CD4 Incorporation into HIV-1 Viral Particles Exposes Envelope Epitopes Recognized by CD4-induced Antibodies

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Abstract

CD4 downregulation on infected cells is a highly conserved function of primate lentiviruses. It has been shown to positively impact viral replication by a variety of mechanisms including enhanced viral release and infectivity, decrease of cell reinfection and protection from antibody-dependent cellular cytotoxicity (ADCC), which is often mediated by antibodies that require CD4 to change envelope (Env) conformation. Here we report that incorporation of CD4 into HIV-1 viral particles affects Env conformation resulting in the exposure of occluded epitopes recognized by CD4-induced antibodies. This translates into enhanced neutralization susceptibility by these otherwise non-neutralizing antibodies but is prevented by the HIV-1 Nef accessory protein. Altogether, these findings suggest that another functional consequence of Nef-mediated CD4 downregulation is the protection of viral particles from neutralization by commonly-elicited CD4-induced antibodies.

Importance

It has been well established that Env-CD4 complexes expose epitopes recognized by commonly-elicited CD4-induced antibodies at the surface of HIV-1-infected cells, rendering them vulnerable to ADCC responses. Here we show that CD4 incorporation has a profound impact on Env conformation at the surface of viral particles. Incorporated CD4 exposes CD4-induced epitopes on Env, rendering HIV-1 susceptible to neutralization by otherwise non-neutralizing antibodies.

55 **Introduction**

56

57 Human immunodeficiency virus (HIV-1) entry, mediated by the trimeric viral envelope
58 glycoproteins (Env), is the first step of the viral replication cycle. The Env trimer is the only virus-
59 specific antigen present on the surface of viral particles; as such, it is the target of neutralizing
60 and non-neutralizing antibodies. Env is a highly dynamic molecule that, upon binding the
61 receptor, CD4, transitions from a "closed" conformation (State 1) to an "open" CD4-bound
62 conformation (State 3). CD4 engagement induces an asymmetric intermediate (State 2)
63 adopted on the pathway to State 3 (1-3). The mature HIV-1 Env trimer is derived by proteolytic
64 cleavage of a trimeric gp160 precursor (4, 5) and is composed of the exterior gp120 and
65 transmembrane gp41 subunits. The gp120 is retained on the trimer via non-covalent
66 interactions with the gp41 ectodomain (6-8). The gp120 glycoprotein is responsible for
67 interactions with CD4 (9, 10). CD4 binding triggers conformational changes in gp120 that
68 promote its interaction with one of the chemokine receptors, CCR5 or CXCR4 (11-18). CD4
69 binding also induces conformational changes within the gp41 ectodomain (19-22). The
70 conformational transition of the gp41 ectodomain into a six-helix bundle composed of the HR1
71 and HR2 heptad repeat regions results in the fusion of the viral and target cell membranes (23-
72 25).

73

74 CD4 downregulation is a highly conserved function of primate lentiviruses (26, 27). It has
75 been shown that HIV-1 uses different mechanisms to downregulate CD4 from the cell surface
76 (reviewed in (28-30)). HIV-1 uses its Nef, Vpu and Env proteins to decrease CD4 cell surface
77 expression. Nef is expressed early during the replication cycle and downregulates CD4 from
78 the plasma membrane by directing the receptor to lysosomal degradation (28, 31-35). Vpu is
79 expressed late in the replication cycle from a bicistronic mRNA also coding for Env. Vpu
80 interacts with newly-synthesized CD4 in the endoplasmic reticulum (ER) and induces its

81 degradation through an endoplasmic-reticulum-associated protein degradation (ERAD)
82 mechanism (36-39). The action of Vpu liberates Env from CD4-dependent retention in the ER
83 (40) allowing trafficking in its unliganded form to the plasma membrane.
84
85 CD4 downregulation appears to be important for viral replication at different levels (28-
86 30) and was shown to be important for Env incorporation into viral particles, viral infectivity (41-
87 44) and to avoid reinfection of the cell (26, 45-47). CD4 downregulation also prevents exposure
88 of otherwise occluded CD4-induced (CD4i) epitopes which are recognized by easily-elicited
89 non-neutralizing antibodies (nnAbs) (48). In HIV-1-infected individuals, CD4i antibodies are
90 present in different biological fluids, including sera, breast milk and cervicovaginal lavages (49-
91 52). Some of these antibodies have been shown to possess potent antibody-dependent cellular
92 cytotoxicity (ADCC) activity against cells expressing Env in its "open" CD4-bound conformation
93 (48, 51, 53-56). This "ADCC susceptible" conformation was recently identified as a fourth Env
94 conformational state named State 2A (57). This new conformation is asymmetric and was
95 shown to be stabilized by a combination of small CD4 mimetics (CD4mc) and two types of CD4i
96 antibodies, anti-coreceptor binding site (CoRBS) and anti-cluster A antibodies. Alternatively, it
97 could be stabilized through Env-CD4 *cis* interactions. Accordingly, Nef-mediated CD4
98 downregulation prevented the spontaneous sampling of this antibody-vulnerable conformation
99 at the surface of infected cells (57). This finding raised the intriguing possibility that another
100 functional consequence of HIV-1-mediated CD4 downregulation is to prevent neutralization by
101 otherwise non-neutralizing CD4i antibodies.
102
103 Here, using a combination of virus capture assay (VCA), infection, neutralization and
104 cold-inactivation assays, we have investigated the functional consequences of CD4
105 incorporation on Env conformation. We report that CD4 incorporation has a significant impact

on Env conformation, stabilizing "open" conformational states and increasing the susceptibility of viral particles to neutralization by commonly-elicited CD4i antibodies.

Results

CD4 interaction exposes CD4i epitopes on viral particles

To investigate the impact of CD4 on Env conformation at the surface of viral particles we adapted a previously-described virus capture assay (58, 59). This virus capture assay relies on the binding of HIV-1 virions by anti-Env Abs that are immobilized on ELISA plates. The viral particles used in this assay are generated by transfecting HEK293T cell with the pNL4.3 Nef-Luc Env- construct (8, 59-61). This construct is co-transfected with a plasmid encoding HIV-1 Env and a plasmid encoding the G glycoprotein from vesicular stomatitis virus (VSV-G), resulting in a virus capable of a single round of infection. Virus-containing supernatants are added to the antibody-coated plate and unbound virions are washed away. Retention of virions on the surface of the plate by anti-Env Abs is visualized by the addition of HEK293T cells that do not express CD4. Infection of the HEK293T cells is mediated by VSV-G and measured by luciferase activity 2 days after infection. A scheme of the assay is depicted in Figure 1A. VSV-G must be present on the virion in order to allow viral infection and subsequent luciferase expression. If only HIV-1 Env is present and that Env is recognized by the capture antibody, the virions are captured but unable to infect HEK293T cells and therefore no signal is obtained (Figure 1B). Similarly, if only VSV-G is present, the anti-Env Abs are unable to capture the virions and therefore no signal is obtained. Only the presence of HIV-1 Env and VSV-G on virions results in a signal when using anti-Env Abs such as 2G12, which recognizes an exposed glycan-dependent epitope on the State 1 Env. Since the epitope recognized by the A32 antibody, which targets the gp120 inner domain, is buried in the closed trimer, it fails to capture the virus (Figure 1B).

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133 Using this virus capture assay (VCA), we evaluated the impact of CD4 incorporation on Env
134 conformation. Briefly, HEK293T cells were co-transfected with pNL4.3 Nef- Luc Env- together
135 with plasmids expressing wild-type (wt) HIV-1_{JRFL} Env or a mutant Env (D368R) unable to
136 engage CD4, VSV-G and wild-type human CD4 (hCD4) or a mutant CD4 (F43H) impaired in its
137 ability to engage gp120 (48, 62, 63). Released viral particles were collected two days after
138 transfection and normalized by reverse transcriptase (RT) content, as described in Material and
139 Methods. Ninety-six well plates were coated with anti-HIV-1 Env monoclonal antibodies
140 recognizing the gp120 outer domain (2G12), the V1V2 glycan trimer apex (PG9), CD4-induced
141 gp120 epitopes (17b, A32, C11), the CD4-binding site (VRC03, b12), CD4i gp41 Cluster I
142 (F240, QA255.072), anti-HIV Immune Globulin (HIVIG, prepared from pooled plasma of
143 asymptomatic HIV+ donors), and the anti-CD4 OKT4 Ab, which binds to the D3 domain of CD4.
144 Normalized amounts of viral particles were added to the plates for 4 hours at 37 ºC and then the
145 plates were washed to remove unbound viruses. HEK293T cells were added to the wells and
146 lysed 48 hours later to measure luciferase activity. Co-transfection of CD4 resulted in its
147 incorporation on viral particles, as measured by the OKT4 antibody. CD4 was able to engage
148 Env in *cis* as suggested by a small but nevertheless significant decrease in virion capture by the
149 VRC03 and b12 CD4BS antibodies (Figure 2A). CD4 incorporation also decreased virion
150 capture by PG9, which preferentially recognizes the “closed” State 1 Env conformation (64).
151 This is expected since CD4 interaction “opens” Env decreasing the sampling of the quaternary
152 epitope recognized by this antibody (48). The F43H change in CD4, which decreases Env
153 interaction but does not completely abrogate it (62), diminished the effect of incorporated CD4
154 on VRC03 and b12 binding. As expected, viral particles bearing the D368R mutation, known to
155 abrogate recognition by CD4BS, were efficiently recognized by PG9 but not by VRC03 or b12
156 (Figure 2B). In the absence of incorporated CD4 none of the CD4i anti-gp120 antibodies tested
157 (17b, A32, C11) were able to capture viral particles, whereas gp41-directed antibodies did

158 (F240 and QA255-072; Figure 2C). The lack of binding of gp120 antibodies is in agreement
159 with the occluded nature of the gp120 epitopes they recognize (48, 57, 65). Strikingly,
160 incorporation of wild-type CD4 but not of its F43H counterpart greatly enhanced the capacity of
161 these antibodies to capture viral particles (Figure 2C). These results suggest that Env-CD4
162 interaction on viral particles can lead to exposure of these CD4i gp120 epitopes. Supporting
163 this observation, the Env D368R variant failed to expose these epitopes despite the
164 incorporation of CD4, as measured by effective capture by OKT4 (Figure 2D). In the absence of
165 incorporated wild-type CD4, the gp41 CD4i epitopes recognized by the F240 and QA255-072
166 antibodies were more available than the gp120 CD4i epitopes (Figure 2C). More viruses were
167 captured by these antibodies when wild-type CD4 was incorporated; this effect was nullified by
168 the F43H change in CD4 (Figure 2C) or by the D368R change in Env (Figure 2D). These results
169 indicate that the incorporation of CD4 into HIV-1 viral particles leads to CD4-gp120 interaction
170 and increased exposure of CD4i epitopes on Env.
171
172 To extend these results beyond the HIV-1_{JRFL} Env, we performed the VCA using viral
173 particles pseudotyped with the HIV-1_{YU2} and HIV-1_{BG505} Envs and obtained similar results
174 (Figure 3A and Figure 3B). CD4 incorporation resulted in a significant increase in the
175 interaction of several CD4i Abs with viral particles. As expected, CD4 competed with CD4BS
176 Abs for binding, resulting in decreased interaction of VRC03 and b12 with HIV-1_{YU2}. Opening of
177 Env by CD4 also decreased recognition by PG9, an antibody that preferentially binds the closed
178 State 1 conformation (64, 66, 67). The gp41 epitopes recognized by the F240 and QA255-072
179 antibodies were exposed in the presence of CD4 on HIV-1_{YU2} more than on HIV-1_{BG505}. This
180 may relate to the differential triggerability of these Envs by CD4 (65). Altogether, these results
181 confirm that incorporated CD4 alters the conformational landscape of Env to sample more
182 “open” conformations.
183

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185 **CD4 interaction sensitizes viral particles to cold inactivation**

186 For some HIV-1 Env isolates, prolonged incubation on ice results in functional inactivation (59).
187 It has been suggested that cold inactivation depends on the ability of the HIV-1 gp120 to sample
188 the CD4-bound conformation (59) and is more efficient for Envs that are prone to undergo
189 conformational changes (68). Accordingly, viral particles bearing Envs in "open" conformations
190 are more susceptible to this ligand-free inactivation (1), which can be modulated by the V1V2
191 and V3 variable regions of gp120 (69). To evaluate whether incorporated CD4 affects the
192 susceptibility of viral particles to cold inactivation, we incubated them on ice for up to 24 h.
193 Briefly, HIV-1 virions encoding a luciferase reporter (pNL4.3 Nef- Env-Luc) and bearing wild-
194 type (wt) Env from HIV-1_{JRFL} or HIV-1_{YU2} were incubated for different amounts of time on ice
195 before being used to infect Cf2Th cells expressing CD4 and CCR5 (70). Luciferase activity was
196 measured 48 h later, as described (8). Env-pseudotyped viral particles produced in the absence
197 of hCD4 were resistant to cold inactivation. CD4 incorporation modestly but significantly
198 enhanced virus susceptibility to cold inactivation. This suggests that Env-CD4 *cis* interaction
199 changes the conformational landscape of Env, resulting in the stabilization of more open and
200 thus cold-sensitive conformations (Figure 4).

201

202

203 **CD4 incorporation sensitizes viral particles to neutralization by CD4-induced antibodies**

204 As our data indicate that incorporation of CD4 into viral particles affects Env conformation, we
205 evaluated whether CD4 incorporation also affected the susceptibility of viral particles to
206 neutralization by ligands that recognize "open" conformations. We used plasmids encoding full
207 proviruses of the transmitted/founder infectious molecular clones HIV_{CH58} and HIV_{CH77}, either
208 wild-type (wt) or deleted in their Nef gene (Nef-), to transfect HEK293T cells in the absence of or
209 with different amounts of plasmids encoding human CD4. By doing so, we generated HIV-1

virion particles enriched in CD4. We used these virions to infect CD4+ CCR5+ TZM-BL cells in the presence of increasing quantities of antibodies. In agreement with previous reports (41-43), we observed that CD4 incorporation decreases viral infectivity (Figure 5A). Interestingly, CD4 incorporation significantly reduced infectivity of HIV_{CH58} wt but not HIV_{CH77} wt viral particles. Thus, HIV_{CH77} is intrinsically more resistant to the detrimental effects of CD4 incorporation on viral infectivity. This phenotype was modulated by Nef since *nef* deletion further impaired viral infectivity of HIV_{CH58} viral particles but also resulted in a significant dose response decrease in viral infectivity for HIV_{CH77}. Nevertheless, under these conditions, a fraction of the viral particles generated in the presence of CD4 remained infectious, allowing us to evaluate their susceptibility to antibodies with different specificities. As shown in Figure 5B and C, Nef-defective viral particles produced in the presence of the highest ratio of CD4 were modestly but significantly more susceptible to neutralization by pooled plasma from asymptomatic HIV-1-infected donors (HIVIG). Because this phenotype is reminiscent of the neutralization mediated by non-neutralizing CD4i Abs such as 17b (anti-CoRBS), 19b (anti-V3), and A32 (anti-cluster A) in the presence of subinhibitory concentrations of CD4mc (71-73), we then tested the susceptibility of viral particles to these antibodies. Figures 6A to 6F show that low CD4 incorporation, at a ratio of 0.1, is sufficient to render HIV_{CH58} Nef- and HIV_{CH77} Nef- viral particles, which bear neutralization-resistant Tier-2 Envs, susceptible to neutralization by anti-gp120 Abs 17b and 19b. At this ratio of CD4, HIV_{CH77} Nef- but not HIV_{CH58} Nef- viral particles were also susceptible to A32. Intriguingly, higher expression of CD4 restored baseline sensitivity neutralization of Nef-defective viral particles. This could be explained by the impact of CD4 incorporation on viral infectivity (Figure 5A). As CD4 incorporation increases, viral infectivity is gradually impaired; thus modifying the nature of the pool of infectious viral particles. At higher levels of CD4, incorporated CD4 abrogates viral infectivity; the remaining infectious viral particles might be those that did not incorporate sufficient CD4 to modulate Env conformation, thus explaining why the neutralization goes to baseline. These results suggest that there is a

fine balance between CD4 incorporation, loss of infectivity, Env-CD4 stoichiometry and its impact on Env conformation and neutralization by CD4i antibodies. Nevertheless, the protective effect of Nef in this system can apparently be surmounted, as co-expression of higher quantities of CD4, at a ratio of 0.5, was sufficient to sensitize the wild-type HIV-1_{CH77} to neutralization by these non-neutralizing antibodies. Altogether, these results indicate that CD4 incorporation enhances the susceptibility of viral particles to neutralization by otherwise non-neutralizing CD4i antibodies.

Nef-mediated CD4 downregulation prevents Env conformational changes and neutralization by CD4i antibodies

Since the HIV-1 Nef accessory protein downregulates CD4 from the cell surface, we evaluated if its expression was sufficient to prevent the Env conformational changes associated with incorporation of CD4 into virions. As described above, the readout of our VCA depends on luciferase expression; in Figures 1-5 we used a provirus that encoded the *luciferase* gene instead of *nef* and therefore these viruses were Nef-defective. To explore the role of Nef, we used a different proviral construct encoding both Renilla luciferase and Nef. In this construct *nef* expression is driven by a modified encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) (74, 75). This full-length provirus and its nef-defective counterpart can express HIV-1_{Bal} Env. As expected, Nef expression was required to efficiently downregulate CD4 from the cell surface (not shown). The impact of CD4 incorporation on Env expression was evaluated by VCA as described above. In the absence of CD4, the Env conformation of both Nef+ and Nef- viral particles was similar. The HIV-1_{Bal} Env was poorly recognized by CD4i Abs A32, C11 and 17b. When viral particles were produced in the presence of CD4, Env at the surface of Nef-defective viral particles exposed CD4i gp120 epitopes and were efficiently captured by these CD4i Abs, consistent with the efficient incorporation of CD4 (Figure 7). By contrast, Nef expression limited the exposure of CD4i epitopes upon CD4 incorporation. Altogether, these

data suggest that Nef-mediated CD4-downregulation might be a mechanism to protect exposure of vulnerable epitopes recognized by CD4i Abs.

Discussion

The presence of receptor molecules on the infected cell surface can present problems for enveloped viruses, leading to viral strategies to minimize potential detrimental effects on virus replication. For example, sialic acid serves as the receptor for the influenza virus and is bound by its hemagglutinin (HA) protein. Sialic acid is present on many glycoproteins but influenza neuraminidase (NA) removes it. If the viral neuraminidase is inactivated, influenza aggregates at the cell surface (76) but also HA conformational changes required for fusion are restricted, leading to premature HA inactivation (77).

HIV-1 also put in place different mechanisms to downregulate its receptor from the cell surface. This function is highly conserved among primate lentiviruses (27) and appears to be important for viral replication in T cells (43, 78). Downregulation of CD4 from the surface of infected cells positively impacts viral pathogenesis by virtue of multiple effects. CD4 downregulation has been shown to enhance viral infectivity by facilitating gp120 incorporation (41-44). CD4 downregulation also prevents superinfection and may facilitate the release of viral particles from the infected cell (26, 45-47). CD4 downregulation may weaken the antiviral immune response by limiting CD4 interaction with the major histocompatibility complex class II, which is involved in T cell activation (79).

Another plausible reason to remove CD4 from the cell surface is to limit Env-CD4 interactions which otherwise expose CD4i epitopes recognized by commonly-elicited CD4i ADCC-mediating antibodies (reviewed in (80, 81)). It is well established that Envs from primary

HIV-1 isolates intrinsically resist sampling the conformations recognized by CD4i Abs. This resistance is likely due to the stability of State 1 in primary Envs, which rarely make spontaneous transitions to conformations recognized by CD4i Abs (2). Soluble CD4 (sCD4) or CD4mc engagement also drive Env into more open States 2 and 3, rendering them susceptible to CD4i Abs (1, 48, 51, 54, 73, 82). Interestingly, CD4 incorporation into viral particles was recently shown to stabilize more open Env conformations, including State 2A, which is vulnerable to antibody attack (57).

The asymmetric State 2A conformation is characterized by the exposure of gp120 inner domain cluster A epitopes (57). A32 and C11 are well-characterized anti-cluster A antibodies (8, 83-85). These antibodies failed to capture viral particles bearing different primary Env unless CD4 was incorporated. Using our VCA, we found that CD4 incorporation into viral particles had a significant impact on the conformational equilibrium of four different primary Envs. Indeed, CD4 incorporation facilitated virus capture by antibodies targeting different CD4i Abs located in the V3, CoRBS, cluster A and gp41 cluster I regions. Exposure of these epitopes was also accompanied by enhanced neutralization sensitivity to different CD4i Abs such as 17b, 19b and A32. It is therefore tempting to speculate that Nef-mediated CD4 downregulation represents a viral mechanism to avoid exposure of vulnerable CD4i epitopes at the surface of viral particles. Importantly, these above mentioned effects were reduced in presence of Nef, further demonstrating the crucial role of CD4 downregulation in avoiding immune responses. Altogether, our results suggest that targeting the ability of Nef to downregulate CD4 or strategies aimed at modifying Env conformation to expose CD4i epitopes could have therapeutic utility.

314 **Materials and Methods**

315 **Cell lines and plasmids**

316 HEK293T human embryonic kidney and Cf2Th canine thymocytes (American Type Culture
317 Collection) were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium
318 (Invitrogen) containing 10% fetal bovine serum (Sigma) and 100 units/ml penicillin / 100 μg/ml
319 streptomycin (Mediatech, Inc.). Cf2Th cells stably expressing human CD4 and CCR5 or CD4
320 and CXCR4 (70) were grown in medium supplemented with 0.4 mg/ml of G418 (Invitrogen) and
321 0.2 mg/ml of hygromycin B (Roche Diagnostics). The E168K mutation was introduced into the
322 previously described pcDNA3.1 expressing codon-optimized HIV-1_{JRFL} envelope glycoproteins
323 (1) using the QuickChange II XL site-directed mutagenesis protocol (Stratagene). Other
324 plasmids used to transfect 293T cells include pcDNA3.1 human CD4 expressor and its F43H
325 variant (48).

326

327 **Virus capture assay (VCA).** Viral particles were produced by transfecting $2x10^6$ HEK293T
328 cells with pNL4.3 Luc Env- (3.5μg), HIV-1_{CH58TF} (3.5μg) and VSV-G (1μg) using standard
329 calcium phosphate protocol. Forty-eight hours later, supernatant containing virions were
330 collected and cell debris was removed by centrifugation (1500 rpm, 10 minutes). To immobilize
331 antibodies on ELISA plates, white MAXISORP ELISA plated (Thermo Fisher Scientific) were
332 incubated with 5 μg/ml of the different anitbodies in PBS overnight at 4ºC. Unbound antibodies
333 were removed by washing twice the plates with PBS. Plates were subsequently blocked with
334 3% BSA in PBS for one hour at room temperature. After two washes with PBS, 200ul of virus-
335 containing supernatant were added to the wells. After 4 to 6 hours incubation, virions were
336 removed and the wells were washed with PBS 3 times. Viral capture by any given antibody was
337 visualized by adding HEK293T cells ($10x10^4$) in full DMEM media per well. Forty-eight hours
338 post infection, cells were lysed by the addition of 30 μl of passive lysis buffer (Promega) and

three freeze-thaw cycles. An LB 941 TriStar luminometer (Berthold Technologies) was used to measure the luciferase activity of each well after the addition of 100 µl of luciferin buffer (15 mM MgSO4, 15 mMKPO4 [pH 7.8], 1mMATP, and 1mM dithiothreitol) and 50 µl of 1 mM D-luciferin potassium salt (Prolume).

Antibodies

The following antibodies were used: anti-HIV-1 gp120 mAbs recognizing gp120 outer domain (2G12) (NIH AIDS Reagent Program), the V1V2 glycan trimer apex (PG9) (Polymun), CD4-induced gp120 epitopes (17b, A32, C11) (NIH AIDS Reagent Program), the CD4-binding site (VRC03, b12), CD4i gp41 Cluster I (F240, QA255.072 (86)), anti-HIV Immune Globulin (HIVIG, prepared from pooled plasma of asymptomatic, HIV+ donors obtained from the NIH AIDS Reagent Program), and the anti-CD4 OKT4 Ab which binds to the D3 domain of CD4 (Invitrogen).

Virus neutralization

CH58 and CH77 transmitted/founder infectious molecular clones HIV-1 were produced by calcium phosphate transfection of 293T cells together with an expressor of CD4 wt at weight ratio of 1 provirus//0.1 CD4 or 1 provirus/0.5 CD4. Two days after transfection, the cell supernatants were harvested. The reverse transcriptase activities of all virus preparations were measured, as described previously (87). Each virus preparation was used immediately and was never frozen. Twenty-four hours before infection, TZM-bl cells were seeded at a density of 5 X 10^4 cells/well in 96-well luminometer-compatible tissue culture white plates (Perkin Elmer). Luciferase-expressing viruses (10,000 reverse transcriptase units) were incubated for 1 hour at 37°C with serial dilutions of Env ligands in a volume of 200 µl. The recombinant viruses were then incubated in quadruplicate with TZM-bl cells. After a 48-hour incubation at 37°C, the medium was removed from each well, and the cells were lysed by the addition of 30 µl of

passive lysis buffer (Promega) and three freeze-thaw cycles. After the addition of 100 µl of luciferin buffer (15 mM $MgSO_4$, 15 mM KPO_4 [pH 7.8], 1 mM ATP, and 1 mM dithiothreitol) and 50 µl of 1 mM D-luciferin potassium salt (Prolume), the luciferase activity in each well was measured with an EG&G Berthold microplate luminometer LB 96V.

Cold-inactivation assay

To assess the effect of cold on virus infectivity, virus preparations equalized for reverse transcriptase activity were incubated on ice for 0, 8 or 24 48 h, as described (69). At the end of the incubation, aliquots were removed and transferred to a -80°C freezer until infection. To measure the infectivity of the virus, aliquots were thawed at 37°C just before infection of Cf2Th-CD4/CCR5 cells in quadruplicate.

Statistical Analyses

Statistics were analyzed using GraphPad Prism version 6.01 (GraphPad, San Diego, CA, USA). Every data set was tested for statistical normality, and this information was used to apply the appropriate (parametric or nonparametric) statistical test. P values of <0.05 were considered significant; significance values are indicated as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p < 0.0001$.

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Figure Legends

Figure 1. Depiction of the virus capture assay (VCA).

(**A**) Ninety-six well plates were coated with anti-HIV-1 Env Abs. Viral particles coding for luciferase and bearing HIV-1 Env and the VSV-G protein were added to the wells. Free virions were washed away and CD4-negative cells (HEK293T) were added to the wells. After 48 hours, cells were lysed and luciferase activity measured. (**B**) Incorporation of both Envs, HIV-1 Env and VSV-G, is required to obtain a signal in the VCA.

Figure 2. CD4 incorporation exposes HIV-1 Env CD4i epitopes.

VSV-G-pseudotyped viral particles expressing HIV-1_{JRFL} Env wild-type (**A**, **C**) or an Env variant unable to engage CD4 (D368R) (**B**, **D**) were produced together with wild-type human CD4 (hCD4) or a mutant CD4 (F43H) that has decreased affinity for gp120. These viral particles were added to plates coated with antibodies targeting different Env epitopes or the anti-CD4 OKT4 antibody. Free virions were washed away and HEK293T cells were added to the wells. After 48 hours, cells were lysed and luciferase activity was measured. Luciferase signals were normalized to those obtained with the 2G12 antibody. Data shown are the mean ± SD of at least three independent experiments. Statistical significance was evaluated using a paired t test (*, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$).

Figure 3. Exposure of CD4i epitopes on additional HIV-1 strains by incorporated CD4.

VSV-G-pseudotyped viral particles expressing HIV-1_{YU2} Env (**A**) or HIV-1_{BG505} Env (**B**) were produced with or without human CD4. These viral particles were added to plates coated with antibodies targeting different Env epitopes or the anti-CD4 OKT4 antibody. Free virions were washed away and HEK293T cells were added to the wells. After 48 hours, cells were lysed and luciferase activity was measured. Luciferase signals were normalized to those obtained with the 2G12 antibody. Data shown are the mean ± SD of at least three independent experiments.

Statistical significance was evaluated using an unpaired t test (A) or Wilcoxon paired t test (B) (*, $P < 0.05$; **, $P < 0.01$).

Figure 4. Incorporated CD4 sensitizes viral particles to cold inactivation.

Viral particles pseudotyped with HIV-1_{JRFL} (**A**) or HIV-1_{Yu2} were produced by co-transfection with or without human CD4. Viral particles were incubated on ice for different amounts of time. At the indicated time points, aliquots were removed and frozen at -80°C. After completion of the longest incubation, all samples were thawed and infectivity on Cf2Th-CD4/CCR5 cells was measured. Data is representative of results from at least three independent experiments, performed in quadruplicate. Data shown are the mean ± SD of at least three independent experiments. Statistical significance was evaluated using an unpaired t test (*, $P < 0.05$, **, $P < 0.01$).

Figure 5. CD4 incorporation sensitizes viral particles to neutralization mediated by HIVIG.

Full-length infectious molecular clones either wild-type (shown in blue) or Nef defective (shown in red) from transmitted/founder CH58 and CH77 viruses were produced by transfection in the absence (circle) or presence of different concentrations of CD4. Reverse transcriptase normalized amounts of viral particles were used to infect TZM-BL cells. Relative infectivity is shown in (**A**). Infectious viral particles of CH58 (**B**) and CH77 (**C**) were incubated with the indicated dilutions of HIVIG before infecting TZM-BL cells. Infection levels were expressed as the percentage of the RLU observed in the condition without serum. Data shown are the mean ± SD of at least three independent experiments. Statistical significance was evaluated using a paired t test (A) or an unpaired t test (B, C) (*, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

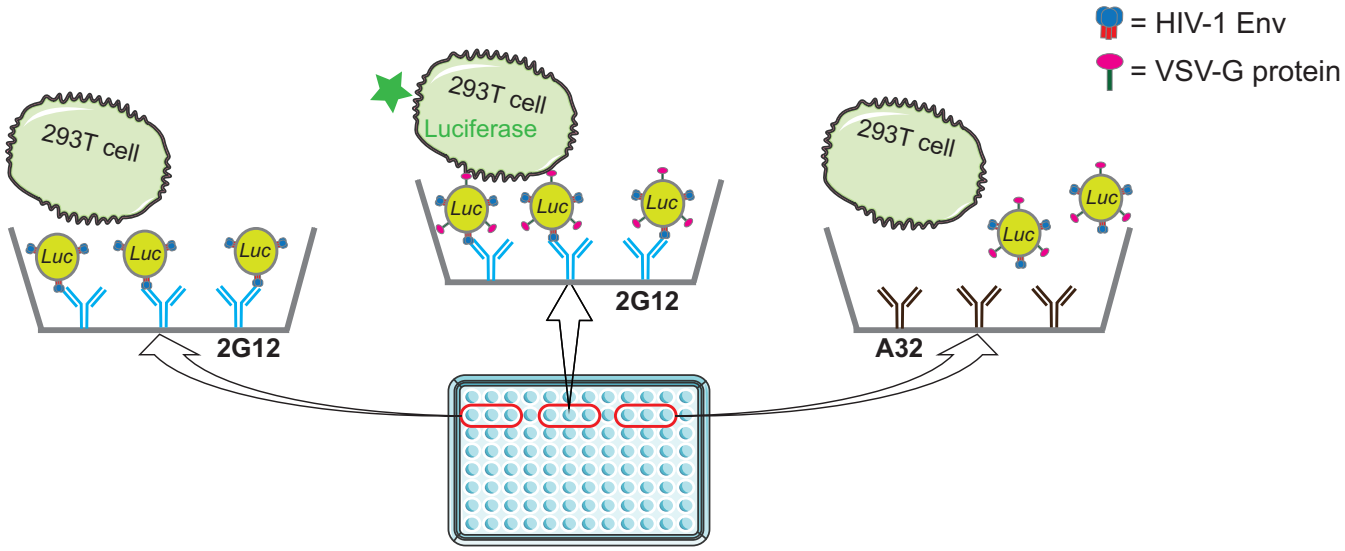
Figure 6. CD4 incorporation sensitizes viral particles to neutralization mediated by CD4i antibodies.

Full-length infectious molecular clones either wild-type (shown in blue) or Nef defective (shown in red) from transmitted/founder CH58 and CH77 viruses were produced by transfection in the absence (circle) or presence of different concentrations of wild-type human CD4 (huCD4) (squares and diamonds). The viruses were incubated with the indicated concentrations of CD4i antibodies 17b (**A** and **B**), 19b (**C** and **D**) and A32 (**E** and **F**) before infecting TZM-BL cells. Infection levels were expressed as percentage of the RLU in the condition without antibody. Data shown are the mean ± SD of at least three independent experiments. Statistical significance was evaluated using an unpaired t test (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

Figure 7. Nef expression limits the exposure of CD4i epitopes on viral particles.

VSV-G-pseudotyped viral particles expressing HIV-1_{Bal} Env coding or not for Nef were produced in the absence or presence of human CD4. Viral particles were added to plates coated with antibodies targeting different CD4i epitopes or the anti-CD4 OKT4 antibody. Free virions were washed away and HEK293T cells were added to the wells. After 48 hours, cells were lysed and luciferase activity was measured. Luciferase signals were normalized to those obtained with the 2G12 antibody. Data shown are the mean ± SD of at least three independent experiments. Data shown are the mean ± SD of at least three independent experiments. Statistical significance was evaluated using the Mann-Whitney unpaired t test (*, $P < 0.05$; **, $P < 0.01$).

A



B