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Quaternary contact in the initial interaction of CD4 with the HIV-1 envelope trimer

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Abstract

Binding of the gp120 envelope glycoprotein to the CD4 receptor is the first step in the HIV-1 infectious cycle. Although the CD4-binding site has been extensively characterized, the initial receptor interaction has been difficult to study because of major CD4-induced structural rearrangements. Here, we use cryogenic electron microscopy to visualize the initial contact of CD4 with the HIV-1-envelope trimer at 6.8-Å resolution. A single CD4 molecule is embraced by a

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Supplementary Information

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Accession Codes

The 7.5 Å and 6.8 Å cryo-EM maps have been deposited under accession code EMD-8427. Fitted coordinates have been deposited under accession code PDB ID: 5U1F.

Author contributions:

Q.L. and P.L. conceived the project and designed biological and virological studies; P.A., T.Z., and P.D.K. conceived and designed structural studies; Q.L., J.L. and D.G. mutagenized gp160 from different HIV-1 isolates and produced infectious pseudoparticles; Q.L., C.G., H.M. and A.K. carried out infectivity assays and antigenicity studies of surface-expressed Envs. Q.L., D.G. and P.Z. produced and purified mutants of SOSIP trimer and gp120 monomer; Q.L. characterized the purified proteins and performed immunogenicity studies, CD4-binding and CCR5-binding assays; A.D. expressed DS-SOSIP proteins for SPR and cryo-EM studies; Q.L. and P.A. purified CD4-BS2 mutants of DS-SOSIP trimer and performed SPR analysis of CD4 binding; P.A. purified DS-SOSIP, prepared the DS-SOSIP-CD4-PGT145 complex, and performed cryo-EM experiments, with W.J.R. and C.W. providing assistance with EM data collection; P.A., T.B., G.Y.C. and T.Z. analyzed the structure; M.A.D. performed docking experiments and molecular dynamics simulations; B.C., C.S.P. and P.D.K. supervised cryo-EM experiments and structural analyses; Q.L., P.A., P.D.K. and P.L. analyzed data and wrote the paper; all the coauthors read and commented on the manuscript.

Competing interests:

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Data and materials availability:

Primary data are available upon request to the authors. Please address requests to: Paolo Lusso at plusso@niaid.nih.gov

quaternary HIV-1-Env surface formed by coalescence of the previously defined CD4-contact region with a second CD4-binding site (CD4-BS2) in the inner domain of a neighboring gp120 protomer. Disruption of CD4-BS2 destabilized CD4-trimer interaction and abrogated HIV-1 infectivity by preventing acquisition of coreceptor-binding competence. A corresponding reduction in HIV-1 infectivity occurred upon mutation of CD4 residues interacting with CD4-BS2. These results document the critical role of quaternary interactions in the initial HIV-1 envelope-receptor contact, with implications for treatment and vaccine design.

INTRODUCTION

The native HIV-1-envelope (Env) spike on the surface of mature virions is the sole functional form that mediates viral attachment and entry¹, and thereby represents the major target for neutralizing antibodies and a central focus for vaccine development. The native spike is a trimer of gp120-gp41 heterodimers constrained into a metastable structure, which evades immunologic control by a variety of mechanisms, including antigenic variability, *N*-linked glycosylation, and conformational masking of the conserved receptor-binding sites^{2,3}. Upon binding to the primary cellular receptor, CD4, the external gp120 Env glycoprotein undergoes major conformational changes, transitioning to a state that is competent for interaction with a coreceptor, CCR5 or CXCR4 (ref. 1). The CD4-binding site (CD4-BS) of gp120, which is a critical target of broad-spectrum neutralizing antibodies, has been finely mapped by both mutagenesis⁴⁻⁷ and co-crystallization in complex with soluble CD4 (sCD4)⁸⁻¹¹. However, most studies were performed with monomeric gp120 subunits, thereby hindering the evaluation of the role of quaternary elements assembled in the pre-fusion trimeric structure that may be involved in the initial CD4 interaction. Quaternary interactions with an adjacent gp120 protomer have indeed been documented for two monoclonal antibodies (mAbs) directed to the CD4 supersite, VRC-PG04 (ref. 12) and VRC01 (ref. 13), by cryogenic electron microscopy (cryo-EM) and crystallography, respectively. In addition, Lyumkis and colleagues used *in silico* docking to model quaternary trimer interactions for additional anti-CD4-BS mAbs and for sCD4 itself, predicting contacts of VRC01 and sCD4 with a limited surface of the $\alpha 0$ helix of an adjacent protomer, as well as more extensive contacts of other mAbs with basic residues in C2 and the V3 loop¹²; however, the functional significance of these interactions was not investigated.

Considerable progress has recently been made in the elucidation of the molecular anatomy of the HIV-1 Env spike, particularly with the design and crystallization of soluble truncated trimers (SOSIP.664) stabilized in a near-native configuration¹³⁻¹⁸ and the increasing resolution of cryo-EM imaging of native, membrane-bound Env spikes¹⁹⁻²¹. Nevertheless, obtaining structural information on the initial contact of CD4 with the pre-fusion trimer has remained a challenging endeavor, primarily due to the rapid transition of the trimer toward an open configuration upon CD4 interaction²². Accordingly, cryo-EM studies have visualized both soluble and membrane-bound Env trimers adopting an open conformation upon CD4 binding, regardless of the presence of bound antibodies directed to CD4-induced epitopes^{21,24} or to epitopes compatible with the pre-fusion trimer state²³.

In this study, we present the cryo-EM structure of a conformationally-constrained HIV-1 Env soluble trimer complexed with sCD4 and a trimer-specific broadly-neutralizing antibody, PGT145, documenting the quaternary configuration of the initial CD4-contact site in the closed, pre-fusion HIV-1 Env trimer. We show that the quaternary interactive site stabilizes CD4-Env binding and plays an essential role in HIV-1 entry into host cells, as its disruption prevents the acquisition of coreceptor-binding competence. Finally, we refine the boundaries of the CD4 antigenic supersite, showing that the quaternary CD4-interactive surface is immunogenic *in vivo*. These results have implications for HIV-1 treatment and vaccine design.

RESULTS

Cryo-EM structure of the initial CD4 contact with the HIV-1 Env trimer

To gain structural insights into the initial interaction of CD4 with the HIV-1 Env trimer, we used a conformationally-constrained soluble trimer, DS-SOSIP.664 (ref. 16), which remains in a pre-fusion, closed conformation even after interaction with CD4, as evidenced by retention of binding to trimer-specific antibodies, including the V1V2 loop-directed mAb PGT145 (ref. 25). Notably, the DS-SOSIP.664 trimer binds sCD4 with an asymmetric 1:1 (CD4:trimer) stoichiometry¹⁶, which likely reflects the earliest Env-receptor interaction. Thus, we formed complexes between DS-SOSIP.664, 4-domain sCD4, and the antigen-binding fragment (Fab) of mAb PGT145, and collected cryo-EM data, yielding a 6.8-Å resolution reconstruction (Fig. 1a,b, Table 1 and Supplementary Figs. 1,2). Known structures of the Env trimer, sCD4 and PGT145 were used to position each molecule into the cryo-EM map (Supplementary Table 1,2 and Supplementary Fig. 2), followed by minimization of the Env-interactive interfaces with PGT145 and CD4, to yield a final coordinate model fitted within the reconstructed electron density map (PDB 5U1F).

The reconstructed map confirmed the trimer to be in a pre-fusion, closed conformation, bound to a single CD4 molecule. The integrity of the pre-fusion trimer structure was reinforced by the presence of the quaternary-specific mAb PGT145, with its extended third heavy chain complementarity-determining region (CDR H3) loop penetrating through the glycan shield and reaching into a hole at the trimer apex formed by the junction of the V1V2 loops, making contacts with all three gp120 protomers, including the apex glycans, and essentially sealing the pre-fusion trimer at its apex (Fig. 1a,c and Supplementary Fig. 2). The highly electronegative CDR H3 loop of PGT145 lodged within an electropositive pocket formed by residues from all three protomers (Fig. 1c, left insert), with a sulfated tyrosine at its tip (Fig. 1c, right insert) reaching into a space often occupied by SO_4^{2-} anions in previously reported structures of the pre-fusion closed trimer^{13,15,16,18}.

Initial contact of CD4 with a quaternary Env surface

The reconstructed cryo-EM structure of the DS-SOSIP.664 trimer revealed domain 1 of sCD4 intrude between two gp120 protomers and to establish contacts not only with the previously defined CD4-BS in the outer domain of one gp120 protomer (henceforth referred to as “primary CD4-contacting” protomer), but also with a second CD4-binding site (CD4-

BS2) located in the inner domain of a neighboring protomer (“quaternary CD4-contacting” protomer), with no interactions with the third (“non-CD4-contacting”) protomer (Fig. 1b).

To define the orientations of the three Env protomers in the single CD4-bound, closed trimer structure, we compared the orientation of each protomer in our EM reconstructed map with those in previously determined structures of the pre-fusion, closed Env trimer (listed in Supplementary Table 1). The structures were superimposed by aligning each previously defined protomer onto the coordinate model of the non-CD4-contacting protomer in our cryo-EM reconstruction and comparing the resulting orientations of the other two protomers (Fig. 1d). We found the position of the primary CD4-contacting protomer in our structure to be shifted by an average translation of 3.4 Å and a rotation of 2.4° relative to the mean coordinate position of other pre-fusion soluble trimer structures (Fig. 1d, top insert), while the quaternary CD4-contacting protomer shifts by a translation of 3.1 Å and a rotation of 1.9°, with both protomers moving outwards from the trimer axis towards the CD4 molecule (Fig. 1d, inserts). The most distal orientation for both primary (difference of 5.6 Å translation and 10.5° rotation) and quaternary (difference of 5.0 Å translation and 8.7° rotation) CD4-contacting protomers occurred with the PGT151 complex²⁰, which also induces an asymmetric conformation, but in the opposite direction. No major change in the secondary structure of either gp120 or gp41 was observed in the 6.8 Å maps versus prior pre-fusion closed structures, reflecting the ability of the HIV-1 Env protomers to sustain up to 5 Å movements in trimer orientation without undergoing substantial structural rearrangements. Although the 6.8 Å resolution of the reconstructed cryo-EM maps precluded a precise experimental definition of individual side chain positions, robust fits of the Env protomer domains to the reconstructed EM map (Fig. 1a,b and Supplementary Table 1), together with a statistically significant difference of domain orientations of 6 σ or greater relative to known trimer structures (Fig. 1d), allowed for the positioning of an asymmetric protomer domain arrangement within the single CD4-bound trimer structure.

To obtain insights into the CD4-trimer interaction, we used a molecular dynamic (MD) simulation that led us to focus on four candidate CD4-contacting residues in the quaternary CD4-contacting protomer, namely, E62, E64 and H66 in the α1-helix, and K207 at the base of the β3-β4 loop (Fig. 2a). In the crystal structure of the unliganded trimer¹⁸, the side chains of these residues project toward the incoming CD4 receptor and are exposed to the solvent (see Fig. 2a). Notably, residues E64, H66 and K207 are nearly universally conserved (>99.7%) across all group-M HIV-1 isolates, while position 62 is occupied by an acidic residue (E or D) in more than 90% of group-M isolates (Fig. 2b).

Critical role of quaternary CD4 interaction in HIV-1 infection

To elucidate the functional relevance of the quaternary CD4 contact to HIV-1 infection, we mutagenized residues E62, E64, H66, and K207 in full-length gp160 from 6 HIV-1 isolates of different genetic subtypes, namely, BG505 (clade A), BaL and JR-FL (clade B), ZM106.9 (clade C), QD435.A4 (clade D), and CM244 (clade E), and generated infectious viral pseudoparticles for each mutant. Both individual and combined charge inversions were introduced into each Env to create a reverse-charged surface; as a control, we mutated by charge inversion a key residue in the primary CD4-BS, D368 (refs. 5, 26). Both wild-type

(WT) and mutated gp160 were efficiently expressed on the surface membrane of transfected human embryonic kidney (HEK) 293T cells and did not show alterations in their folding and trimeric structure, as determined by reactivity with a wide panel of anti-envelope mAbs, including trimer-preferring and cleavage-sensitive mAbs such as PG9 (ref. 27), 35O22 (ref. 28), and PGT151 (ref. 20) (Supplementary Table 3). Charge inversions of E62 E64, H66 and K207 virtually abrogated the infectivity of all the HIV-1 Env tested, regardless of their genetic clades, as did the control D368R mutation (Fig. 2c,d).

A set of additional mutations was introduced into the BaL Env in order to obtain a more complete topological and functional mapping of the CD4-BS2 region. Alanine substitutions of E62, E64, H66, and K207, either individually or in combination, consistently reduced HIV-1 infectivity, albeit less markedly than charge inversions; nevertheless, a triple alanine substitution (D62A E64A K207A) caused a complete loss of infectious capacity (Supplementary Fig. 3). Charge inversions or alanine substitutions of neighboring solvent-exposed residues either had no effects (K59E, E102A or E102K) or even enhanced infectivity (Y61A, presumably due to removal of the bulky side chain, allowing for a closer approach of CD4 to CD4-BS2), corroborating the specificity of the effects observed upon disruption of CD4-BS2. These results suggest a critical role of the quaternary trimer interaction with CD4 for the progression of the HIV-1 fusogenic process.

Stabilization of CD4 binding to the trimer by quaternary interaction

To elucidate the mechanism underlying the complete loss of infectivity induced by disruption of CD4-BS2, we initially tested whether the mutations caused a reduced gp120-gp41 association, but the levels of gp120 shedding from cell surface-expressed gp160 were unaltered (data not shown). Next, we mutagenized the four CD4-BS2 residues (E62, E64, H66 and K207) in both the DS- and WT soluble SOSIP trimers and measured the ability of the mutants to bind sCD4. The trimers were expressed in HEK 293FS cells and purified to homogeneity (Supplementary Fig. 4b). As expected from the outward orientation and complete solvent exposure of the side chains of the CD4-BS2 residues, their mutation by simple charge inversion had no effect on the correct folding and trimeric configuration of the mutants, as shown by reactivity with a panel of mAbs including several trimer-preferring, conformation-dependent and cleavage-sensitive mAbs (Supplementary Fig. 4a). By surface plasmon resonance (SPR) analysis, all of the DS-SOSIP-based CD4-BS2 mutants showed a markedly reduced sCD4-binding affinity (K_D), which was primarily due to an accelerated off-rate, with K207E exhibiting the most pronounced decrease (K_D = 262.0 nM compared to 8.0 nM for the unmutated DS-SOSIP), followed by H66E (K_D =127.2 nM) and E62K E64K (K_D =57.7 nM); no detectable binding was seen with the control D368R mutant (Fig. 3a). Likewise, all the WT-based mutants showed a similarly reduced ability to bind sCD4 in both ELISA (Supplementary Fig. 5a) and SPR assays (Fig. 3b), thus excluding that the reduced CD4-binding affinity could be specific for the stabilized DS-SOSIP trimer. Since soluble SOSIP trimers display some structural and antigenic differences from native Env trimers²⁹, we also tested CD4 binding to full-length native gp160 expressed on the surface membrane of 293T cells. All CD4-BS2 mutants for both BG505 and BaL Envs exhibited reduced binding to CD4-Ig, when compared to their respective WT proteins (Supplementary Fig.

5b). Altogether, these results demonstrate that CD4-BS2 plays an important role in stabilizing the interaction of the HIV-1 Env trimer with CD4.

Quaternary CD4 interaction confers coreceptor-binding competence

Next, we studied the events downstream of CD4 binding, which induce gp120 to acquire coreceptor-binding competence and eventually to dissociate from gp41, leading to activation of the fusogenic process. After pre-activation with sCD4, the BG505 SOSIP.664 soluble trimer efficiently bound to cell surface-expressed CCR5; in contrast, none of the CD4-BS2 mutants were able to bind to CCR5 regardless of sCD4 treatment (Fig. 3c). Likewise, little, if any, induction of the 48d epitope, which extensively overlaps the CCR5-binding surface³⁰, was detected in any of the CD4-BS2 mutants upon treatment with sCD4 (Fig. 3d). These results indicate that the quaternary interaction of CD4 with the gp120 inner domain is essential for triggering conformational transitions that lead to coreceptor binding and progression of the fusogenic process.

Limited effects of CD4-BS2 mutagenesis on monomeric gp120

Since mutation of specific residues in the gp120 inner domain, including H66, was previously reported to reduce sCD4 and coreceptor binding to monomeric gp120^{7,31}, we investigated the effects of CD4-BS2 mutations in the context of gp120 monomers. The correct folding of the mutated gp120 proteins was confirmed by their reactivity with a panel of anti-gp120 mAbs (Supplementary Fig. 6c). Among all mutants, only H66E showed a slight reduction in sCD4-binding activity in both BG505 and BaL gp120, whereas K207E showed reduced binding only in BG505 but not in BaL gp120 (Supplementary Fig. 6a), indicating that CD4-BS2 is not a functionally relevant receptor-binding site in the context of the monomer. Mutation of CD4-BS2 residues resulted in decreased CCR5 and 48d binding in the absence of sCD4 (Supplementary Fig. 6b), confirming previous mutagenesis studies that attributed this effect to reduced monomer flexibility caused by altered inter-layer interactions within the inner domain^{7,31}. In contrast, in the presence of sCD4, binding to 48d was unaffected by CD4-BS2 mutations, demonstrating that sCD4 was able to induce the full range of conformational changes in the context of the mutated monomer, while binding to CCR5 was only moderately reduced, with the exception of mutant K207E that, as expected, lost the ability to bind CCR5 since this residue is part of the coreceptor-contact surface³² (Supplementary Fig. 6b). Overall, the effects observed by mutation of monomeric gp120 were only partial and limited, unlike the complete loss of CCR5 and 48d binding observed in the context of the trimer (Fig. 3c,d).

To interpret the above results, we examined the available crystal structures of monomeric gp120, which invariably revealed a disassembled CD4-BS2 region due to the opposite spatial orientation of the E62, E64, H66 and K207 side chains, which in the monomer point inwards to establish intramolecular contacts with residues in the underlying layer 2 of the gp120 inner domain^{8–11}; conversely, in the structures of soluble Env trimers, the same residues point outwards forming a continuous, electrostatically charged surface crest^{13,16–18} (Supplementary Fig. 7). Thus, as suggested^{7,31}, the slight reduction of CD4 and CCR5 binding observed in the context of monomeric gp120, as well as the reduced binding of mAb 48d in the absence of sCD4, may be due to reduced molecular flexibility and distant

allosteric effects secondary to altered inter-layer adherence within the flexible inner domain of the gp120 glycoprotein.

Identification of CD4 residues involved in quaternary interaction

To validate further the role of CD4-BS2 in the envelope-receptor interaction, we investigated the CD4 residues that make contact with CD4-BS2 (Fig. 4a). On CD4, two discontinuous surfaces mirror the quaternary binding site on the Env trimer, with one surface interacting with the primary CD4-contacting protomer, and the other interacting with the quaternary CD4-contacting protomer (Fig. 4b). To provide an estimate of surface areas of interaction, we used the Env trimers fitted to the EM map listed in Supplementary Table 1 as well as previously determined 2-domain sCD4 (residues 1–176) fitted to the EM density (Fig. 4c and Supplementary Table 2). Fittings of CD4 to the cryo-EM reconstructed map revealed a ~ 6 Å translation and a $\sim 8^\circ$ rotation for the median position of CD4 in the initial contact structure relative to the position of CD4 in the monomeric post-fusion structure. A total of 7 Env trimers and 29 CD4 domains produced a matrix of 203 interactive surfaces, yielding 526 ± 52 Å² of buried surface area for the primary CD4-contacting gp120 and 159 ± 41 Å² of buried surface area for the quaternary CD4-contacting gp120 (Supplementary Table 4). Examination of the contact site in the fitted model and in the MD simulation revealed four residues in CD4 domain 1 (D1), namely, K21_{CD4} and K22_{CD4} in the CDR1-like loop, and D63_{CD4} and Q64_{CD4} in the DE loop, as potentially interactive with CD4-BS2 in the fitted cryo-EM structure and in the molecular dynamic simulation; none of these residues had been previously reported to contact gp120 (refs. 8–11).

To assess the functional role of the CD4 residues predicted to interact with CD4-BS2, each residue was mutagenized by charge inversion, and full-length CD4 mutants were expressed on the surface of Cf2Th/syn-CCR5 cells and tested for HIV-1 receptor function. The correct expression and folding of the mutants was verified using a panel of anti-CD4 mAbs (Supplementary Fig. 8b); of note, mAbs RPAT4, 13B8 and 379 showed a significant loss of reactivity with mutant K22E_{CD4}, suggesting that the mutations directly overlap their binding footprint or, less likely, that charge inversion may have caused subtle conformational changes. Charge inversion of two CD4 residues, K22_{CD4} and D63_{CD4}, caused a dramatic loss of HIV-1 receptor function, as seen with both the BG505 and BaL envelopes, while charge inversion of K21_{CD4} had more limited effects, and alanine substitution of Q64_{CD4} had no effects; as expected, alanine substitution of the critical F43_{CD4} residue caused a complete loss of receptor function (Fig. 4d). Analysis of a more extended panel of CD4 mutants showed that alanine substitutions of K22_{CD4} and D63_{CD4} also reduced HIV-1 infectivity, albeit less markedly than charge inversions (Supplementary Fig. 8a). These results confirmed that alterations on both sides of the CD4:CD4-BS2 interface disrupt the functional interaction between the HIV-1 envelope and the CD4 receptor.

Role of quaternary interaction in neutralizing antibody binding

To assess the immunogenicity of CD4-BS2 and its functional relevance to antibody-mediated neutralization and immune evasion, we examined the potential interaction of this site with a panel of anti-CD4 supersite mAbs isolated from HIV-1-infected individuals. Numerous structures of these mAbs have been determined in complex with monomeric

gp120^{33–37} and in the context of the Env trimer^{12,13}. Docking onto the pre-fusion closed Env trimer revealed that all of the anti-CD4 supersite mAbs reach deep into the inter-protomer groove, although only some of them (most notably, VRC03, VRC06, and VRC-CH31) appear to make direct contact with CD4-BS2 (Fig. 5a). Optimization of quaternary contacts appeared to occur by somatic hypermutation-induced alteration of the framework regions of these antibodies (Supplementary Fig. 9). We note that quaternary interactions were predicted for several anti-CD4 supersite antibodies, including VRC01 (ref. 12), and the crystal structure of the VRC01-bound X1193.c1 SOSIP.664 trimer, but not of the JR-FL trimer, indeed documented quaternary contacts with residues Y61 and E64 in the gp120 inner domain¹³. These observations may relate to strain-specific modes of interaction between Env and broadly neutralizing antibodies.

Mutation of the key residues in CD4-BS2 had diverse effects on binding of anti-CD4 supersite mAbs to the BG505 SOSIP.664 trimer (Fig. 5b). Specifically, charge inversion of K207 significantly decreased recognition by VRC03 and, to a lesser extent, VRC-CH31 and 3BNC117, but not VRC06; in contrast, mutation of E62 E64 selectively affected VRC06 binding, while none of these mutations significantly reduced the binding of VRC01 and VRC-PG04. These results were confirmed on a more extended panel of mAbs and envelope mutants tested on cell-surface-expressed gp160 from both BG505 and BaL (Supplementary Table 3). In line with their dependence of CD4-BS2 and consistent with previous data³⁶, both VRC03 and VRC06 exhibited trimer-preferring reactivity, with no detectable binding to monomeric BG505 and BaL gp120 in ELISA (Supplementary Fig. 6c), even though binding and even crystallization in complex with monomeric gp120 have been reported for other strains^{37,38}. Overall, these results demonstrate that certain neutralizing human mAbs establish quaternary contacts with the HIV-1 Env spike, providing inferential evidence that the CD4-BS2 region is immunogenic *in vivo*.

DISCUSSION

Our work delineates the initial contact of the HIV-1 Env with the CD4 receptor, documenting the quaternary configuration of a retrovirus receptor-binding site, a feature previously recognized for *Picornaviruses*^{39–41}, but never hitherto for enveloped viruses. We found that the initial CD4-binding site on the pre-fusion HIV-1 Env trimer spans conserved regions on two neighboring gp120 protomers, which embrace the receptor with an extended quaternary surface complementary to discontinuous elements located on two faces of the N-terminal domain of CD4. This initial interaction with CD4 represents the first event in a multi-step process during which the trimer undergoes sequential structural transitions toward downstream intermediate states (i.e., a requisite intermediate high-FRET state²², a 3-CD4-bound state^{21,42}, and coreceptor-bound states^{22,43}) before reaching a final low-energy post-fusion state^{1–3}. To prevent CD4-induced conformational changes in the trimer, which have impeded until now the study of the initial events of receptor interaction, we took advantage of a conformationally-stabilized soluble trimer, DS-SOSIP, which binds to CD4 with an asymmetric 1:1 (CD4:trimer) stoichiometry¹⁶. Accordingly, our cryo-EM structure showed a structural asymmetry in the initial Env-CD4 complex, with a ~3 Å outward shift of both CD4-interactive protomers towards the incoming CD4 molecule. This shift represents perhaps the earliest conformational change to take place within the HIV-1 Env trimer. Its

marked asymmetry appears to be maintained in the requisite transition intermediate observed by smFRET²², before binding of two additional CD4 molecules to yield the 3:1 (CD4:trimer) stoichiometry commonly observed with the CD4-bound open trimer^{21,24,42}.

The dramatic loss of HIV-1 infectivity that we documented upon mutation of key residues involved in the quaternary Env-CD4 contact both on the trimer and on the receptor sides indicates that binding to the full quaternary Env surface is critical for the functional interaction of CD4 with the HIV-1 virion. The more pronounced effect induced by charge inversion versus alanine mutagenesis suggests that CD4 interaction with CD4-BS2 is predominantly driven by electrostatic forces. The quaternary site in the inner gp120 domain appears to be essential for stabilizing the Env-receptor interaction and for triggering Env activation, which in turn leads to coreceptor binding and progression of the fusogenic process. Consistent with this model, the inner domain has been implicated in regulating the transition from the prefusion ligand-free to the CD4-activated conformation in the context of monomeric gp120⁷. However, the orientation of the CD4-BS2 region is dramatically different in the trimer and the monomer. In the prefusion metastable trimer, the key residues that form CD4-BS2 point outwards and are well exposed to the solvent, facing the incoming CD4 receptor; in contrast, the same residues display a peculiar inward orientation and are almost completely buried in the fusion-transitioned structure of the monomeric gp120 core⁸⁻¹¹. Thus, only in the context of the monomer, CD4-BS2 residues establish intramolecular interactions of with layer-2 residues, which affect inter-layer adherence within the inner domain and, consequently, the monomer flexibility and spontaneous access to the coreceptor-binding-competent state. This can explain the allosteric effects on CD4 binding that have been previously reported upon mutation of H66 and other inner-domain residues in the context of the monomer^{7,31}. The cryptic configuration of the CD4-BS2 region in monomeric gp120 also suggests that the quaternary CD4-contact site may be a region of conformational masking³, facilitating immune evasion of this critical surface.

Major efforts aimed at developing a protective vaccine are currently focused on the design of HIV-1 Env-based immunogens that display the major epitopes recognized by broad and potent neutralizing antibodies^{14,16,44}. The definition of the initial CD4-contact surface in the HIV-1 Env trimer allowed us to reassess the supersite of vulnerability formed by the CD4-binding site, with potential implications for HIV-1 vaccine development. Our observation that selected neutralizing antibodies to the CD4-BS, like VRC03 and VRC06 which preferentially recognize the trimeric envelope, develop contact with CD4-BS2 through somatic hypermutation provides evidence that this region is immunogenic *in vivo* and, therefore, should be considered in the design of vaccine immunogens. Furthermore, the CD4-BS2 region may provide a new molecular target for the development of HIV-1 entry inhibitors.

Methods

Sample preparation for electron microscopy analysis

DS-SOSIP.664, 4-domain CD4, and PGT145 Fab were expressed and purified as described previously¹⁶. Purified DS-SOSIP.664 at 1 mg/ml was incubated with 20 μ M of 4-domain sCD4 for 16 hours at room temperature; PGT145 Fab was then added at 3-fold molar excess

of the trimer concentration, and the sample was incubated for another 3–4 hours before vitrification.

Specimen vitrification for cryo-electron microscopy

To avoid aggregation during vitrification, the sample was incubated with a small amount of dodecyl-maltoside (DDM) at a final concentration of 0.085 mM (2x below the critical micelle concentration). Specimens were prepared for cryo-EM by applying 3 μ L of sample to a freshly plasma-cleaned holey gold grid, allowing the sample to adsorb to the grid for 30 sec., followed by blotting with filter paper and plunge-freezing into liquid ethane using the CP3 cryo-plunger (Gatan, Inc.) (20 °C, 85–90% relative humidity).

Cryo-electron microscopy data collection and raw-frame alignment

As summarized in Table 1, data were acquired using the Legion system⁴⁵ installed on a Krios electron microscope operating at 300kV, with a dose of $\sim 86 \text{ e}^-/\text{\AA}^2$ and an estimated defocus ranging from 1.0–4.0 μm underfocus (distributed in an approximately Gaussian manner and centered at $2.5 \pm 0.8 \mu\text{m}$). The dose was fractionated over 40 raw frames collected over a 10-sec. exposure time (250 msec. per frame) on the Gatan K2 Summit direct detection device, with each frame receiving a dose of $\sim 9.4 \text{ e}^-/\text{pixel}/\text{sec}$. A total of 1,900 such “movies” were automatically collected over a span of 4 data collection sessions, recorded at a nominal magnification of 22,500 x, corresponding to a pixel size of 1.1 $\text{\AA}$ at the specimen level. The individual frames were globally aligned using a GPU-enabled frame-alignment program (generously provided by Y. Cheng and X. Li)⁴⁶.

Image processing and refinement

Unless otherwise noted, all of the following image-processing steps were performed within the Appion pipeline^{47,48}. The CTF for all micrographs was estimated using the GCTF package⁴⁹. All micrographs were also manually masked with the “manual masking” tool in Appion to remove regions that were over gold and/or containing large amounts of disordered or aggregated particles. To obtain a clean stack of particles on which to perform the final refinement, we first performed an iterative stack cleaning procedure, described below. Particles were initially selected using DoG Picker⁵⁰ from 800 micrographs (Supplementary Fig. 1a). A phase-flipped, contrast-inverted, and 2x binned stack of 10k particles was created from these picked particles using a box size of 160 pixels and pixel size of 2.2 $\text{\AA}$, and subjected to reference-free 2D alignment and clustering using CL2D⁵¹ to obtain 256 2D classes. The classes were inspected visually, and 15 of these classes (representing different views) were used as templates to select particles over the entire set of micrographs using the Template picking algorithm within Appion^{52,53}. Following 2D alignment and clustering using CL2D and RELION⁴⁸ (outside of Appion) to eliminate class averages that did not display identifiable features (Supplementary Fig. 1b), 100,500 particles were retained for all subsequent analyses, which were performed outside of Appion. A map of unliganded BG505 SOSIP.664 (EMDB ID 5782), low-pass filtered to 60 $\text{\AA}$ was used as the starting point of 3D classification/sorting to eliminate trimer particles that were not bound to both CD4 and PGT145, as well as to exclude badly formed particles. After 2 rounds of 3D classification separating the particles into 4 classes the first round and 3 classes in the second round, the class that showed both PGT145 and CD4 bound (class I) (Supplementary

Fig. 1c) was selected for 3D refinement in RELION (Table 1 and Supplementary Fig. 1d,e). Automasking in RELION yielded an 18 Å resolution map. We truncated the CD4 density from this map using the volume eraser tool in Chimera, and used the `relion_mask_create` program to make masks that extended from this CD4-truncated map. 3D refinement was then performed using these manual masks resulting in maps with incrementally truncated density for the bound CD4 with resolutions of 11 Å (mask 1) and 9 Å (mask 2) (Supplementary Fig. 1e). Next, dose fractionation was performed by incrementally excluding movie frames, and the best resolutions of 9 Å (mask 1) and 7.5 Å (mask 2), respectively were achieved for the two maps that were generated using masks truncated at different lengths, by using the first 30 frames, which corresponded to a total dose of $\sim 51 \text{ e}^{-}/\text{Å}^2$. In a second masking strategy we generated masks extending from the 3D map from Class I (Supplementary Fig. 1c) followed by auto-refine within RELION using these manual masks, resulting in a best resolution of 6.8 Å following post-processing within RELION (Supplementary Fig. 1e). This 6.8 Å resolution reconstruction showed the best resolved density for the bound 4-domain CD4 molecule. This map was used for all model fitting unless otherwise noted.

Model fitting

First, all HIV-1 Env soluble trimer models available in the PDB were fitted onto the 7.5 Å cryo-EM map (Supplementary Table 1). Next, each fitted trimer was separated into its gp120-gp41 protomers, and the protomers were fitted to optimize map-to-model correlation. The trimer with optimal fit was assembled by combining the protomers that fit the map with highest correlation. On examination of the protomers, all three of which came from PDB ID 5ACO, it was found that the protomers were missing residues 59–67 in the gp120 chains. This stretch of residues was added back into each of the protomers by superposing with the trimer in PDB ID 5FYL¹³ and grafting residues 57–70 from each protomer of 5FYL to each equivalent protomer of the fitted trimer. This resulted in a small increase in map-to-model correlation from 0.84 to 0.85. Finally, the fits of the protomers were optimized within the 6.8 Å cryo-EM map. Once the fit of the trimer to the map had been optimized, the coordinates of free PGT145 (PDB ID 3U1S)⁵⁶ were fit to the density visible in the map near the V1V2 region of the trimer. The Fab was first fit as a whole; then, fits for the variable and constant domains were optimized separately to maximize map-to-model correlation. Two orientations of the antibody Fab fragment, rotated by 180° along the longitudinal axis of the Fab, were tested and the orientation that fit with higher correlation (0.75) was also visually found to fit better to the map (Supplementary Fig. 2) compared to the Fab orientation that fit the EM map with reduced correlation (0.66). To fit CD4, we used an approach similar to that used for the trimer, utilizing all the PDB deposits containing CD4 (Supplementary Table 2). For gp120-bound CD4, we superposed the gp120 in the gp120-CD4 complex with the gp120 protomer in the trimer that had its outer-domain CD4-binding loop facing the CD4 density in the cryo-EM map. We designated the resulting orientation as the “post-fusion” orientation of CD4. For CD4 structures that were not bound to gp120, CD4 residues 2–176 were superimposed on residues 2–176 of CD4 from PDB ID 2NY4 that had already been placed in the post-fusion orientation. From this starting position, we fit each CD4 molecule using rigid-body docking within Chimera (Supplementary Table 2, Fig. 4c). Following model fitting, the PGT145-trimer and CD4-trimer interfaces were minimized using the steepest

descent protocol in Chimera, and the interactive areas were optimized by varying side chain rotamer conformations. The 7.5 Å map obtained using Mask2 in RELION auto-refine (Supplementary Fig. 1e) showed better definition of glycan density around the trimer, and was used to place the glycans. Both the 7.5 and 6.8 Å maps have been deposited in the electron microscopy database (ID: EMD-8427).

Structural analyses

To calculate the relative orientations of the CD4-bound protomers in the single CD4-bound Env trimer structure, we first superposed all trimer structures with PDB IDs listed in Supplementary Table 1 to the single CD4-bound structure, by aligning a protomer of each trimer to the protomer of the single CD4-bound Env trimer that did not contact CD4. For trimers where each protomer had been determined independently (i.e., where EM maps were refined using C1 symmetry or where the entire trimer was part of one crystallographic asymmetric unit), each protomer was fit to the protomer of the single CD4-bound Env trimer that did not contact CD4. From this starting orientation, for each pairwise comparison between the single CD4-bound trimer and an Env trimer from the PDB, the rotation and the translation needed to superpose the two CD4 contacting protomers to the respective protomers of the aligned Env trimer were calculated (Fig. 4c). All superpositions were performed using the Superpose module⁵⁷ within the CCP4 suite⁵⁸ and rotation angles were obtained using Superpose, MATLAB (MATLAB 2016a, The MathWorks Inc., Natick, MA.) and Origin (OriginLab, Northampton, MA).

For estimating surface area buried at the interfaces between trimer and CD4, starting from the Env trimers listed in Supplementary Table 1 that had been fitted to the EM density map, trimers missing any of the following CD4 contacting residues were removed: 281, 368, 371, 280, 428, 62, 64, 65, 66, 207, resulting in 7 Env trimer structures. Similarly for CD4, starting from Supplementary Table 2 with CD4 structures that had been fitted to the EM density map, CD4 missing any of the following Env trimer contacting residues were removed: 20, 21, 22, 40, 43, and 64, resulting in 29 CD4 structures. Complex structures for all pairwise Env trimer:CD4 combinations were minimized using Chimera (only CD4 D1 domain and Env residues that are 10 angstroms within CD4 were allowed to move). The side chain rotamers of the interface residues were further refined using SCAP⁵⁹. Buried surface areas were calculated using PISA⁶⁰.

To define the CD4 supersite that is targeted by CD4-binding site antibodies on the pre-fusion closed HIV-1 Env trimer, PDB coordinates of HIV-1 gp120 in complex with CD4-binding site antibodies isolated from 14 human donors³⁵ were superposed onto the BG505.SOSIP (PDB ID 4TVP) by aligning the outer domain residues 255:392, 412:422 and 437:459. The CD4 supersite for antibodies was defined as a set of HIV-1 Env atoms that are within 5.5 Å of docked CD4-binding site antibodies, including the VH1-2 germline derived VRC01-class antibodies, the VH1-46 germline derived 8ANC131 class antibodies as well as the CDR H3-dominated antibodies (VRC13, VRC16, CH103 and HJ16). In addition to the previously defined primary antibody contact region on gp120, the CD4 supersite for CD4-binding site antibodies also extended to the neighboring gp120 protomer in the trimeric pre-fusion closed Env and overlapped with CD4-BS2. Electrostatic potential molecular surfaces for antibodies

and HIV-1 gp120 were generated using the APBS and PDB2PQR server (http://nbc-222.ucsd.edu/pdb2pqr_2.1.1/).

Molecular dynamics (MD) simulation

A model of the structure of HIV-1 BG505-SOSIP.664 trimer with a single CD4 molecule bound was subjected to an all atom, isobaric-isothermal (1 atm, 310 K) molecular dynamics (MD) simulation. The simulation was performed using the AceMD⁶¹ program (v.3212) on the Biowulf Linux cluster at the NIH, Bethesda, MD (<http://biowulf.nih.gov>). The complex was explicitly solvated with TIP3P water, neutralized through addition of counterions (Na⁺, Cl⁻ to 0.15 M), with periodic boundary conditions applied. Electrostatic interactions were calculated using the Particle-Mesh Ewald summation with grid spacing of 1 Å along with a 9 Å cutoff. The CHARMM27 force field was used⁶¹ for protein. The system was energy minimized, warmed to 310 K, and submitted to a production run. A 4 fs integration time step was used by making use of a hydrogen mass repartitioning scheme and data was collected for 668 ns. Langevin dynamics were used to maintain temperature and a Berendsen barostat was used to control pressure.

Sequence conservation analysis

Sequence alignment of a segment of the C1 region (centered around the $\alpha 1$ -helix) and of the C2 region (a segment of the $\beta 3$ - $\beta 4$ loop) of the gp120 inner domain from all the available group-M HIV-1 and SIVcpz isolates (including both subtypes A-K and recombinant forms) was obtained from the Los Alamos HIV database (<http://www.hiv.lanl.gov>). The sequence logo was created using WebLogo (<http://weblogo.berkeley.edu>). The height of each stack indicates the degree of conservation for each residue; the relative height of each letter within individual stack represents the frequency of the indicated amino acid at that site.

Mutagenesis, protein expression and purification

Site-directed mutagenesis was carried out using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). The BG505-SOSIP.664 trimer was expressed by co-transfecting the relevant plasmid (a kind gift of John P. Moore) with a plasmid expressing the cellular protease furin into human embryonic kidney (HEK) 293 free-style (FS) cells. Cell-free supernatants were harvested after 7 days, passed through a 0.22 μ m filter, and loaded onto a *Galanthus nivalis* (GNA) lectin column (Vector laboratories). After repeated washing with PBS, bound proteins were eluted with 1 M methyl α -D-mannopyranoside in PBS, followed by dialysis toward PBS. Dialyzed samples were concentrated to 2 ml using Amicon Ultra-15 centrifugal filter units (MWCO 50,000, Millipore) and applied to a PBS pre-equilibrated Superdex 200 column. The peak corresponding to the trimeric form was collected and concentrated, followed by a second round of Superdex 200 purification. Finally, purified trimers were concentrated to 0.5–1 mg/ml and stored at –80 °C. Monomeric HIV-1 BG505-T332N gp120 and BaL gp120 (gene synthesized by GeneArt) were expressed in HEK 293FS. After 7 days, the supernatants were harvested and passed through a 0.22 μ m filter. Purification of gp120 was carried out by loading filtered supernatants onto a lectin column as described above. The eluted proteins were concentrated using Amicon Ultra-15 centrifugal filter units (MWCO 10,000, Millipore) and applied to a Superdex 200 column.

DS-SOSIP.664-based trimers were purified as described previously¹⁶. Briefly, cell-free supernatants were loaded onto a VRC01 or 2G12 affinity column, followed by gel filtration. The trimeric form was collected and passed through a 447-52D affinity column for negative selection. A further clean-up was performed by using a column of anti-V3 antibodies (1006-15D, 2219, 2557, 2558, 3074, 50.1).

Expression of full-length HIV-1 gp160 or CD4

The full-length CD4 gene and the gp160 gene from BG505-T332N and BaL (both from Edward Berger) were mutagenized as described above for the SOSIP trimer. HEK 293T cells were used to express CD4 or gp160 on the cell-surface membrane. The cells were seeded into 6-well plates at 300,000 cells/well in 2 ml of DMEM containing 10% fetal bovine serum (DMEM 10%). After overnight incubation at 37 °C in humidified atmosphere with 5% CO₂, the culture medium was replaced with 1.5 ml of fresh 10% DMEM. DNA-FuGENE 6 complex was prepared by mixing 2 µg DNA with 10 µl FuGENE 6 Transfection Reagent (Promega) in Opti-MEM (Gibco) to a final volume of 150 µl and incubated for 15 min at room temperature (RT). The cells were transfected by adding the DNA-FuGENE 6 complex dropwise to each well and harvested after 48–60 hours. The same protocol was used for CD4 expression on the surface membrane of Cf2Th/syn-CCR5 cells (AIDS Reagent Program).

Flow cytometry

HEK 293T cells expressing CD4 or gp160 were harvested by mechanical shaking and pipetting, washed with PBS and incubated with anti-CD4 or anti-gp120 antibodies (5 µg/ml) or serially diluted human Fc-chimeric CD4-Ig (produced in 293FS cells; plasmid provided by Michael Farzan) at RT for 30 min. After washing with PBS twice, the cells were incubated with PE-conjugated sheep anti-mouse IgG (Sigma) or goat anti-human IgG (Southern Biotech) at RT for 15 min. The cells were then washed once with PBS, fixed in 2% paraformaldehyde (PFA) and analyzed on a BD FACSCanto™ (BD Biosciences). Data analysis was performed using the FlowJo software.

Pseudovirus preparation and infectivity assays

Viral pseudoparticles expressing WT or mutated gp160 from HIV-1 BG505-T332N or BaL were produced in HEK 293T cells by co-transfecting Env-expressing plasmids together with a backbone plasmid, pSG3^{env}, expressing a full-length HIV-1 clone with a defective Env gene. To produce the pseudoparticles, 2 µg of each Env-expressing plasmid and 4 µg of the backbone plasmid were mixed in Opti-MEM medium (Gibco), and 24 µl of FuGENE 6 Transfection Reagent was added, followed by 15-min incubation at RT. DNA-FuGENE 6 complex was then added to the cells and incubated overnight at 37 °C. After replacing the culture medium with 2 ml fresh 10% DMEM, the cells were incubated for 48 hours and the supernatants containing the pseudoviruses were harvested by centrifugation. For pseudovirus infection, TZM-bl cells (AIDS Reagent Program) were harvested and seeded into 96-well flat bottom plates at 10,000 cells/well in 100 µl 10% DMEM. After 30 min at 37 °C, the pseudovirus stock (normalized to 1 ng of p24_{GAG} protein per mutant) was added to the cells in a total volume of 200 µl/well. Reporter gene activation was detected 2 days later using the Luciferase Assay kit (Promega) with a luminometer (PerkinElmer). Relative Light Units

(RLU) were recorded, and the final values were normalized against the values obtained with the WT envelope set at 100%. All the samples were tested in duplicate wells.

Pseudoviruses expressing firefly luciferase were produced by co-transfecting Env-expressing plasmids (WT BaL or BG505-T332N) together with a backbone plasmid, pNL4-3.Luc.R⁻E⁻ (AIDS Reagent Program), at a ratio of 1:1. Supernatants containing pseudoviruses were harvested by centrifugation after 48 hours of incubation. Target cells were prepared by transfecting WT or mutated CD4-expressing plasmids to Cf2Th/syn-CCR5 cells. After incubation for 24 hours, the cells were harvested and seeded to 96-well flat-bottom plates at 10,000 cells/well in 100 µl 10% DMEM. Pseudovirus stocks were added to the cells to a total volume of 200 µl/well. The reporter gene activity was detected 2 days later using Luciferase Assay kit (Promega) and expressed as relative Light Units (RLU). Serial dilutions of the WT CD4 plasmid were used to generate a reference curve which allowed to extrapolate accurate infectivity values for each mutant based on expression levels, as measured by flow cytometry using mAb OKT4. All samples were performed in duplicate.

Enzyme immunoassays

All the antibodies and envelope proteins used in ELISA experiments were diluted in 0.02% casein in PBS. All samples were washed 3 times after each step with 1x wash buffer (R&D Systems). For assessing sCD4 binding to the BG505 SOSIP.664 trimers or gp120 monomers, 96-well ELISA plates (Corning) were coated with 2 µg/ml of mAb 2G12 at 4 °C overnight. After blocking with 0.2% casein in PBS, purified trimers or monomers (2 µg/ml) were added and incubated for 2 hours at RT. After washing, serially diluted 4-domain sCD4 (R&D Systems) was added and incubated for 1 hour at RT. After washing, the samples were incubated with biotin-labeled mAb OKT4 (eBioscience) for 1 hour; after additional washing, horseradish peroxidase (HRP)-conjugated streptavidin (R&D Systems) was added for 1 hour at RT. The reaction was revealed by incubation with the appropriate substrate for 10 min before addition of the stop solution. Light absorption was measured at a wavelength of 450 nm. All samples were tested in duplicate or triplicate wells. To evaluate antibody binding, ELISA plates were coated with 5 µg/ml of an anti-gp120 C-terminus hyperimmune sheep antiserum (D7324) for monomeric gp120 or with 0.5 µg/ml of rabbit anti-His tag antiserum for trimers. Monomeric gp120s or trimers were captured on the plates, and various anti-gp120 antibodies (0.5–5 µg/ml or serial dilutions starting from 5–20 µg/ml) were added to the wells and incubated for 1 hour, followed by 1-hour incubation with HRP-conjugated goat anti-human IgG antibody (Sigma) at RT. The subsequent steps were the same as described above. For all the mutants, relative binding compared to the WT was calculated. To evaluate antibody binding to DS-SOSIP.664, 10 µg/ml lectin from *Galanthus nivalis* (Sigma) was coated on the ELISA plate. DS-SOSIP.664 proteins were added at 1 µg/ml for capture. Various anti-gp120 antibodies were tested at 1–5 µg/ml or with serial dilutions starting from 5–20 µg/ml.

Surface plasmon resonance (SPR) analyses

Single-cycle kinetics analysis of 2-domain sCD4 binding to the BG505 SOSIP.664 and DS-SOSIP.664 trimers was performed at 20 °C on a Biacore T200 (GE Healthcare) as described previously¹⁶. Briefly, MAb 2G12 was immobilized onto four flow cells of a CM5 sensor to

~2,000 response units (RU) using the amine-coupling method. Purified BG505 SOSIP.664 trimers were injected to two sample flow cells at a flow rate of 5 μ l/min for two minutes, and captured to 300–400 RU. The other two flow cells were left blank as reference. Series dilutions of sCD4 (180 nM, 90 nM, 45 nM, 22.5 nM, 11.25 nM) were injected to both the reference and sample flow cells at 50 μ l/min in a single cycle, starting from the lowest concentration. Ten minutes of dissociation phase was allowed after the last sCD4 injection. The same injections were carried out using HBS-EP buffer in order to obtain a reference curve. Data were fitted to 1:1 Langmuir model with Biacore T200 evaluation software. By SPR analysis, we verified that the differential CD4 binding of CD4-BS2 mutants was not due to altered binding to mAb 2G12 (data not shown).

CCR5-binding assay

Cf2Th/syn-CCR5 cells, which express high levels of CCR5 on their surface membrane, were used to assess binding of soluble BG505-SOSIP.664 trimers or BaL gp120 monomers to CCR5. The cells were harvested at ~80% confluency with enzyme-free cell dissociation buffer (Gibco). His-tagged BG505-SOSIP.664 trimers or BaL gp120 monomers were pre-incubated with or without 2-domain sCD4 for 1 hour at 4 °C and incubated with the cells for 1 hour at 4 °C, followed by washing with PBS. For BG505-SOSIP.664 trimers, PE-conjugated mouse anti-His tag antibody (Milenyi Biotec) was added to the cells for 1-hour at 4 °C. For gp120 monomers, D7324 was added to the cells for 1-hour at 4 °C, followed by 15-min incubation with CFTM 488A-conjugated chicken anti-goat antibody (Sigma). The cells were then washed once with PBS, fixed with 2% PFA and analyzed on a BD FACSCanto instrument. Specificity of binding was assessed by abrogation of the signal with an anti-CCR5 mAb (Becton Dickinson). Data analysis was performed using the FlowJo software.

Structure alignments and figures

All graphs were plotted using Graphpad Prism and structural figures were displayed using Pymol⁶². PDB ID numbers are referenced in individual figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cryo-EM structure of the initial interaction between HIV-1-envelope trimer and CD4 receptor. **(a)** Side view of the cryo-EM structure of a ternary complex comprising DS-SOSIP HIV-1 Env trimer, 4-domain sCD4 (yellow) and Fab from the broadly neutralizing mAb PGT145 (green), all shown in ribbon representation, with a 6.8 Å resolution EM-reconstructed density in gray mesh. Each gp120 protomer is displayed in a different color, with gp41 in gray. **(b)** View from the viral membrane of the complex rotated 90° from panel A. CD4 contacts two gp120s, indicated as primary (purple) and quaternary (pink) CD4-contacting protomers, but not the third gp120 (cyan). **(c)** Zoom-in of the PGT145

interaction, with middle inset showing a 90° view and electrostatic surface (electropositive, blue; electronegative, red) display for HIV-1 Env (left insert) and for PGT145 (right insert). **(d)** Orientation of Env protomers in the pre-fusion, closed Env conformation recognized by a single CD4, displayed on the Env trimer in ribbon representation (left). Trimeric Env structures were aligned to the non-contacting protomer of the single CD4-bound trimer, and the resulting orientations of the primary and quaternary CD4-contacting protomers were plotted (right). The distance and angular measurements are absolute values measured from the single CD4-bound trimer. With asymmetric trimers, such as the PGT151-bound trimer (PDB ID 5FUU), each protomer was aligned to the non-contacting protomer of the single CD4-bound trimer, and the alignment displayed is the orientation with median positional displacement relative to the primary CD4-contacting protomer.

Figure 2.

Quaternary configuration of the CD4-binding site in the initial interaction with HIV-1 Env trimer. **(a)** Top view of the cryo-EM structure of a single-CD4-bound HIV-1 Env trimer showing the quaternary interaction of CD4 (yellow) with two neighboring gp120 protomers; gp41 was removed for clarity. Inset shows a close-up of the CD4-binding site (CD4-BS2) on the quaternary contacting gp120. Residues in the C1 (α I-helix) and C2 (β 3- β 4 loop) regions of the quaternary CD4-contacting gp120 protomer (pink) are highlighted in stick representation (E62 and E64, negatively charged; H66 and K207, positively charged). The location of K65, which points away from CD4, is shown for reference. **(b)** Amino acid

conservation within the CD4-BS2 region. Residues E64, H66 and K207 are nearly universally conserved (>99.7%) across all group-M HIV-1 isolates; position 62 is occupied by an acidic residue (either E or D) in more than 90% of group-M isolates, with non-conservative mutations essentially limited to genetic subtype D (58% E→K) and subtypes G and J (100% E→S). (c) Disruption of CD4-BS2 by charge inversion abrogates HIV-1 infectivity. Infectious pseudoparticles bearing the WT or mutated BG505-T332N (clade A) or BaL (clade B) Env were tested in TZM-bl cells. Infectivity values for each mutant were calculated relative to the value obtained for the WT. (d) Infectivity of viral pseudotypes carrying mutated HIV-1 Env from different genetic subtypes. The infectivity of each mutant was calculated relative to that of the respective WT Env. The data shown represent the mean $\pm$ range of duplicate wells from a representative experiment out of three performed which yielded similar results.

Figure 3.

Charge inversions in CD4-BS2 reduce the CD4-binding capacity of the BG505 SOSIP.664 trimer. **(a)** Kinetics of sCD4 binding to the DS-SOSIP-based trimers were measured by single-cycle surface plasmon resonance. **(b)** Kinetics of sCD4 binding to the WT and mutated trimers were measured by single-cycle surface plasmon resonance. **(c)** Charge inversions in CD4-BS2 abrogate the CCR5-binding capacity of BG505 SOSIP.664 trimers after sCD4 treatment. Binding of WT and mutated soluble BG505 SOSIP.664 trimers to CCR5 expressed on the surface of Cf2Th/syn-CCR5 cells was assessed by flow cytometry. Mean fluorescence intensity (MFI) values were calculated relative to the positive control (sCD4-bound WT). The data shown represent the mean $\pm$ range of two independent experiments. **(d)** Charge inversions in CD4-BS2 abrogate the mAb 48d-binding capacity of BG505 SOSIP.664 trimers after sCD4 treatment, as assessed by ELISA. The data shown represent the mean $\pm$ range of duplicate wells from a representative experiment out of three performed which yielded similar results.

Figure 4.

CD4 surface interacting with CD4-BS2 in the initial contact with Env. **(a)** Top view of the cryo-EM structure of a single-CD4-bound HIV-1 Env trimer showing the quaternary interaction of CD4 (yellow) with neighboring gp120 protomers; gp41 was removed for clarity. Inset shows a close-up representation of CD4 residues (highlighted by sticks representation and color) at the interface with the second CD4-binding site (CD4-BS2) in gp120 (D63, negatively charged; K21 and K22, positively charged). **(b)** Open-book surface representation of the quaternary HIV-1 Env-CD4 interaction in the optimized cryo-EM structure. The CD4 footprint on the HIV-1 Env trimer is shown in yellow (left middle), while

the trimer footprints on CD4 are shown in purple for the primary CD4-contacting gp120 and in pink for the quaternary CD4-contacting gp120 (right middle). Insets show zoomed-in views of the contact sites (within 5.5 Å). (c) Left, top view of the cryo-EM structure of a single-CD4-bound HIV-1 Env trimer showing CD4 molecules fitted into the cryo-EM density map in yellow and CD4 molecules in the monomeric gp120 (gp120m)-bound (post-fusion) orientation (listed in Supplementary Table 2) in grey. CD4 fits were performed with CD4 domains 1 and 2 (D1D2); CD4 domains 3 and 4 were added to the CD4 molecule showing the best fit by alignment with 4-domain CD4 (PDB ID: 1WIO) and by transplanting domains 3 and 4 onto the fitted D1D2. Gp41 was removed for clarity. Right, orientation of the CD4 molecules fitted into the cryo-EM density map (yellow squares) relative to the gp120m-bound orientation (grey squares). The red square shows the CD4 fit that was chosen for the final DS-SOSIP-CD4-PGT145 model fitted to the cryo-EM density. This fit showed the highest map-to-model correlation among all the CD4 molecules. (d) Disruption of the CD4-BS2-interactive site reduces the HIV-1 receptor function of CD4. Pseudoviruses carrying the WT BG505-T332N or BaL envelopes were tested for infectivity in Cf2Th/syn-CCR5 cells expressing different CD4 mutants. Infectivity values for the mutants were calculated relative to the value obtained with WT CD4. The data shown represent the mean $\pm$ range of duplicate wells from a representative experiment out of two performed which yielded similar results.

Figure 5.

Quaternary interactions of selected anti-CD4-BS antibodies with the HIV-1 envelope trimer.

(a) Fabs for select neutralizing mAbs against the CD4-binding site (i.e., VRC03, VRC06, VRC-CH31) are shown in the top row of panels docked to a surface representation of the HIV-1 Env trimer in its pre-fusion closed conformation. The lower row of panels shows close-up views of the putative contact area with CD4-BS2, with key residues (E62, E64, H66, K207) highlighted by stick representation with Env colored by electrostatics, electropositive in blue and electronegative in red. The right panel shows a surface representation of the CD4 supersite of vulnerability (magenta), with CD4-contact sites highlighted in yellow. Glycans are shown in stick representation. (b) Disruption of CD4-BS2 affects binding of neutralizing mAbs specific for the CD4-binding site. Binding of anti-CD4-BS mAbs to WT and mutated soluble cleaved HIV-1 envelope trimers (BG505 SOSIP. 664) was assessed by ELISA. The data shown represent the mean $\pm$ range of duplicate wells from a representative experiment out of two performed which yielded similar results.

Table 1

Cryo-EM data collection and refinement information for the DS-SOSIP.664 trimer in complex with sCD4 (4-domain) and PGT145 Fab.

DS-SOSIP.664-sCD4-PGT145	
Data collection	
Software	Leginon 3.1
Pixel Size (Å)	1.1
Defocus range (μm)	1.0–4.0
Voltage (kV)	300
Electron dose (e ⁻ /Å ²)	86
Magnification	22,500
Refinement	
Software	RELION 1.4
Particles	55,000
Resolution (Å)	6.8
Accuracy of rotation (°)	4.63
Accuracy of translation (pixels)	2.81
Map sharpening B-factor (Å ²)	−172