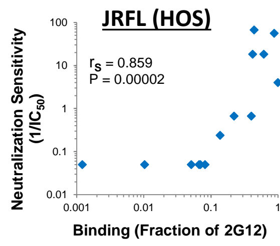
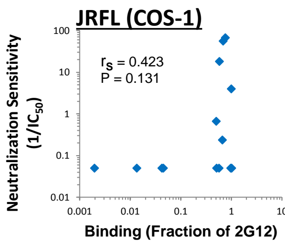
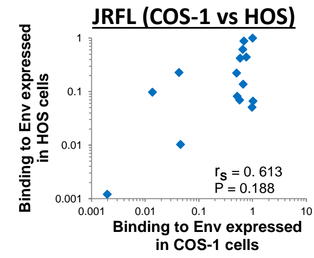
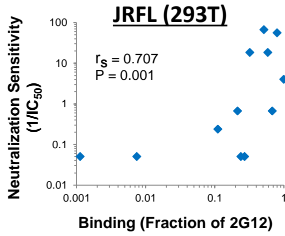
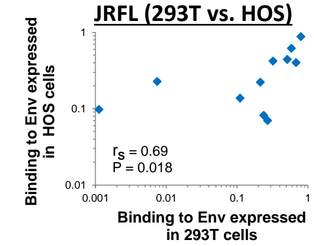
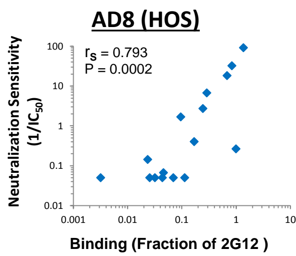

A
JRFL (HOS)
rS = 0.859
P = 0.00002
Neutralization Sensitivity (1/IC50)
Binding (Fraction of 2G12)

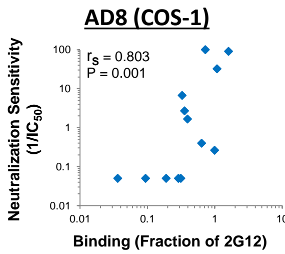

JRFL (COS-1)
rS = 0.423
P = 0.131
Neutralization Sensitivity (1/IC50)
Binding (Fraction of 2G12)

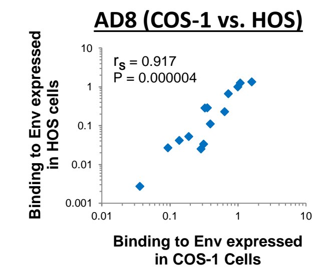

JRFL (COS-1 vs HOS)
rS = 0. 613
P = 0.188
Binding to Env expressed in HOS cells
Binding to Env expressed in COS-1 cells

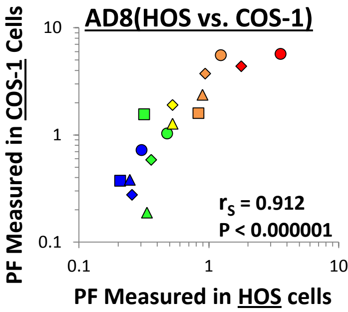

JRFL (293T)
rS = 0.707
P = 0.001
Neutralization Sensitivity (1/IC50)
Binding (Fraction of 2G12)

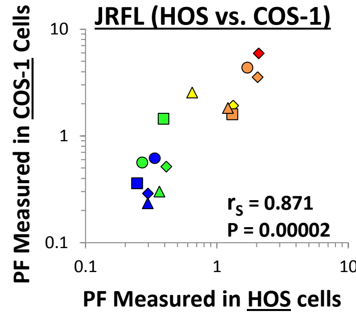

JRFL (293T vs. HOS)
rS = 0.69
P = 0.018
Binding to Env expressed in HOS cells
Binding to Env expressed in 293T cells

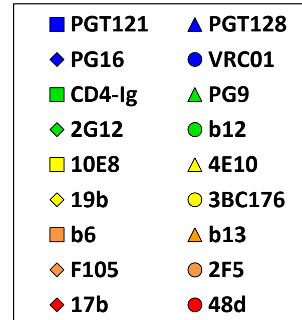

AD8 (HOS)
rS = 0.793
P = 0.0002
Neutralization Sensitivity (1/IC50)
Binding (Fraction of 2G12)

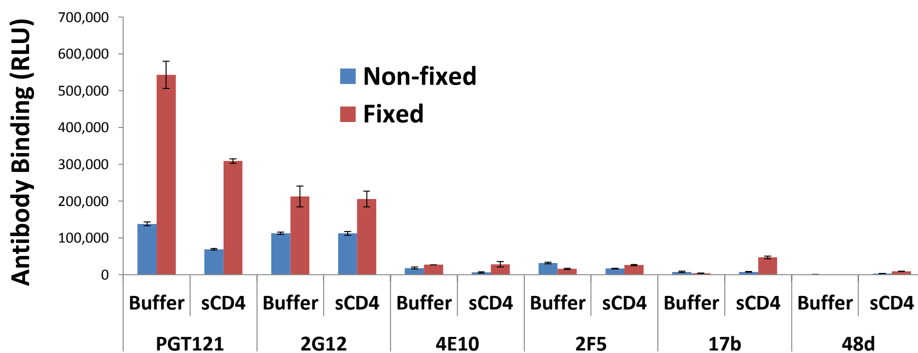

AD8 (COS-1)
rS = 0.803
P = 0.001
Neutralization Sensitivity (1/IC50)
Binding (Fraction of 2G12)

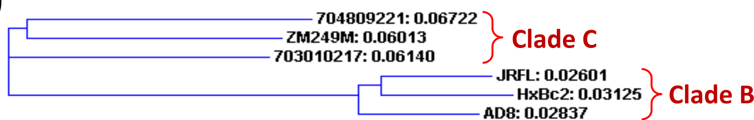

AD8 (COS-1 vs. HOS)
rS = 0.917
P = 0.000004
Binding to Env expressed in HOS cells
Binding to Env expressed in COS-1 Cells

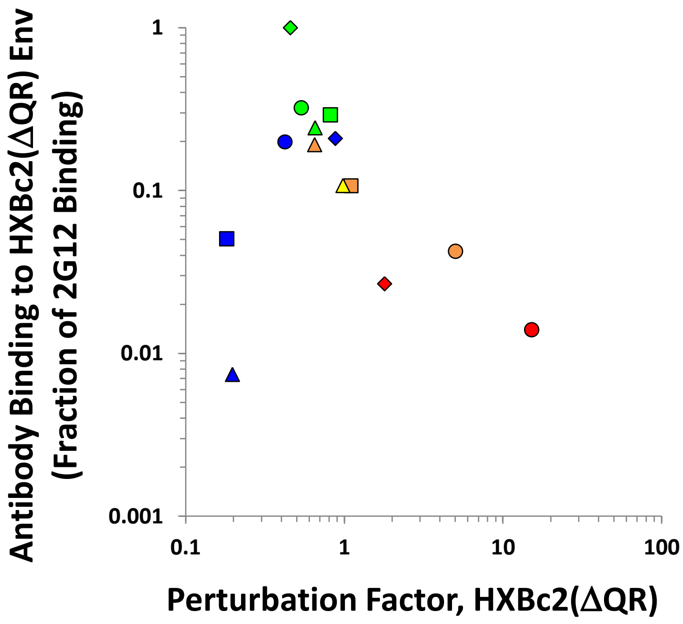

B
AD8(HOS vs. COS-1)
rS = 0.912
P < 0.000001
PF Measured in COS-1 Cells
PF Measured in HOS cells

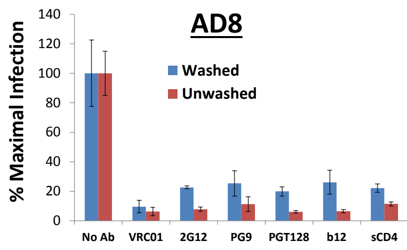

JRFL (HOS vs. COS-1)
rS = 0.871
P = 0.00002
PF Measured in COS-1 Cells
PF Measured in HOS cells

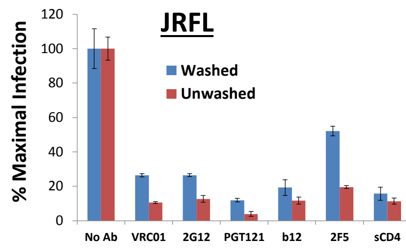

PGT121
PGT128
PG16
VRC01
CD4-Ig
PG9
2G12
b12
10E8
4E10
19b
3BC176
b6
b13
F105
2F5
17b
48d

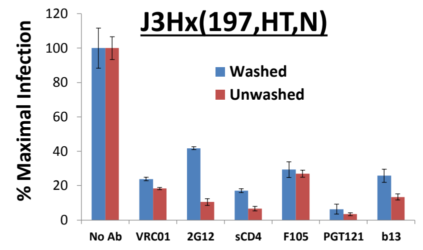

C
Antibody Binding (RLU)
Non-fixed
Fixed
Buffer
sCD4
PGT121
2G12
4E10
2F5
17b
48d

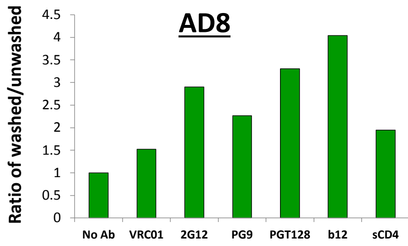

D
704809221: 0.06722
ZM249M: 0.06013
703010217: 0.06140
Clade C
JRFL: 0.02601
HxBc2: 0.03125
AD8: 0.02837
Clade B

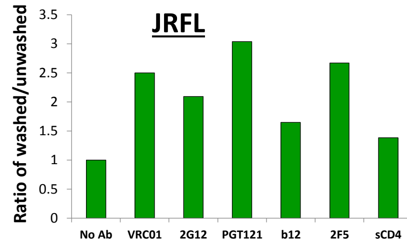

Antibody Binding to HXBc2(ΔQR) Env
(Fraction of 2G12 Binding)
1
0.1
0.01
0.001
0.1
1
10
100
Perturbation Factor, HXBc2(ΔQR)
PGT121
PGT128
PG16
VRC01
CD4-Ig
PG9
2G12
b12
10E8
4E10
19b
3BC176
b6
b13
F105
2F5
17b
48d

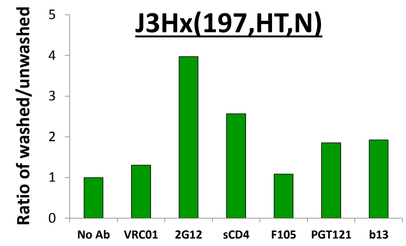

AD8
% Maximal Infection
Washed
Unwashed
0
20
40
60
80
100
120
140
No Ab
VRC01
2G12
PG9
PGT128
b12
sCD4


JRFL
% Maximal Infection
Washed
Unwashed
0
20
40
60
80
100
120
No Ab
VRC01
2G12
PGT121
b12
2F5
sCD4


J3Hx(197,HT,N)
% Maximal Infection
Washed
Unwashed
0
20
40
60
80
100
120
No Ab
VRC01
2G12
sCD4
F105
PGT121
b13


AD8
Ratio of washed/unwashed
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
No Ab
VRC01
2G12
PG9
PGT128
b12
sCD4


JRFL
Ratio of washed/unwashed
0
0.5
1
1.5
2
2.5
3
3.5
No Ab
VRC01
2G12
PGT121
b12
2F5
sCD4


J3Hx(197,HT,N)
Ratio of washed/unwashed
0
1
2
3
4
5
No Ab
VRC01
2G12
sCD4
F105
PGT121
b13

Supplemental Figure Legends

Supplemental Figure S1. Binding efficiency, neutralization potency and sampling frequency of the binding-competent conformation of a panel of monoclonal Abs to HIV-1 Envs. **(A)** Relationship between neutralization potency and binding efficiency measured in different cell types. Binding of different Abs and CD4-Ig to COS-1 or HOS cells transiently expressing the indicated Envs was measured using the standard cell-based ELISA protocol described in the Experimental Procedures. To measure Ab binding to 293T cells, which easily detach from the plate, cells were initially cultured and transfected with Env and Tat in 6-well plates. Three days later, the cells were washed twice with washing buffer and then added to protein-binding 96-well plates (6×10^4 cells per well). After attachment of the cells to the plate, the wells were blocked using 3% BSA for 2 hours at room temperature. The cell-based ELISA was then carried out using the standard protocol. Correlations are shown between binding efficiency and neutralization sensitivity. Each data point represents the mean value of the results obtained in at least two independent experiments. Spearman rank-order correlation coefficient, r_S;P-value, two-tailed T-test. **(B)** Sampling frequency of the binding-competent conformation of different Abs was measured in COS-1 and HOS cells expressing the AD8 or JRFL Envs. Each data point represents the mean value of the results obtained in at least three independent experiments. **(C)** Effect of GA fixation on binding of Abs to native Env or after pre-treatment with sCD4. HOS cells expressing the AD8 Env were pretreated with sCD4 (15 mg/ml) or buffer for 30 minutes at room temperature. Cells were then washed, and half of the samples were subjected to GA fixation. The binding efficiency of each Ab to fixed and non-fixed Envs, with or without initial sCD4 incubation, was measured and is expressed as the mean relative light units (RLU). The data points represent the means of the binding values obtained in a representative experiment performed with two replicate samples. As previously observed (Walker et al., 2011), sCD4

decreased the binding of PGT121. However, a proportionate decrease was observed for both the fixed and non-fixed Env, and thus the PGT121 sampling frequency was not changed by sCD4 binding. Data are represented as mean +/- SEM. The binding ratios are shown in **Figure 1D**. **(D)** Phylogenetic tree analysis of the Envs used in this study. Phylogenetic trees were generated by the neighbor-joining method using ClustalW2. The distance value of each *env* sequence from the hypothetical common ancestor (node) is indicated as the number of substitutions relative to the length of the alignment. **Figure S1** is related to **Figure 1**.

Supplemental Figure S2. Relationship between the PF of each Ab, as measured for the HXBc2(ΔQR) Env, and binding efficiency of the Ab to the HXBc2(ΔQR) Env . Each data point represents the means of the Ab binding and PF values obtained in at least two independent experiments. Note that the PGT121 and PGT128 Abs bind very inefficiently to the HXBc2(ΔQR) Env. Nevertheless the PF of both Abs is similar to that measured with the AD8 and JRFL Envs, which bind the Abs very efficiently (see **Figure 4D**). **Figure S2** is related to **Figure 4**.

Supplemental Figure S3. Reversibility of HIV-1 Inhibition by Abs. Viruses containing the indicated Envs were purified by ultracentrifugation and bound to protein-binding plates, as described in the Extended Experimental Procedures. After blocking the plate, the plate-bound viruses were incubated with the indicated Abs for 2 hours at 37°C at a concentration that achieves 90% inhibition of the virus. Viruses were then extensively washed over the course of 2 hours and then Cf2Th cells that express human CD4 and CCR5 were added. As controls, some of the samples were not washed (cells were added directly to the virus-Ab mix). Data shown in the top panel represent the infectivity measured in an experiment performed with three replicate samples and are presented as percent infection measured in the absence of Ab (+/- SEM). The

bottom panel shows the ratios between the infectivity measured in the washed samples relative to the infectivity measured in the unwashed samples. **Figure S3** is related to **Figure 6**.

EXTENDED EXPERIMENTAL PROCEDURES

Antibodies and CD4-Ig

The 2G12 Ab, which targets a carbohydrate-dependent gp120 epitope (Trkola et al., 1996), and the 2F5 Ab, which recognizes the membrane-proximal external region (MPER) of gp41 (Muster et al., 1993), were provided by Hermann Katinger. The IgG1 b12 Ab, which recognizes the CD4-binding site of gp120 (Burton et al., 1994; Zhou et al., 2007), was a kind gift from Dennis Burton. The F105 Ab, which also recognizes the gp120 CD4-binding site, was kindly provided by Marshall Posner (Posner et al., 1991). The CD4-binding site-directed Abs b6, b13 and VRC01 were provided by Dennis Burton, Peter Kwong and John Mascola, respectively (Chen et al., 2009; Roben et al., 1994). James Robinson kindly provided the 17b and 48d Abs, which recognize gp120 epitopes that are induced by CD4 binding (Thali et al., 1993). The PG9, PG16, PGT121 and PGT128 Abs, which recognize glycan-dependent epitopes on gp120 (Pejchal et al., 2011; Walker et al., 2011; Walker et al., 2009) were kindly provided by the International AIDS Vaccine Initiative (IAVI) Neutralizing Ab Consortium. Plasmids encoding the heavy and light chains of the Ab 3BC176 were provided by Michel Nussenzweig and Florian Klein. The Ab was purified as previously described (Mouquet et al., 2011). The 10E8 Ab was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Mark Connors (Huang et al., 2012).

The CD4-Ig fusion protein is composed of the Fc region of human IgG1 linked to two copies of the two N-terminal domains of the CD4 molecule. The CD4-Ig protein was produced and purified as previously described (Si et al., 2004). Four-domain sCD4 was purified from the culture medium of a 293F producer cell line (Yang et al., 2004).

Envelope Glycoprotein Constructs

The Envs of the AD8, JR-FL and HXBc2 isolates of HIV-1 (accession numbers AF004394, U63632 and K03455, respectively) were expressed from the pSVIIIenv vector (Sullivan et al., 1995). The vectors were constructed by replacing the *KpnI*(5893)-B*am*HI(8021) fragment of the HXBc2 *env* sequence of the original pSVIIIenv plasmid with the corresponding sequences from AD8 or JR-FL *env* (Nucleotide numbering is based on that of the HXBc2 provirus, with the RNA start site designed +1) (Yang et al., 2004). The J3Hx(197) variant is a chimera between the Envs of the AD8 and HXBc2 strains (Haim et al., 2011) and was generated by using the SspI restriction site located in the AD8 *env* gene at nucleotide position 7822 (numbered as described above (Korber et al., 1998)). The N-terminal portion of the J3Hx(197) Env is derived from the AD8 Env and the C-terminal portion from the HXBc2 Env. This construct contains an Asn to Ser change at position 197 (Haim et al., 2011; Kolchinsky et al., 2001). The J3Hx(197,HT,N) construct is identical to J3Hx(197) except for additional changes: Env residues 625 and 626 are changed from Asn-Met to His-Thr and Env residue 674 from Asp to Asn (Haim et al., 2011). Residues 625/626 of gp41 are located in the loop between the heptad repeat 1 (HR1) and heptad repeat 2 (HR2) regions of gp41. The Asn-Met to His-Thr changes are associated with a shift of a potential N-linked glycosylation site from

position 625 to 624. Based on its location, this change may influence the formation of the extended pre-hairpin intermediate that characterizes the CD4-bound state. Indeed, this change is associated with an increased capacity to infect CD4-negative cells (Haim et al., 2011). The Asp residue at position 674 is located in the membrane-proximal external region (MPER) of gp41 (Huang et al., 2012). The Asp to Asn change introduces a potential N-linked glycosylation site and increases the level of ER by an unknown mechanism.

The Hx(ΔQR) construct contains a deletion of the amino acid residues Gln-Arg 310-311 at the tip of the V3 loop of the HXBc2 Env. The Envs of three transmitted/founder HIV-1 subtype C viruses (Salazar-Gonzalez et al., 2008) were studied: isolate ZM249M from Zambia, isolate 704809221 from South Africa and isolate 703010217 from Malawi (accession numbers EU166862, FJ444116 and FJ443589, respectively). The clade C transmitted/founder HIV-1 Envs were a kind gift from Beatrice Hahn.

Cell-based ELISA to Measure Binding of Abs to Cell-surface Expressed Env

Binding of Abs and CD4-Ig to HIV-1 Env trimers expressed on human osteosarcoma (HOS) or COS-1 cells was measured using a modified version of the cell-based enzyme-linked immunosorbent assay (ELISA) system described previously (Haim et al., 2009; Haim et al., 2011). Briefly, HOS cells were seeded in 96-well plates (9×10^3 cells per well) and were transfected the next day with 0.06 μg of an Env-expressing plasmid and 0.008 μg of a Tat-expressing plasmid per well using Effectene transfection reagent (Qiagen). COS-1 cells (1.4×10^4 cells per well) were transfected

with 0.06 µg of a plasmid expressing the envelope glycoproteins and 0.006 µg of a Tat-expressing plasmid per well. Three days later, cells were washed twice with blocking buffer (35 mg/ml BSA, 10 mg/ml non-fat dry milk, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM Tris, pH 7.5 and 140 mM NaCl) and incubated with the indicated primary Ab in blocking buffer for 30 min at 37°C. Cells were then washed four times with blocking buffer and four times with washing buffer (140 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM Tris, pH 7.5). A horseradish peroxidase (HRP)-conjugated Ab specific for the Fc region of human IgG was then incubated with the samples for 45 min at room temperature. Cells were washed 5 times with blocking buffer and 5 times with washing buffer. HRP enzyme activity was determined after addition of 35 µl per well of a 1:1 mix of Western Lightning oxidizing and luminol reagents (Perkin Elmer Life Sciences) supplemented with 150 mM NaCl. Light emission was measured with a Mithras LB 940 luminometer (Berthold Technologies).

Glutaraldehyde Fixation of Cell-surface Env

Three days after transfection of HOS cells with the Env- and Tat-expressing plasmids as described above, cells were fixed using glutaraldehyde. Briefly, cells were washed twice with fixation buffer (140 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes, pH 7.4) and then incubated with fixation buffer containing 5 mM glutaraldehyde for 15 min at room temperature. As controls, some samples were incubated with fixation buffer with no glutaraldehyde added. Glutaraldehyde activity was halted by addition of 25 mM glycine in fixation buffer, which was added to all samples.

Cells were then washed twice with fixation buffer containing 25 mM glycine, twice with fixation buffer containing 12.5 mM glycine and then twice with washing buffer. Subsequently, fixed and non-fixed samples were examined for binding of Abs using the cell-based ELISA method described above.

Preparation of Recombinant Luciferase-expressing Viruses and Measurement of Sensitivity of Infection to Ab Neutralization

Single-round, recombinant HIV-1 viruses that express the luciferase gene were generated by transfection of 293T cells using Effectene transfection reagent. Briefly, cells were seeded in 6-well plates (approximately 7×10^5 cells per well) and transfected the next day with 0.2 μg of the HIV-1 packaging construct pCMVΔP1ΔenvpA, 0.6 μg of the firefly luciferase-expressing vector pHIvec2.luc and 0.2 μg of plasmid expressing the Env and HIV-1 Rev proteins, as previously described (Haim et al., 2009). The next day, transfection medium was changed to culture medium (DMEM/10% FBS). Virus-containing supernatants were collected on the following day, cleared of cell debris by low-speed centrifugation and filtered through 0.45-μm filters. For binding and neutralization assays in which viruses were bound to plates, viruses were further purified by centrifugation at 100,000 x g for two hours at 10°C through a 30% sucrose cushion (in washing buffer). Pelleted viruses were resuspended in binding buffer and virus particle content was quantified by measuring reverse transcriptase (RT) activity.

Infection by the recombinant viruses was measured using Cf2Th cells that express CD4 and coreceptor (CCR5 or CXCR4). Target cells for infection were seeded approximately 16 hours before infection at a density of 8×10^3 cells/well in 96-well luminometer-compatible tissue culture plates (PerkinElmer). For infectivity assays, virus input was normalized for RT unit content and added to the cells in a final volume of 150 µl per well. For neutralization assays, viruses were incubated with the inhibitor for one hour at 37°C and then added to $CD4^+CCR5^+$ Cf2Th cells. All infections were carried out by incubation of virus with cells for two days at 37°C. Medium was then removed and cells were lysed with 35 µl of passive lysis buffer (Promega). Cultures were then subjected to three freeze-thaw cycles. To measure luciferase activity, 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 (BD Pharmingen) were added to each well. Luminescence was recorded using a Berthold LB 960 microplate luminometer.

Reversibility of Ab Inhibition and Binding

Supernatants containing recombinant viruses that express the luciferase gene were purified by ultracentrifugation through a 30% sucrose cushion at 100,000 x g for 2 hours at 10°C. Control virus particles that do not contain Env were generated by replacing the Env-expressing plasmid with the AD8-stop plasmid, which contains the AD8 *env* gene with a stop codon in place of Env codon 46 (numbered according to

convention (Korber et al., 1998)). Virion pellets were then resuspended in TS buffer and viral particle content was determined by RT assay.

To measure reversibility of inhibition, purified luciferase-expressing viruses were bound to 96-well opaque protein-binding plates (PerkinElmer) by spinoculation at 10°C for 2 hours at 2,000 x g. Plates were then blocked overnight with BSA buffer (30 mg/ml BSA suspended in TS buffer). The next day, plates were washed twice with BSA buffer and twice with DMEM/10% FBS, and then incubated for 2 hours at 37°C with Ab at a concentration that results in 90% virus neutralization. Subsequently, plate-bound virions were washed three times every 20 minutes with DMEM/10%FBS for 2 hours. As controls, some samples were incubated with Ab for 2 hours but were not washed. Cf2Th cells that express human CD4 and CCR5 were then added to the plate-bound virions (6×10^4 cells per well) and the plate was centrifuged for 2 minutes at 1,000 x g to increase virus-cell contact. The plate was then incubated for 1 hour at 37°C to allow virus entry into cells. To halt entry, samples were incubated with 0.25% Trypsin/0.1% EDTA for 6 minutes at 37°C. Samples were then incubated at 37°C and 5% CO_2 for 2 days and infectivity measured by luciferase assay. The reversibility of inhibition is represented by the ratio of the infectivity measured for washed samples relative to that measured for the unwashed samples.

The binding of Abs to plate-bound virus was examined. After normalization for virus input by RT activity, samples were spinoculated onto the protein-binding plates as described above and blocked overnight with BSA buffer. As a control, virions that do not

contain Env were generated by substituting the AD8-stop plasmid for the Env-expressing plasmid. Abs were adjusted to a concentration that achieves 90% neutralization of free virus and incubated with HRP-conjugated Protein G (1 μg/ml) for two hours at 37°C. The Ab-Protein G-HRP mix was then added to the plate-bound virions and incubated for an additional two hours at 37°C. Viruses were then washed three times with blocking buffer every 20 minutes for 2 hours. Binding of the Ab-Protein-G-HRP complex to the virions was measured using a luminometer, as described above. The normalized reversibility of inhibition is described by:

$$\frac{Reversibility\ of\ Inhibition}{Reversibility\ of\ Binding} = \frac{\dfrac{Infection\ by\ washed\ virus}{Infection\ by\ unwashed\ virus}}{\dfrac{Antibody\ bound\ before\ washes}{Antibody\ bound\ after\ washes}}$$

Since the level of inhibition for all Abs was maintained constant at 90%, we assume that whether inhibition is reversible or irreversible, the initial level of binding was similar for this given level of inhibition. Under these conditions, the normalized reversibility of inhibition can be described by:

$$\frac{Infection\ by\ washed\ virus}{Infection\ by\ unwashed\ virus} \times \frac{Antibody\ bound\ after\ washes}{Constant}$$

Measurement of the On-rate of Ab Binding to Cell-surface Env

HOS cells cultured in 96-well plates were transfected with the AD8 Env-expressing plasmid, as described above. Three days later, cells were incubated with different Abs at 5 μg/ml for different time periods, extending between two and 45 minutes. After Ab was added to the last sample, all samples were washed four times with blocking buffer and four times with washing buffer. An HRP-conjugated secondary Ab was then added to all samples and binding was quantitated by luminescence.

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