V2; bnD.2: JR-FLgp120; bnD.3: trimeric BG505-SOSIP). Each DARPin/Env pair was competed with increasing concentrations of the BG505 V3 peptides indicated in the legend.
strongest competition was observed for mimetic V3-IF, and bnD.3 favored V3-RY, V3-IY, and V3-IF.

Crystal structures of the V3-crown DARPin: epitope complexes reveal two distinct V3 conformations linked to broad neutralization. In order to define V3-crown epitopes that lead to broad neutralization, we co-crystallized bnDs with V3-IF (BG505), i.e., the V3-crown mimetic that all bnDs bound best (Fig. 3, Supplementary Fig. 9 and Supplementary Data 5). 5m3_D12 and 63_B7 were co-crystallized with V3-IY (MN). Since 63_B7 also bound well to the linear peptide, it was additionally crystallized in complex with linear V3-crown peptide (MN). The canonical DARPin structure with the adjacent ankyrin repeats was maintained in all analyzed complexes (Supplementary Fig. 9a, c, e, g).
As expected from the pronounced preference of 5m3_D12 for V3-1Y register mimetics (Supplementary Fig. 3c27), the structure of the isolated V3-1Y mimetic was also retained in complex with 5m3_D12 (Fig. 3a and Supplementary Fig. 9a). In contrast, V3-1Y and V3-IF mimetics in complex with the three bnDs and 63_B7 adopted conformations that were different from their original design (Supplementary Fig. 3a, b). In all cases, a loss of the mimetic’s β-hairpin conformation was observed (Fig. 3a). Two distinct V3-crown conformations emerged, each captured by two different DARPins (Fig. 3a, Supplementary Fig. 9e, g). The V3 conformity adopted in complex with bnD.1 and bnD.3 proved highly similar to the structure of linear V3 peptide bound by mAb 3074 (2, PDB ID: 3mlx, Fig. 3a and Supplementary Fig. 9g). In contrast, V3-IF in complex with 63_B7 and bnD.2 adopted a conformation strongly resembling the cryo-EM structure of the V3-loop on gp120 when bound to co-receptor CCR5 (2, PDB ID: 6meo) which features a helical turn in the C-terminal strand (Fig. 3a, b and Supplementary Fig. 9e). Considering this similarity, the conformation of V3 bound to bnD.2 will hereinafter be referred to as Post-CCR5-V3. Since bnD.1 and bnD.3 also target the V3 post-CD4 engagement, the conformation adopted by V3 in complex with these DARPin must either be a transitory intermediate state that occurs prior to CCR5 binding or an induced fit upon binding an intermediate. This conformation will hereinafter be referred to as Inter-CCR5-V3.

The V3-crown conformational space was investigated by performing a molecular dynamics (MD) simulation of fully glycosylated gp120 bound to CD4 (based on the CD4 and CCR5 bound gp120 PDB ID: 6meo). A principal component analysis (PCA) of the conformations sampled by the V3-crown was then carried out. The V3-crown structures determined in complexes with DARPins or antibodies were projected into the eigenspace defined by the first two principal components (Fig. 3c). MD simulation suggested a high conformational flexibility of the V3 loop with β-hairpin variants being adopted most frequently and covering all experimentally determined V3 structures. Note-worthy, we observed that the Post-CCR5-V3 conformation is occasionally sampled spontaneously, possibly promoting the interaction with either CCR5 or bnD.2 (Fig. 3b). In contrast, the Inter-CCR5-V3 conformation was sampled less often in the MD simulations, suggesting that bnD.1 and bnD.3 must have the means to efficiently interact with V3 prior to the adoption of this V3 state.

The capacity of the bnDs to induce a fit or capture a transient conformation (conformational selection) was supported by additional observations. MD simulations showed that the conformational space of the V3-crown mimetics and the linear V3-crown peptide is strongly reduced when compared to the V3 loop on gp120 (Supplementary Fig. 10d–h). This is in agreement with Nuclear Magnetic Resonance (NMR) data of the V3-crown mimetics27 and confirms that mimetics only rarely adopt the Post-CCR5-V3 conformation. Despite this observation, the different V3-crown mimetics, as well as linear V3-crown peptide, were able to adopt the Post-CCR5-V3 conformation when in complex with 63_B7 and bnD.2 (Fig. 3b and Supplementary Fig. 9c, e). It should be noted that 63_B7 and bnD.2 were selected using different panning targets (V3-1Y mimetic and CD4-bound gp120, respectively), thus highlighting again that a common transient conformation of the V3 must exist.

bnDs engage conserved residues in the V3-crown. The analysis of the buried surface area (BSA) of the V3-crown mimetics across DARPin-complexes revealed several important features in the interaction motifs (Fig. 3d and Supplementary Data 6). Despite different preferences for the Inter- or Post-CCR5-V3 conformation, the bnDs overlapped in their contact patterns, engaging mainly with R/H308, I309, P313, F317, and Y318. All these residues are highly conserved across HIV-1 strains (>79%) except for position 308 where both arginine (42%) and histidine (31%) are frequently present (Supplementary Fig. 6a). The R308 side chain engages via a salt bridge with bnD.1 and bnD.2 (Fig. 3d, Supplementary Fig. 9h, h). Despite this contact, the bnDs maintained neutralization capacity against viruses with different residues in position 308 (Supplementary Data 1, Supplementary Figs. 5 and 6b), indicating the energetic importance of the other interactions. 63_B7 was the only DARPin that made substantial contact with the side chain of R315, which is prevalent only in clade B strains41. This would explain the restriction of 63_B7 for this clade (Supplementary Data 1). H-bonds formed with V3-crown peptide side chains and backbone differed substantially across DARPins (Fig. 3d and Supplementary Fig. 9). However, a striking commonality found across all DARPins was H-bonding with Y318.

Overall, we observed that bnD.3 forms eight H-bonds, distributed across six V3 amino acid residues, thus potentially leading to the comparatively high binding affinity observed for this DARPin (Supplementary Fig. 3c, and Supplementary Data 3 and 4). Six of these bonds were established with the V3-crown peptide backbone, and as a result, this should make bnD.3 less sensitive to V3 sequence variations. These features probably contribute collectively to the observed higher neutralization breadth of bnD.3 compared to bnD.1, which covers a BSA similar to bnD.3, but only forms four H-bonds with V3. In addition to
the hydrogen bonds, additional hydrophobic contacts add to the interaction strength (Supplementary Fig. 9b, d, f, h, i).

bnDs approach the V3-crown loop from opposite sides. The bnDs make extensive contacts with Y318 and F317 which are exposed to opposite sides of the V3 loop in the Inter- and Post-CCRS-V3 conformations, due to the presence or absence of the helical turn. This enables bnD.2 to approach the V3-crown from the opposite side compared to bnD.1 and bnD.3, and to interact with several shared conserved contact residues on the V3-crown (Fig. 3d and e).

In order to compare the relative orientations of the bnDs, Abs and Env, we carried out docking experiments incorporating the structural flexibility of the V3 loop sampled by the MD simulation of the glycosylated gp120 bound to CD4 (Fig. 3f and Supplementary Fig. 11). The bnD.3/bnD.1/3074 epitope is exposed to the outside of the trimer with mAb 3074 approaching the trimer from the top similar to DH753. In contrast, the epitopes of mAbs 2219 and F425-B4e8 face the two neighboring protomers. This allows only for a shallow approach angle of the Abs which, nevertheless, allows access for notable neutralization activity (Fig. 1c, d and Supplementary Fig. 8c). This provides further support for the accessibility of the bnD.2 epitope on functional Env, as bnD.2 binds the V3 from the same side as V3-crown Ab 2219 (and 447-52D)19,25.

Functional mapping of the V3 DARPin epitopes. To probe the functional relevance of individual amino acids for neutralization by the V3-crown DARPin Abs, we conducted an Env mutational scanning analysis encompassing 130 point mutants within gp120 (Fig. 4, Supplementary Data 7). V3-crown mAb 447-52D was included for comparison. A range of Env single-residue mutants showed enhanced neutralization sensitivity to V3-crown directed agents, indicating a more open Env trimer structure with increased V3 exposure (e.g., I309A, F317A, Y318A, Q422A, and K432A, see Fig. 4a and Supplementary Data 7). Notably, mAb 447-52D benefited from improved access to a much higher extent than the bnDs, reminiscent of the effect of V1V2 deletion (Supplementary Fig. 8b) suggesting that antibody access may have a greater dependence on complete trimer opening than bnDs.

Resistance mutations, defined by a > 5-fold increase in IC50 of mutant over wild-type virus, were mapped onto the crystal-structure of gp120 complexed with CD4 and CCR52 (Fig. 4b–e). Despite shared contact residues (Fig. 3d), identified resistance mutations showed comparatively little overlap across DARPinbs. Only Y318 proved to be critical for most DARPinbs, but also for this residue influence varied depending on the Env context (Fig. 4a).

Five shared resistance mutations highlighted the similarity between bnD.2 and 63_B7 (Fig. 4a and Supplementary Data 7). These positions (T257, K282, P470, M475, W479) localize outside V3 near the interface of the gp120 inner and outer domains and involve residues of the CD4 binding site and/or layer 3, a structural element previously implicated in Env conformational transitions42. Mutation of the neighboring residues N280 and D474 also showed an effect on bnD.2 and 63_B7, although the 5-fold threshold was not reached in both cases. The P470A mutation also influenced bnD.3 potency but stayed below the 5-fold threshold for bnD.1.

The removal of glycosylation sites surrounding the V3 loop (N197, N332, N386) had hardly any effect on bnD activity (Fig. 4a and Supplementary Data 7). Observed decreases in sensitivity were less than 5-fold. Removal of N332 led to higher sensitivity to all three bnDs but the increase was less than 5-fold. We only noted a potential effect of glycosylation on 5m3_D12: Introduction of a glycan at N197 strongly decreased sensitivity, conversely the T388A substitution (and to a lower extent also N386A) led to a decrease in sensitivity, suggesting that loss of this glycan may lead to altered structural dynamics of the V3 loop that do not favor 5m3_D12 binding. In support of this, the V3-IY conformation preferred by 5m3_D12 was only rarely sampled in a V3 MD simulation on deglycosylated gp120 (Supplementary Fig. 10).

Accessibility of V3-crown bnD epitopes. Due to their smaller size, DARPinbs (~16 to 20 kD, depending on the number of ankyrin repeats, see Supplementary Fig. 4) may access restricted sites more easily than Abs (~150 kDa). In order to assess the influence of space constraints, we generated bivalent Fc fusion proteins of each bnD (Fig. 5a; bnD.2-Fc: ~80 kD; bnD.1-Fc and bnD.3-Fc: ~85 kD). Like the monovalent DARPinbs, bnD-Fcs required CD4 triggering to bind cell surface-expressed JR-FL Env (Fig. 5b) while V3-crown mAbs display a low-level binding also in the absence of CD4 at equivalent concentrations (Supplementary Fig. 12). We next compared bnD-Fc binding to a recombinant native-like Env trimmer, BG505-SOSIP8,43,44, which is known to partially expose V344 (Fig. 5c, Supplementary Data 9). The effect of bivalency on binding V3 on BG505-SOSIP varied considerably across bnDs. While the binding for the bnD.3-Fc improved substantially, bnD.1-Fc binding activity remained unchanged and bnD.2-Fc even recorded a strong loss in binding capacity. Binding to trimeric BG505-SOSIP lacking V1V2 improved for bnD.1-Fc and to a lesser extent for bnD.2-Fc over the monovalent DARPin version, while bnD.3-Fc gained less in activity compared to binding to the wt protein (Fig. 5c, Supplementary Data 9). Collectively, this suggests that bnD.1 epitopes are arranged such that a bivalent engagement across the trimer is not possible, and that the bnD.2 epitopes are not accessible for the larger sized bnD.2-Fc in its current design. Neutralization efficiency of the bnD-Fcs against wt and V1V2-deleted viruses revealed a similar pattern (Fig. 5d, Supplementary Fig. 13, and Supplementary Data 9). Unlike bnD.2-Fc, bnD.1-Fc showed a moderate, bnD.3-Fc a stronger consistent increase in potency compared to their monovalent counterparts against V1V2-deleted viruses and the neutralization sensitive (Tier-1) strain SF162. Remarkably, when probing neutralization of difficult-to-neutralize (Tier-2) wt viruses, bnD.3-Fc fully retained its activity while bnD.1-Fc and bnD.2-Fc showed less activity than their monovalent counterparts. Interestingly, bivalent V3-crown mAbs had a modestly higher potency against wild-type Env compared to their monovalent Fab version while bivalent binding strongly improved potency against V1V2-deleted Env (Fig. 5d). Thus, although wild-type Env not only restricts access to the V3 but also bivalent binding, full-sized V3-crown Abs performed on average better than corresponding Fab molecules.

Collectively this indicates that both for V3-crown Abs and bnDs the exposure of the epitope during the entry process rather than the size of the inhibitor regulates neutralization efficacy. The analysis of bivalent bnD-Fc binding and neutralization activity highlights the overlapping influences of avidity gain and steric constraints. The observed differences amongst the three bnD-Fcs are in agreement with our docking studies which placed the bnD.1/bnD.3 epitopes to the outside of the trimer and thus more accessible than the bnD.2 epitope located between two protomers. Further space constraints for bnD.2-Fc may result from the C-terminal linked Fc facing the host-cell membrane (Fig. 3e and f). These constraints may in part be specific to the design of the bnD.2-Fc construct, as the bnD.2 epitope is accessible for larger sized inhibitors such as mAb 10A37 (Fig. 5d and Supplementary Fig. 8c) which recognizes a similar V3 conformation than...
Distinct patterns of V3-crown plasma antibody reactivity are linked with neutralization breadth. We next investigated whether naturally occurring V3-crown Abs exist that share with bnDs a preference for distinct V3-crown conformations. Probing a range of V3-crown mAbs we found that most, but not all, bind better to the linear V3-crown peptide than the four V3-crown mimetics (Supplementary Fig. 14a). Reactivity of the mAbs towards the mimetics differed, with most mAbs binding the V3-crown mimetics V3-IY and/or V3-IF better than V3-RF and V3-RY, further illustrating conformational preferences. Only mAb 2442 bound all mimetics with similar affinity.

We next leveraged the well-characterized patient cohort of the Swiss 4.5 K Screen13,47 to assess a potential impact of V3 reactivity on neutralization breadth. This cohort comprises 4281

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\text{Env-mutant} & \text{region} & \text{Feature} & \text{5m3_D12} & \text{SE4} & \text{SB11} & \text{63_B7} & \text{bnD.1} & \text{bnD.2} & \text{bnD.3} & \text{447-52D} \\
\hline
C2 & PNGS & 2.4 & 1.4 & 1.0 & 0.3 & 0.0 & 0.5 & 1.0 & 0.5 & 1.0 \\
C2 & CD4-bs & 2.4 & 1.4 & 1.0 & 0.3 & 0.0 & 0.5 & 1.0 & 0.5 & 1.0 \\
C2 & CD4-bs & 2.4 & 1.4 & 1.0 & 0.3 & 0.0 & 0.5 & 1.0 & 0.5 & 1.0 \\
C2 & CD4-bs & 2.4 & 1.4 & 1.0 & 0.3 & 0.0 & 0.5 & 1.0 & 0.5 & 1.0 \\
C2 & CD4-bs & 2.4 & 1.4 & 1.0 & 0.3 & 0.0 & 0.5 & 1.0 & 0.5 & 1.0 \\
C2 & CD4-bs & 2.4 & 1.4 & 1.0 & 0.3 & 0.0 & 0.5 & 1.0 & 0.5 & 1.0 \\
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**Fig. 4 Mutational scanning reveals key residues that determine sensitivity to V3 bnDs.** Mutational scanning of gp120 was performed across a 130 Env-pseudovirus mutant panel (Supplementary Data 7 and 8). a Overview of Env mutants that lead to >5-fold increase in IC50 compared to wt Env for at least one DARPin. For the DARPin, IC50 values for each mutant represent geometric means from at least two independent experiments (Supplementary Data 8). IC50 ratios (IC50mutant/IC50wt) are depicted. Light orange 5 < IC50-ratio<10; orange IC50-ratio>10; light grey 0.01 < IC50-ratio<0.2; dark grey IC50-ratio<0.01. PNGS: potential N-glycosylation site; CD4-bs: CD4-binding site; BrS: bridging sheet; C1-C5: Env constant regions 1-5; V1-V5: Env variable regions 1-5. b-e Structure of gp120 as in complex with sCD4 and CCR5 (PDB ID: 6meo; gp120 inner domain (dark grey), gp120 outer domain (light grey), V1V2 base (cyan), V3-base (dark blue), V3-stem (green), V3-crown (orange). Amino acid substitutions leading to a > 5-fold increase in the respective DARPin IC50 compared to the wt Env are indicated in red. (*/§): Mutations with >5-fold increase in IC50 identified in different Env's: BG505 (*) or JR-FL (§). JR-FL naturally lacks the glycosylation site at position 197.
**Fig. 5 Activity of bnDs as bivalent molecules.**

**a** Model of a bivalent DARPin-Fc generated by fusing the DARPin (violet with orange interaction surface) to the N-terminus of the Fc-region of human IgG1 (light grey and dark grey for the paired chains) with a preserved hinge region. **b** Binding of bivalent DARPin-Fc fusions to cell surface-expressed Env (JR-FL) measured by flow cytometry. DARPin-Fc fusions were titrated and incubated with cells in the presence or absence of 2 μM sCD4. Mulv Env served as a negative control. Histograms of normalized fluorescence intensities (top panel) and dose-response curves with mean fluorescence intensities (MFI, bottom panel) are depicted. See also linked experiments in Supplementary Fig. 12. **c** Binding of increasing concentrations of monovalent DARPin and bivalent DARPin-Fc fusions to wt (BG505-SOSIP) and V1V2 deleted Env trimer (BG505-SOSIPΔV1V2) in ELISA. Representative relative luminescent unit (RLU) data from one of two independent experiments are shown. **d** Comparison of monovalent and bivalent V3 inhibitors. Log IC₅₀ ratios (Fabs/mAbs and bnDs/Fc-bnDs) from a panel of 9 Tier-2 viruses (top) and corresponding V1V2-deleted viruses (bottom) are depicted. Values > 1 indicate higher potency of the bivalent inhibitor version. Individual data points are shown as circles and were calculated from geometric means of two independent experiments for wt and V1V2-deleted virus respectively (n = 2). Pairs with resistant strains were not included in the plots. Box plot limits extend from the 25th to 75th percentiles; centerline: median; whiskers indicate the minimum and maximum values. See Supplementary Data 9 and Supplementary Fig. 13 for a full data set.
Fig. 6 The V3-crown IgG1 response in HIV-1 infection recognizes distinct V3-crown conformations and is linked with the development of neutralization breadth. Relative plasma IgG1 binding activity (distributed uniformly in [0,1]) to linear V3 and structurally constrained BG505 V3-crown mimetics (V3-IY, V3-IF, V3-RF, V3-RY) from 4281 chronic HIV-1 infected individuals enrolled in the Swiss 4.5 K Screen were obtained and assessed in the context of neutralization activity data of the cohort (defined as a cross, broad and elite neutralization, or no neutralization) available from source. Data are provided as Supplementary Data. Unweighted average linkage hierarchical clustering based on the Spearman correlation of relative V3-crown peptide IgG1 binding activities across the cohort indicates reactivity clusters dependent on V3-crown peptide conformation. A two-dimensional representation (t-SNE map) of all 4,281 plasma samples based on relative IgG1 binding activities to the five V3 peptides. Red color denotes high binding activity and blue color indicates low binding activity to the V3 peptide indicated on the top of each panel. B Comparison of relative V3 binding activity in plasma from patients with (n = 909) and without (n = 2250) heterologous virus-neutralizing activity by multivariable linear regression analysis. Grey bars indicate the mean difference in relative binding activity for individual peptides (two-tailed p-values from t-test are provided; they are not adjusted for multiple testing). Black error bars indicate the 95% confidence intervals. These data belong to a comprehensive analysis of host, virus, and disease parameters, shown in Supplementary Fig. 14c and Supplementary Data 11, where only the n = 3159 patients with complete information on all parameters were included. C The top 105 neutralizing plasmas of the cohort were stratified by bnAb epitope specificity (using neutralization fingerprinting) and their mean relative V3 binding activities were compared.

individuals with chronic HIV-1 infection for whom detailed information on plasma neutralization activity, anti-HIV binding Abs, patient demographics, and disease parameters have been established. First, we compared the binding reactivity of plasma IgG1 to linear V3-crown peptide (strain BG505) and the four corresponding V3 mimetics (V3-IY, V3-IF, V3-RF, and V3-RY) across the cohort (Fig. 6 and Supplementary Fig. 14b). Correlation analysis showed that the relative V3-crown binding activities formed two reactivity clusters, one comprising the linear V3-crown peptide together with V3-IY and V3-IF and the other comprising V3-RY and V3-RF (Fig. 6a), underscoring that V3-crown antibodies with different conformational binding preferences occur in HIV-1 infection.

Two-dimensional V3 response maps based on the dimensionality reduction algorithm t-SNE showed distinct areas with high V3 reactivity that overlapped between V3 probes (Fig. 6b). Visualization of V3 patterns in sub-groups based on neutralization activity revealed no distinct clustering (Supplementary Fig. 15). Cross, broad, or elite plasma neutralization capacity and top 105 neutralizers stratified according to their predicted bnAb specificity as defined in showed overall no strong accumulation in V3 dense areas of the t-SNE plots. A degree of clustering was however evident amongst certain bnAb specificities amongst top neutralizers, in particular for trimer-apex predicted plasmas (Supplementary Fig. 15b). As we previously observed that trimer-apex (V2) responses are more prevalent outside subtype B and these plasma should react better with the subtype A (BG505) based antigens in binding, this accumulation in the t-SNE plots has to be expected and alone cannot define an association between bnAb activity and V3 binding responses.

To control for influences of co-variables we next conducted a multivariable linear regression analysis analyzing the influence of patient and disease parameters on relative binding activities to V3 probes (linear V3 and mimetics) and Env targets (gp120 and trimer) (Supplementary Fig. 14c). Previous analyses of the Swiss 4.5 K Screen identified an influence of infection length, viral load, virus diversity, and black ethnicity on the development of neutralization breadth and IgG responses to HIV-1 Env antigens. Here, we found that length of untreated infection and transmission by injection drug use (which entails prolonged periods of untreated HIV-1 infection), correlate positively with IgG1 V3 binding activity across all tested V3 probes.
(Supplementary Fig. 14c). Viral load, which correlated strongly with trimer and gp120 IgG1 responses was not associated with increased V3 antibody responses, highlighting epitope-specific differences in the parameters that steer binding antibody responses, as also noted by Kadelta et al. As noted before, the reactivity of plasma with the Env antigens proved to be subtype-dependent, with subtype B plasmas showing higher reactivity with subtype B derived antigens (MN and JR-FL) and non-subtype B plasmas showing higher reactivity with BG505 antigens. Likewise, we confirmed black ethnicity as a driver of IgG1 binding responses including V3 responses (Supplementary Fig. 14c and Kadelta et al. Most intriguingly, when comparing Swiss 4.5 K patients with and without neutralizing activity by multivariable linear regression, we found that neutralizing plasma activity was associated with higher IgG1 binding activity for V3-crown peptides, with the strongest association observed for reactivity with the V3-IY mimetic (Fig. 6c).

This higher prevalence of V3-crown-specific antibody responses among broad neutralizers is intriguing and suggests that V3 responses are influenced by the same disease parameters as bnAbs, but may evolve independently and can serve as surrogate markers for the evolution of bnAbs. Alternatively, V3-crown responses may be functionally linked to the evolution of bnAbs. For example, escape of V3-crown Abs may drive the evolution of different bnAb types. In addition, rare V3-crown mAbs may themselves exhibit notable neutralizing breadth.

To gain further insight into the potential functionality of V3-crown responses, we next examined whether V3 reactivity was uniformly high among bnAb inducers or whether differences existed depending on the bnAb specificity elicited again utilizing the top neutralizers with predicted bnAb specificity (Fig. 6d). Intriguingly, we found that V3 reactivity differed depending on the bnAb specificity of the plasma with CD4bs bnAb predicted plasma recording the highest V3 reactivity. V3-glycan bnAb reactivity was associated likewise with high V3 peptide reactivity across all probes, whereas V2-glycan bnAb plasmas showed high, but differential reactivity across V3 probes. Plasmas with yet unidentified bnAb specificity and MPER bnAb predicted plasmas showed lower V3 reactivity than the other bnAb plasmas but also for these plasmas the reactivity was higher than in non-neutralizers in agreement with the analysis in Fig. 6c.

In conclusion, the population-wide survey of V3-crown responses in the Swiss 4.5 K cohort (n = 4281) lends support to the idea that the naturally occurring antibody response in HIV-1 infection is shaped to distinguish conformational differences of V3 (Fig. 6a). The V3-crown response appears to co-evolve with neutralization breadth (Fig. 6c and d), highlighting the importance of deciphering which V3 responses are an irrelevant by-product and which are a direct or indirect component of bnAb evolution.

Discussion

Harnessing the potential of the V3-crown for HIV-1 inhibition has been attempted for several decades. Terminally the principal neutralization domain, it rapidly became evident that V3-crown neutralization by antibodies is rendered inefficient due to its conformational masking. Moreover, the immunodominance of the V3-crown is thought to distract the antibody response from known bnAb epitopes by competition for T follicular helper cells during affinity maturation in germinal centers. For these reasons current Env-immunogen design seeks to avert the immunodominant V3-crown response as exemplified by the new generations of well-shielded, stabilized, soluble Env trimer immunogens. Nonetheless, exploiting the V3-crown remains a tempting target because of its functional importance, sequence conservation, and high immunogenicity — potentially all key factors for efficient inhibition. V3-crown antibodies show exceptional potency and cross-reactivity in the absence of V1V2-shielding. Thus, the potential for effective inhibition is present, provided shielding can be bypassed.

In this study, we demonstrate that broad neutralization, reaching up to 90% of breadth, via the V3-crown can indeed be achieved by creating novel DARPin-based inhibitors. The large breadth of the V3-crown bnDs is even more astonishing as they are currently still substantially less potent than bnAbs targeting the Env trimer. The discovery of the bnDs provides proof that the V3-crown, although only transiently exposed on the open Env, during viral entry, is widely targetable across divergent strains. The bnDs further define two conformations, Inter-C5R5-V3 and Post-C5R5-V3 that expose conserved residues and are accessible on the CD4-triggered Env trimmer.

MD simulations suggest that the V3-crown samples various conformations before CCR5 engagement. Molecular docking suggests that the Inter-C5R5-V3 conformation, defined by bnD.1 and bnD.3, may thereby be exposed already on a partially open Trimeric Env (Supplementary Fig. 11b). bnD.2 does not access V3 on this Env conformation due to steric hindrance by V1V2. This bnD requires CD4-triggered Env with a more exposed V3 to bind.

The capacity of bnD.2 to bind Env similarly to CCR5 in the Post-C5R5-V3 conformation may, however, be a key component of its extraordinary neutralization breadth. All three bnDs benefit less from V1V2 deletion than nAbs, supporting the notion that bnDs can access a comparatively early - partially - opened state of the trimmer. This notion is further strengthened by the fact that bnD potency does not benefit from point mutations opening the trimmer to the same extent compared to V3 mAb 447-52D, for which we noted strongly enhanced neutralization potency. While V3-crown mAbs in part also access partially open Env, space constraints appear to restrict bivalent binding unless V1V2 shielding is completely released. Overall, this indicates that access is less of a limiting factor for neutralization by V3-binding bnDs than V3 antibodies, with the latter likely requiring complete and prolonged opening of Env to be fully effective.

The majority of bnAbs function by neutralizing free virus. A notable exception is a group of bnAbs targeting the membrane-proximal-external-region (MPER) on gp41 that are known to require CD4 triggering to efficiently bind Env. MPER bnAbs, like V3-crown bnDs, are remarkably broad, but not very potent. Other bnAbs also exhibit post-CD4 activity, as demonstrated during cell-to-cell transmission, where likewise a broad but not very potent activity is seen. The overall lower potency of post-CD4 neutralizing activity is with great certainty a consequence of the transient and brief exposure of these Env states. This makes the activity of V3 bnDs all the more remarkable. Intriguingly, bnAb VRC01 which failed to protect against HIV acquisition in the AMP trial has high potency against the free virus but not post attachment and during cell-cell transmission. Combining bnAbs with high potency against the free virus with inhibitors that provide inhibition after binding to CD4, such as the novel V3-crown bnDs described here, would increase the options for effective entry inhibition and is worth exploring in forthcoming studies. Therapeutic development of DAR Pins has been demonstrated in other disease models. DARPins are being evaluated in late-stage clinical trials for, e.g., COVID (ensovibler, neovascular age-related macular degeneration (abicipar). DARPins can be refined by a large variety of engineering strategies increasing valency, potency, and half-life. Access of the V3-crown bnDs to early opening Env intermediates, as our results
suggest, maybe decisive for their activity, as it elongates their window of opportunity. Certain post-receptor engagement conformations may also be sampled by Env spontaneously and might be trapped by the bnDs. We observed no exposure of the bnD epitopes in the absence of CD4 in our experimental setup using a relatively neutralization-resistant Env (Tier-2 strain JR-FL). However, spontaneous conformational sampling may differ among strains and needs to be taken into consideration. Collectively, these observations demonstrate that post-CD4 conformations of Env may be more widely accessible for neutralization before and during the entry process than was previously thought and could be exploited. The description of inhibitor-targetable V3-crown conformations defined here is, therefore, an important step in this direction.

Although bnDs benefit from their smaller size in accessing the distinct V3 conformations post-CD4 engagement, the analysis of bivalent Fc-fusion constructs together with mAb-Fab-pairs highlights that larger molecules can also access these regions. Spatial constraints inflicted by the V1/V2, host cell membrane, and neighboring Env protomers however differentially affect access to epitopes depending on the size and epitope of the inhibitors. As demonstrated by bnD3, high affinity and avidity effects can counterbalance the access limitations of larger molecules. Therefore, antibodies could also benefit from these effects to attack this domain efficiently.

In this study, we linked strong V3-crown responses with neutralization breadth in chronic HIV-1 infection (Fig. 6b, c). Whether these binding reactivities are a surrogate response that evolves alongside bnAbs without having a direct effect, whether they are indicative of the evolution of rare V3-crown bnAbs, or whether they resemble a contribution of V3-crown Abs as helper lineages for other bnAbs, remains to be defined. A proof of principle that the V3 conformations recognized by the bnDs are also targetable by antibodies comes from structure analysis of two V3-crown mAbs that neutralize some Tier-2 viruses but lack targetable epitopes. The bnDs are indicative of the evolution of rare V3-crown bnAbs, or effects can counterbalance the access limitations of larger molecules. Therefore, antibodies could also benefit from these effects to attack this domain efficiently.

In this study, we linked strong V3-crown responses with neutralization breadth in chronic HIV-1 infection (Fig. 6b, c). Whether these binding reactivities are a surrogate response that evolves alongside bnAbs without having a direct effect, whether they are indicative of the evolution of rare V3-crown bnAbs, or whether they resemble a contribution of V3-crown Abs as helper lineages for other bnAbs, remains to be defined. A proof of principle that the V3 conformations recognized by the bnDs are also targetable by antibodies comes from structure analysis of two V3-crown mAbs that neutralize some Tier-2 viruses but lack overall breadth. MAb 10A37 targets a conformation closely related to Post-CCR5-V3 bound by bnD245,46. MAb 3074 recognizes a conformation of V3 similar to Inter-CCR5-V3 recognized by bnD1 and bnD3 and approaches V3 from the same side as these DARPin5,19,20,32. mAb 3074-like Abs may therefore benefit in particular from better accessibility of their epitope, similar to bnD3/bnD3-Fc.

Collectively, our findings strongly suggest that directing antibody responses to distinct V3-crown conformations accessible after CD4 attachment should be feasible, thus opening roads for developing vaccine strategies to elicit bnD-like V3-crown bnAbs. Exploiting this knowledge will likely require the design of appropriate epitope scaffolds to be used as immunogens in order to channel the polyclonal V3-crown response to the desired specificity. These responses will likely not be potent, but due to their high breadth and capacity to recognize an entry state in which bnAbs targeting prefusion Env have less activity, may add important functionality to multivalent vaccines.

**Methods**

**Cells.** HEK 293-T cells were obtained from the American Type Culture Collection and TZM-bl cells through the NIH AIDS Reagent Program. Both cell lines were cultured in DMEM containing 10% FCS. HEK 293 T Freestyle™ suspension (293 F and Expi293F) cells (Thermo Fisher) for protein expression were maintained in suspension in serum-free Freestyle™ 293F and Expi293F Expression Media (both from Thermo Fisher) according to the manufacturer’s instructions. Media were supplemented with Penicillin and Streptomycin and cell lines were regularly checked for the absence of mycoplasma. No specific cell line authentication was performed.

**Reagents.** Properties of monoclonal antibodies used in this study are listed in Supplementary Data 12. We thank the individuals and agencies listed there as providers of antibodies and/or Ab expression plasmids for this study either directly or via the NIH AIDS Research and Reference Reagent Program (NIH ARP). Soluble four-domain CD4 (CD4)36,38 was provided by W. Olson (Progenics Pharmaceuticals Inc., Tarrytown, New York, USA).

**Peptides and mimetics.** CD4M47 was synthesized as described35. Linear V3-crown peptides and structure-constrained V3-crown mimetic peptides of strains MN, JR-FL, and BG505.W6 and ENV.C2 were synthesized as described39. The V3 mimetic peptides (Supplementary Fig. 3a, b) were designed to build anti-parallel β-strands that differ in the formation of inter-strand hydrogen bonds (mimetic peptides). Four strands with different registers were cyclized by a D-Pro-L-Pro (PPP) dipeptide which stabilizes the hairpin conformation. The codes of the registers (IY, IF, RY/HY, RF/HF) refer to the amino acids forming the respective hydrogen bond37,39. The IY register was defined in complex with mAb F425-B4e85, IF in complex with mAb 221940, RF/HF in complex with mAb 537-10D8, and RY/HY with mAb 447-S2D35. See Supplementary Fig. 3a, b for a full overview of peptides and mimetics used.

Peptides were biotinylated for use in ELISA, Luminox-based assays, and Ribosome Display. The linear V3 (MN) peptide was biotinylated on the N-terminus of a four-glycine N-terminal spacer (Supplementary Fig. 3a). For V3-IV (MN), a PEG8 linker was introduced between the L-proline in the D-Pro-L-Pro template of the mimetic and biotin. In the same way, a PEG4 linker was introduced between the peptid chain and biotin in all V3 mimetics based on the sequence of strain BG505 (see also36). In the case of linear V3 (BG505), a biotinylated PEG-4 linker was attached to an additional N-terminal glutamic acid residue. All synthetic peptides were ≥95% pure by analytical HPLC and displayed electrospray MS spectra consistent with the expected masses.

**Protein expression, purification and modification**

CD4-Two-domain scDm_183 protein was expressed in E. coli and purified as described36.

**Envs proteins.** Codon-optimized sequences of strain JR-FL gp120 wild-type and V1V2 loop deleted JR-FL67 were custom synthesized (GeneArt, Germany). The BG505-SOSIP source plasmid was kindly provided by J.P. Moore (Weill Cornell University, New York, USA) and Rogier Sanders (Academic Medical Center, Amsterdam, Netherlands)34. All Env constructs were fused to a C-terminal AviTag and cloned into a CMV/RI expression vector48 to allow in vitro biotinylilation. Env proteins were produced by transient transfection of HEK 293 T Freestyle suspension (FS) cells. BG505-SOSIP was expressed by transient transfection using a furin-expressing helper plasmid at a 3:1 ratio49. All HIV-1 Env proteins were purified from culture supernatants using Galanthus nivalis lectin resin (Vector Laboratories) as described in36. Mono-biotinylation with BirA enzyme was performed according to the manufacturer (Avidity, Aurora, USA). Proteins were subjected to Superdex 200 size exclusion chromatography (GE Healthcare, USA) to derive pure monomer or trimer.

**Monoclonal antibody and Fab production.** DNA-strings encoding the Fab regions of Abs 3074, DE753, F425-B4e8, 2219, 10A37 were synthesized (Geneart, Thermo Fisher Scientific) and cloned into human IgG1, human IgG kappa, and human IgG lambda expression vectors (AbVec) using In-Fusion methodology (Takara). Abs were expressed in Exp293 F cells (Thermo Fisher Scientific) by transient transfection using Transit-PRO transfection reagent (Mirus Bio LLC) according to the manufacturer’s instructions. Supernatants were harvested six days after transfection, sterile filtered, and supplemented with Protease Inhibitor tablets (Roche). Antibodies were purified from supernatants using AmmMag Protein A Agarose beads (Genscript) according to the manufacturer’s protocol. After elution of the Abs by 0.1 M glycine, pH 2.7, the eluate was neutralized with 1 M Tris, pH 8.7 using a 20μl of the eluate volume. Abs were further purified on a HiLoad 16/600 Superdex 200 pg size exclusion chromatography column (GE Healthcare) equilibrated in 20 mM NaHPO4/NaH2PO4, 100 mM NaCl, pH 6.0, and concentrated to 6 mg/ml (40μM) using Amicon centrifugal filter units (Millipore).

Fab fragments were prepared from purified mAbs by digestion with papain-agarose resin (Thermo Fisher Scientific) overnight at 37 °C using 50 μl settled resin/mg IgG in 20 mM NaHPO4/NaH2PO4, 10 mM EDTA, 20 mM cysteine, pH 7.0. To remove Fc-fractions and non-digested IgG the reaction was subsequently incubated with AmMag Protein A magnetic beads (Genscript) according to the manufacturer’s protocol. After elution of the Abs by 0.1 M glycine, pH 2.7, the eluate was neutralized with 1 M Tris, pH 8.7 using a 20μl of the eluate volume. Abs were further purified on a HiLoad 16/600 Superdex 200 pg size exclusion chromatography column (GE Healthcare) equilibrated in 20 mM NaHPO4/NaH2PO4, 100 mM NaCl, pH 6.0, and concentrated to ≥16 μM concentration using Amicon centrifugal filter units (Millipore).

Concentrations of mAbs and Fabs were estimated by measuring OD280 and applying specific absorption coefficients determined by ProtParam tool (www.expasy.org) using the respective amino-acid sequences. When converting concentrations, the molecular weight of the mAbs and Fabs was approximated to be 150 kDa and 50 kDa, respectively.
**DARPin selection by ribosome display.** General description of the methodology:

The principal methods of ribosome display, DARPin library design, and DARPin selection and purification have been previously described in detail26,28,31,33,91. An overview of the methods is provided in Supplementary Fig. 1.

Ribosome display is an in vitro translation method, where genetic information (i.e., mRNA) and translated protein remain linked in a ternary complex to the ribosome26. Since the DARPin sequence is fused to a C-terminal additional sequence (tether), it emerges from the ribosome and folds, while the C-terminus is still linked to the peptidyl-tRNA and thus trapped in the ribosome. From the DARPin library, DARPins of interest are enriched, based on their binding properties, and simultaneously their coding sequence is obtained from the mRNA23,31,92. DARPin library screening in combination with ribosome display has been successfully used in diverse settings62,91.

DARPins 5m3_D12 was selected in a previous study27. The selection of novel V3-crown specific DARPins in the present study was performed by Ribosome Display (RD) as described previously27,33,31,33,91. In the five RD selections performed (selections A-E, Fig. 1a and Supplementary Fig. 2), the following proteins were used as panning targets: (i) the V3-crown mimetic peptide V3-IY-(MN)27, (ii) monomeric gp120 arrested in the CD4-bound state, on which V3 is highly accessible (JR-FL-gp120 (clade B) in complex with the CD4 mimetic CD4MAM25 and 2Z6S1-gp120 (clade C) complex with soluble CD4 (sCD4)), and (iii) BG505-SOSIP (clade A), an engineered trimeric Env gp140 ectodomain93 that has been successfully used in diverse settings26,32,91.

The pool of soluble target (500 nM) was added to select for high-affinity DARPins with one N-capping, one C-capping and three internal ankyrin repeats (Supplementary Fig. 2). In successive RD rounds either a single panning target was used or two different targets were employed in alternating fashion to promote the selection of cross-reactive DARPins (Supplementary Fig. 2). Three versions of starting DARPin libraries were employed, each encoding N3C DARPin libraries with one N-capping, one C-capping and three internal ankyrin repeats (Supplementary Figs. 1, 2, and 4a). DARPin selections A and B were conducted using a C-cap library that encodes N3C DARPin libraries with one N-capping, one C-capping, and three internal ankyrin repeats25. Selection C used an improved second-generation N3C library, while selection D and E used a combination of this library with an N5c-loop DARPin library25. In the second-generation N3c library and N5c-loop DARPin library, additional positions in both the N3C cap and the N5c-loop were randomized (Supplementary Figs. 1 and 4a), and a more stable construct was used25. The N5c-loop library furthermore contains, in addition to the randomized positions in the DARPin structure25, the insertion of a flexible loop with randomized residues91, potentially allowing additional types of target interaction, similarly to the complementary determining region 3 of Ab heavy chains94. The second-generation N3C library and N5c-loop DARPin library was also used in a version without randomized caps.

Manual Ribosome Display selection: Selection A and B were performed as described in27. Briefly, the respective biotinylated target (Supplementary Fig. 3a and b) was bound to streptavidin-coated magnetic microbeads (MyOne™-Dynabeads, Invitrogen) and panning of ternary DARPin-mRNA-ribosome complexes was performed in 1.5 ml microtubes. Heparin, which is commonly used in RD to prevent non-specific mRNA binding, was omitted from the panning buffer in all selections because of known interference with gp120 recognition by DARPins25. Three to five consecutive rounds of RD selection were conducted to derive DARPin-specific RD DARPin libraries. The off-rate selection was performed to select clones with improved affinity. For this purpose, DARPin-ribosome complexes were first allowed to bind to the bead-immobilized target, before a 100- to 1000-fold molar excess of the non-biotinylated target was added as a competitor for 1-2 h, during which time DARPin binders with a fast off-rate are lost. The supernatant was removed, beads washed extensively (10-15 times) and a further RD round without competitor was conducted to amplify the remaining binders27.

High-throughput Ribosome Display selection (Selections C-E): Bead coupling was performed as described above. Panning of ternary DARPin-mRNA-ribosome complexes was randomized in 1.5 ml microtubes with a KingFisher Flex magnetic particle processor (Thermo Fisher Scientific). Five RD rounds were conducted with decreasing immobilized target concentrations in the first four rounds (250 nM, 125 nM, 30 nM, 5 nM). In the fourth off-rate selection round, a large molar excess of the soluble target (300 nM) was added to select for high-affinity DARPins in the fifth round (Supplementary Fig. 2). The immobilized target concentration was increased (50 mM) to amplify the remaining high-affinity DARPins.

**DARPin expression and purification.** Production of small-scale DARPin batches (1 ml cultures) for primary screens: DARPin pools obtained after the final RD round were used to perform 1 ml规模的基于表达的生物医学研究，以评估其在抗病毒治疗中的潜力。
at 4 °C, followed by three PBST wash steps. Mono-biotinylated target (20 nM) was added for 1 h at RT and residual target removed by three PBST wash steps. DARPin expression plasmids and the pCMV-expression helper plasmid in a 4:1 ratio and stained 36 h later with biotinylated DARPin. To this end, DARPin were cloned into the pBD02 expression vector in-frame with an N-terminal AviTag and a C-terminal polyhistidine-tag. For expression and biotinylation, E. coli XL1 blue bacteria were co-transformed with the AviTag DARPin vector and with the pCMV-expression helper coding for the N-terminal biotinylation enzyme and selected on agar plates containing 100 µg/ml carbenicillin and 10 µg/ml chloramphenicol. DARPins were then expressed in T96-medium supplemented with 0.5% glucose. Expression was induced at OD600 = 0.8 by adding 50 µM IPTG together with 50 µM D-biotin (Applichem). Biotinylated DARPins were purified as described above on a Ni-NTA column (Qiagen) and by SEC.

Cells were incubated with biotinylated DARPins or DARPin-Fc fusions in the presence or absence of scCD4-183 for 20 min at RT. Bound DARPins were detected via APC-Cy7-conjugated streptavidin (BD Biosciences, San Jose, CA, USA). Cells were fixed in 1:500 in FACS-buffer (PBS, 2 mM EDTA, 2% FCS) and 1:500 in FACS-buffere (PBS, 2 mM EDTA, 2% FCS) and washed three times with PBS-1% BSA. All cell samples were acquired on a FACSVerse system (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo 10 software (FlowJo LLC, Ashland, OR, USA). Propidium iodide staining (BD Biosciences, San Jose, CA, USA) diluted 1:500 in FACS-buffer was used to gate for live cells.

Cryocrystallography. Monomeric DARPin fractions were isolated by size-exclusion chromatography on AKTA Pure or Prime systems (GE Healthcare) with HiLoad 16/60 Superdex 75 pg or Superdex 200 10/30 GL columns (GE Healthcare) and 10 mM Tris pH 7.4, 100 mM NaCl (for bnD.1, bnD.2 and bnD.3) or 20 °C (63_B7 or 5m3_D12) as running buffer. Monomeric fractions were concentrated (Amicon Ultra Centrifugal Filters, Merck Millipore) to 20 mg/ml and supplemented with 1:2–fold molar excess of V3 mimetic.

Molecular dynamics. Molecular dynamics (MD) simulations were conducted to characterize the conformational space sampled by the gp120 V3 loop. In total, seven systems were investigated: a fully glycosylated and a fully deglycosylated structure of gp120, and five V3 mimetic peptides. The coordinates for the starting structure of gp120 bound to CD4 were extracted from a cryo-electron microscopy structure of gp120 bound to CD4 and the CC5 co-receptor (pdb: 3l0m). For the fully glycosylated structure, N-linked mannose-5 glycans (Man5) were modeled onto each sequence, using the python package GlycoVal 1.0.1. All glycans were removed in the fully deglycosylated structure. The initial coordinates for the V3 mimetics were extracted from previously determined structures27. All structures were solvated in a 17 Å padding water box and neutralized with 150 mM NaCl (see Table 1 for details).

The simulations were performed with the CHARMM36 force field115,116, including CMAP corrections for the protein. TIP3P water parameterization was
used to describe the water molecules. The simulations were carried out using the ACEMD 3.0 software. The periodic electrostatic interactions were computed using particle-mesh Ewald (PME) summation with a damping coefficient of 1.0 ps. The constant temperature was imposed using Langevin dynamics with a damping coefficient of 1.0 ps. The constant pressure of 1 atm was maintained with the Berendsen barostat. The systems were first minimized by 2000 conjugate gradient steps, followed by a free molecular dynamics simulation with a hydrogen mass repartitioning scheme to achieve a timestep of 4 fs. Snapshots from each simulation were extracted at 1 ns time intervals for structural analysis.

**Table 1. List of performed molecular dynamics simulations.**

| System | Size of unit cell (Å) | Simulation time [μs] |
|--------|-----------------------|----------------------|
| gp120 glycosylated (mannose 5) bound by scD4 | 131 × 130 × 125 | 0.7 |
| gp120 deglycosylated bound by scD4 | 130 × 130 × 124 | 0.7 |

**Protein-protein docking.** Protein-protein docking was used to investigate the binding of bnD.2, bnD.3 and six V3 targeting nAbs in context of the full gp120 (F425-B4e8 (PDB ID:2qsc), 2219 (PDB ID:2b0s), 3074 (PDB ID:3mlx), 447-542D (PDB ID: 4m1d), DH753 (PDB ID:6mnr), PGT135 (PDB ID: 4jm2)). For each structure, the co-crystallized V3 peptide was aligned to the different conformations sampled by the V3 loop during the MD simulation of the fully glycosylated gp120. The frame with the smallest root-mean-square deviation (RMSD) between the peptide and the V3 loop was extracted and used as the initial pose for the docking procedure in the context of an open (CD4 and mAb 17b-bound) Env trimer structure (PDB ID: 5vn3). In addition, bnD.3 was also docked to the partially open trimer (PDB ID: 5vn1). The RosettaDock (2018.33.60351) procedure was used for the protein-protein docking. For each structure, 700 poses were generated. The structure with the lowest energy and an RMSD to the initial pose smaller than 5 Å were considered as successful docking.

**Swiss 4.5 K Screen study population and ethics information.** In the current study, 4,281 plasma samples from HIV-1 infected individuals included in the Swiss 4.5 K Screen were re-analyzed for binding to V3-crown peptides. A detailed description of sample/patient selection and study design of the Swiss 4.5 K Screen has been described previously. All analyzed plasma samples were derived from specimens stored in the biobanks of the Swiss HIV Cohort Study (SHCS) and the Zurich Primary HIV Infection Study (ZPHI). The SHCS, founded in 1988, is highly representative of the HIV epidemic in Switzerland as it includes an estimated 53% of all HIV cases diagnosed in Switzerland since the onset of the epidemic, 72% of all patients receiving ART in Switzerland, and 69% of the nationwide registered AIDS cases. The SHCS is registered under the Swiss National Science Foundation longitudinal platform: http://www.snf.ch/en/funding/programmes/longitudinal-studies/Pages/default.aspx?Current%20supported%20longitudinal%20studies. Detailed information on the study is openly available on http://www.shsc.ch.

The Zurich Primary HIV Infection Study (ZPHI) is an ongoing, observational, non-randomized, single-center cohort founded in 2002 that specifically enrolls patients with documented acute or recent primary HIV-1 infection (www.clinicaltrials.gov; ID NCT00337966) [21].

The SHCS and the ZPHI have been approved by the ethics committee of the participating institutions. The SHCS Ethikkommission Bern, Ethikkommission des Kantons St. Gallen, Comité départemental d’éthique des spécialités médicales et de médecine communautaire et de premier recours, Kantonale Ethikkommission Zürich, Repubblica e Cantine Ticino - Comitato Ethico Cantonale, Commission cantonale d’éthique de la recherche sur être humain, Ethikkommission beider Basel für die SHCS und Kantonale Ethikkommission Zürich for the ZPHI and written informed consent had been obtained from all participants.

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**Proﬁling of the Swiss 4.5 K cohort for V3-crown speciﬁc HIV-1 binding antibodies.** We used a customized multiplex bead assay using the Luminox technology to measure the plasma IgG1 binding antibody activity to the four V3-crown mimitic peptides and mimitics in 4,281 individuals included in the Swiss 4.5 K cohort as described. In addition to the reactivities measured here we utilized binding data established for three linear V3 peptides (based on BG505, MN, and JR-FL sequences), JR-FLg9120, BG505g9140, and trimeric BG505-SOSIP determined for the same patient in a previous study from our group. Based on the high prevalence of IgG1 antibodies to V3 and the optimal plasma dilution for the binding assay that we previously established, we assessed V3-binding IgG1 antibody proﬁles at a ﬁxed plasma dilution of 1:6000. Otherwise, the assay was conducted as described. Recorded binding intensities were transformed into relative binding data by performing ordinal ranking of a plasma sample relative to all samples tested on the same day (ranging between 122 to 354 samples tested per day) to rule out intra- and inter-assay variability as described.

**Dissection of neutralization, host, viral and disease parameters that are linked with V3-IgG1 binding.** Data on eight host, viral, and disease parameters of Swiss 4.5 K individuals was available from our prior studies on this cohort. Neutralization breadth was used as a categorical response variable. Patients were deﬁned to have neutralization breadth if their plasma reached cross-, broad- or elite-neutralization activity score as determined in [7,12]. Patients with no or weak neutralization activity scores were categorized as having no neutralization.

We investigated the association of these parameters with V3-IgG1 reactivity by univariable and multivariable linear regression using Python 2.7 and its library statsmodels 0.8.0-3 as described. Log10 viral load and CD4 level (both measured at the time of sampling); viral pol diversity and infection length were included as continuous variables. The remaining four factors were used as categorical variables and analyzed in relation to the reference category (sex: reference male; mode of transmission: reference men having sex with men (MSM); ethnicity: reference White; HIV-1 clade: reference clade B, neutralization breadth: reference no neutralization).

The dimensionality reduction method t-SNE was used to display the reactivity with V3 peptides across the entire cohort in Fig. 6b and Supplementary Fig. 15a. Supplementary Fig. 15b utilizes the same t-SNE plot to visualize the similarity of V3 peptide-binding responses in the top 105 bnAb plasma samples identiﬁed in the Swiss 4.5 K Screen. Dominant bnAb specificities in these 105 bnAb plasma samples were previously determined by neutralization ﬁngerprinting. The location of bnAb plasma samples with distinct speciﬁcities is visualized on the t-SNE map.

**Statistics.** Statistical analyses were performed in Python 3.7 using the packages scipy.stats, statsmodels, and tune.

The dimensionality reduction method t-SNE was used to display the plasma samples in two dimensions. As a result of the large cohort size, we used the well-established Barnes-Hut-SNE approximation (with 1000 iterations and parameters theta = 0.5 and perplexity = 200) instead of the exact t-SNE method.

All reported statistical analyses about the V3 reactivity in the Swiss 4.5 K cohort are of explorative, descriptive nature. We therefore opted, by default, not to formally adjust for multiple testing since false positives are less of a problem in explorative studies than false negatives. In addition, owing to the large size of our cohort, almost all the associations we focus on exhibit a very low p-value, even lower than a Bonferroni-corrected p-value.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The structural data on DARPin:V3 complexes generated in this study (Fig. 3 and Supplementary Fig. 9) have been deposited in the Protein Data Bank (PDB) database under accession codes 7DNE (5m3_D12:V3-IF), 7DNE (63_B7:V3-IF), 7DNG (63_B7:V3), 7B4T (bnD.1:V3-IF), 7B4U J06 and 7B4V J32 (bnD.2:V3-IF) and 7B4W (bnD.3:V3-IF). Supplementary Data 5 with corresponding data collection and refinement statistics is included in the source data file. Other publicly available datasets from the PDB used in this study (Figs. 3 and 4, Supplementary Figs. 3, 10, 11, and 14) are accessible under PDB IDs 6EMO (CCHR5p120:CD4), 5VNR (b12 Fab:Fab41-SOSIP trimer), 3GHE (337:10D Fab:V3), 2QSC (F425-B4e8 Fab:V3), 2B08 (2219 Fab:V3), 5MEX (3074 Fab:V3), 4MID and JESS (447:52D Fab:V3), 6DMN (DH753 Fab:V3), 4JM2 (PGT135 Fab:V3) and 17b Fab:CD4.

Source Data is provided in Supplementary Data. Additional source data related to Ruset et al. and Kadelka et al. can be found online under https://doi.org/10.1038/s41467-021-27075-0.

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**Author contributions**

A.T. directed this work. N.F., P.R. and A.T. conceived and designed the study and analyzed data. N.F., E.S., M.G., S.H., Y.W., P.E., T.L.e., L.M., C.F., T.Li., B.I., A.M. designed and performed experiments and analyzed data. T.R., M.E., U.K., and S.U. conducted experiments and analyzed data. C.K. analyzed data. J.V.S. and A.P. provided access to the DARPin technology, directed the High-Throughput Binder Selection Facility, and helped design selection strategies and analysis. M.M. and J.A.R. designed and produced V3 peptide mimetics. R.D.K. and H.F.G. managed the SHCS and ZPHI cohorts and contributed patient samples and analyzed patient-related data. N.F. and A.T. wrote the manuscript, which all co-authors commented on.

**Competing interests**

AP is a co-founder and shareholder of Molecular Partners AG who are developing DARPin as therapeutics. HFG has been an advisor/consultant for Merck, Gilead Sciences, ViV Healthcare and is a member of data safety monitoring boards outside of the submitted work. AT has been a consultant for Roche outside of the submitted work. All other authors declare no competing interests.

**Additional information**

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