SUPPLEMENTAL FIGURES

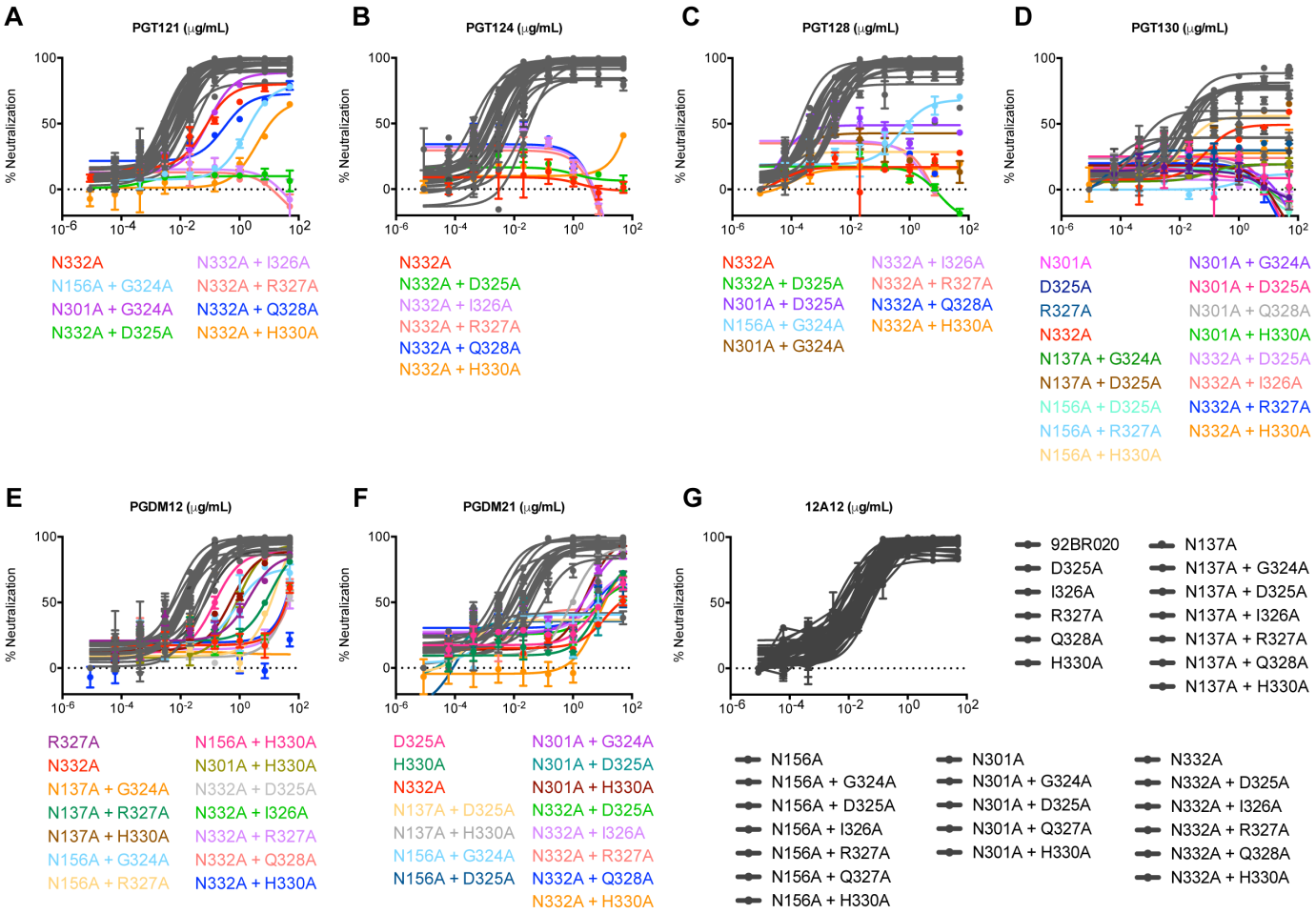

Figure S1. Related to Figure 4; Mapping of overlapping GDIR-glycan antibody epitope footprints on isolate 92BR020. Pairwise alanine mutants of 92BR020 were created for N137, N156, N301, and N332 glycans and the ^{324}GDIRQAH^{330} residues of the V3 loop. Virus mutants were then tested for neutralization by (**A**) PGT121, (**B**) PGT124, (**C**) PGT128, (**D**) PGT130, (**E**) PGDM12, (**F**) PGDM21 and, as a control, the CD4 binding site antibody (**G**) 12A12. The virus mutants listed with 12A12 were tested for neutralization by all antibodies, but for glycan-GDIR bnAbs only those that showed an effect on neutralization IC_{50} are listed beneath the indicated antibody.

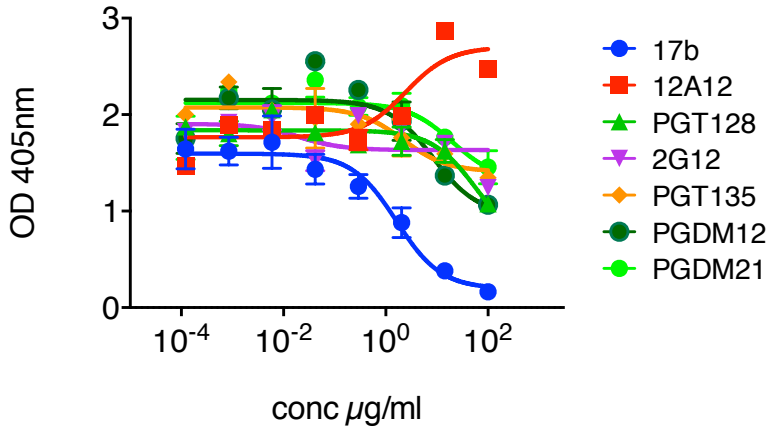

Figure S2. Related to Figure 6C; CD4i antibody 17b does not compete strongly with GDIR-glycan broadly neutralizing antibodies. Biotinylated 17b was tested for competition with GDIR-glycan broadly neutralizing antibodies by ELISA on monomeric 92BR020 gp120. The CD4i antibody 17b was included as a positive control and enhanced binding for 12A12 was observed as expected for CD4 binding site antibodies.

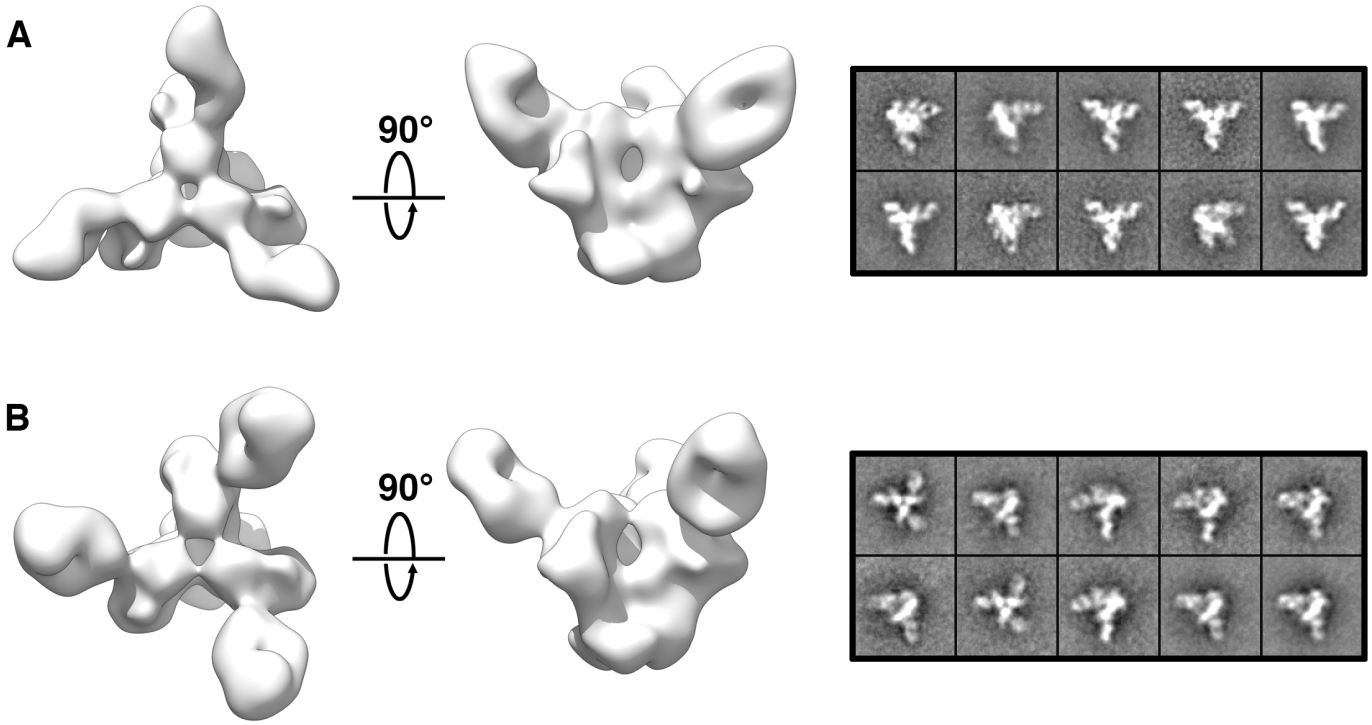

Figure S3. Related to Figure 7; Negative stain EM reconstructions of PGDM14 and PGDM21 bound to BG505 SOSIP.664. We determined negative stained EM densities of PGDM14 (**A**) and PGDM21 (**B**) Fabs bound to BG505 SOSIP.664 trimers. The left panels show respective 3D reconstructions at ~21 Å resolution; the right panels depict 2D class averages.

Table S1. Related to Figure 2; PGDM11-12 and PGDM21 antibodies can only neutralize viruses with a glycan naturally present at the N332 position. Antibodies were tested for neutralization of a cross-clade panel of pseudoviruses (n = 106) in a TZM-bl assay. Viruses are separated by those that naturally contain a glycan at the N332 site (76 viruses), at the N334 site (25 viruses) or no glycan at either N332 or N334 sites (5 viruses). Neutralization was measured at an IC_{50} cut-off of 50 μg/ml.

n = 106	Glycan site	% Neutralized of 106 virus panel	% Neutralized of glycan site	Median IC_{50}
PGDM11	N332 (76)	45%	63%	0.40
PGDM12		54%	75%	0.18
PGDM21		48%	67%	0.10
PGDM11	N334 (25)	0%	0%	0.00
PGDM12		0%	0%	0.00
PGDM21		0%	0%	0.00
PGDM11	None (5)	0%	0%	0.00
PGDM12		0%	0%	0.00
PGDM21		1%	20%	26

Table S2. Related to Figure 2; PGDM11-12 and PGDM21 antibodies can only neutralize viruses when a glycan is present at the N332 position. Antibodies were tested for neutralization on a cross-clade panel of pseudoviruses with a glycan present at the N332 position (WT) and with the glycan site removed by alanine mutagenesis (N332A) in a TZM-bl assay. Neutralization was measured at an IC_{50} cut-off of 50 µg/ml.

		WT		N332A	
CLADE	VIRUS	PGDM12	PGDM21	PGDM12	PGDM21
B	6535.3	0.004	0.011	>50	>50
	QH0692.42	0.348	1.06	>50	>50
	SC422661.8	0.563	0.113	>50	>50
	TRO.11	0.066	0.020	>50	>50
	AC10.0.29	0.916	8.90	>50	>50
	WITO4160.33	>50	0.069	>50	>50
	WEAU_d15_410_5017	0.316	0.048	>50	>50
B (T/F)	1006_11_C3_1601	0.027	0.016	>50	>50
	1054_07_TC4_1499	>50	5.75	>50	>50
	1056_10_TA11_1826	0.192	1.73	>50	>50
	1012_11_TC21_3257	0.033	>50	>50	>50
C	6240_08_TA5_4622	>50	0.377	>50	>50
	6244_13_B5_4576	0.444	0.372	>50	>50
	SC05_8C11_2344	0.087	0.087	>50	>50
	Du172.17	2.43	0.051	>50	>50
	Du422.1	0.274	0.154	>50	>50
	ZM249M.PL1	0.091	>50	>50	>50
	CAP210.2.00.E8	5.06	>50	>50	>50
	HIV-001428-2.42	0.081	>50	>50	>50
C (T/F)	7030102001E5(Rev-)	0.003	0.010	>50	>50
	1394C9G1(Rev-)	>50	1.07	>50	>50
	Ce704809221_1B3	>50	1.80	>50	>50
BC	CNE19	0.214	0.007	>50	>50
	CNE20	0.017	>50	>50	>50
	CNE21	0.014	0.028	>50	>50
	CNE17	1.25	1.59	>50	>50
	CNE52	3.87	7.04	>50	>50
	CNE53	0.066	0.080	>50	>50
A	Q23.17	0.018	0.018	>50	>50
	0330.v4.c3	>50	0.998	>50	>50
CRF02_AG	0260.v5.c36	1.35	0.141	>50	>50
	T251-18	2.62	0.180	>50	>50
G	X1193_c1	0.280	0.228	>50	>50
	X1254_c3	>50	0.114	>50	>50
CD	P1981_C5_3	0.055	0.014	>50	0.153
	6811.v7.c18	0.303	0.055	>50	>50
	Neutralization IC_{50}	10	1	0.1	0.01

Table S3. Related to Figure 3; PGDM12 and PGDM21 show some dependence on the N301 glycan, but no other glycan site. Antibodies were tested for neutralization on glycan site mutants for clade B isolate 92BR020 and clade A isolate BG505. Values listed are fold increases in neutralization IC_{50}. LT = low titer viruses.

92BR020	PGDM12	PGDM21
N88A	1	1
N135A	3	2
N136A	5	2
N141A	5	2
N142A	3	6
N189A	2	2
N197A	2	3
N234A	2	4
N262A	LT	LT
N295A	2	5
N301A	9	9
N332A	> 1000	> 1000
N397A	1	1
N407A	2	4
N411A	1	3
N448A	2	3
N463	1	1
N611	1	1
N616	1	1
N625	1	1
N637	1	1

BG505	PGDM12	PGDM21
N133A	1	1
N137A	1	1
N156A	1	4
N160A	2	3
N185eA	0	2
N197A	3	1
N301A	2	8
N332T	> 1000	> 1000
N339A	1	2
N386A	0	1

Fold difference in neutralization IC_{50} (µg/mL)

Table S4. Related to Figure 3; PGDM11-12 and PGDM21 depend on high-mannose type glycans for neutralization. Effects of different glycosidase inhibitors on the neutralizing activity of listed antibodies against the 6-virus indicator panel. 293S cells yield viruses with $Man_{5-9}GlcNAc_2$ glycans, treatment of 293F cells with kifunensine results in viruses with $Man_{8-9}GlcNAc_2$ glycans, treatment of 293F cells with swainsonine results in hybrid-type glycans, and treatment of 293F cells with NB-DNJ results in a terminal glucose carbohydrate on the D1 arm of $Man_9GlcNAc_2$ glycans. Antibody PGT128, which binds to the terminal mannose of the D1 arm, was included for comparison. Results show isolate-specific effects with PGDM11 and PGDM12 preferring $Man_{8-9}GlcNAc_2$ glycans and PGDM21 depending on a terminal mannose on the D1 arm of an N-linked glycan.

		94UG103	92RW020	92BR020	JR-CSF	92TH021	IAVI C22
PGDM11	293S	-	2	3	4	-	> 50
	Kifunensine	-	1	1	1	-	2
	Swainsonine	-	1	> 10	> 100	-	3
	NB-DNJ	-	-	1	-	-	-
PGDM12	293S	1	1	3	3	-	1
	Kifunensine	1	1	2	1	-	1
	Swainsonine	> 20	1	2	4	-	1
	NB-DNJ	-	-	1	-	-	-
PGDM21	293S	-	1	1	2	-	1
	Kifunensine	-	1	1	1	-	1
	Swainsonine	-	1	0	1	-	0
	NB-DNJ	-	-	-	> 100	-	-
PGT128	293S	4	4	0	4	1	1
	Kifunensine	3	3	0	3	1	2
	Swainsonine	2	2	0	2	1	1
	NB-DNJ	-	-	> 100	-	-	-

Fold difference IC_{50}	> 50	50-10	10-1	1-0

Table S5. Related to Figure 4; GDIR-glycan broadly neutralizing antibodies were tested for neutralization on alanine mutants of isolate JR-CSF. Antibodies were tested for neutralization on single and double alanine mutants on isolate JR-CSF. Antibody 12A12 was included as a control. Values listed are fold increases in neutralization IC_{50}. LT = low titer viruses.

JR-CSF	PGT121	PGT124	PGT128	PGT130	PGDM12	PGDM21	12A12
D325A	3.3	1.0	1.0	> 1000	0.1	> 1000	0.4
I326A	0.6	1.0	1.0	1.2	2.8	10.1	2.7
R327A	1.9	0.5	1.0	1.3	> 1000	0.7	1.2
Q328A	0.1	1.0	1.0	0.4	0.0	0.7	0.2
H330A	0.1	1.0	1.0	0.2	5.9	2.0	1.0
N156A	LT	LT	LT	LT	LT	LT	LT
N156A + D325A	> 1000	20	1.0	> 1000	7.0	> 1000	0.8
N156A + I326A	20	6.8	3.2	> 50	> 1000	1.3	0.4
N156A + R327A	LT	LT	LT	LT	LT	LT	LT
N156A + Q328A	0.3	1.0	1.0	1.4	> 1000	0.7	enhance
N156A + H330A	LT	LT	LT	LT	LT	LT	LT
N301A	0.2	1.0	1.0	> 1000	2.2	1.7	0.3
N301A + G324A	LT	LT	LT	LT	LT	LT	LT
N301A + D325A	> 100	1.0	> 1000	> 1000	5.5	> 1000	0.9
N301A + I326A	6.0	4.6	> 500	> 1000	11.3	3.6	0.4
N301A + R327A	> 100	4.8	> 1000	> 1000	> 1000	7.9	1.4
N301A + Q328A	2.2	2.4	9.7	> 1000	18.0	9.7	0.7
N301A + H330A	9.5	1.0	> 1000	> 1000	> 1000	> 100	0.5
N332A	> 1000	> 1000	1.0	0.5	> 1000	> 1000	2.1
N332A + G324A	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	0.7
N332A + D325A	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	1.0
N332A + I326A	> 1000	> 1000	> 1000	> 100	> 100	> 1000	1.2
N332A + R327A	LT	LT	LT	LT	LT	LT	LT
N332A + Q328A	LT	LT	LT	LT	LT	LT	LT

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Human Specimen

Human PBMC and serum samples were acquired from HIV-1 infected donors of the Protocol G cohort (Simek et al., 2009) under written consent using clinical procedures approved by the Republic of Rwanda National Ethics Committee, the Emory University Institutional Review Board, the University of Zambia Research Ethics Committee, the Charing Cross Research Ethics Committee, the Uganda Virus Research Institute Science and Ethics Committee, the University of New South Wales Research Ethics Committee, St. Vincent's Hospital and Eastern Sydney Area Health Service, Kenyatta National Hospital Ethics and Research Committee, University of Cape Town Research Ethics Committee, the International Institutional Review Board, the Mahidol University Ethics Committee, the Walter Reed Army Institute of Research Institutional Review Board, and the Ivory Coast Comité National d'Éthique des Sciences de la Vie et de la Santé.

Pseudovirus Neutralization Assays

Plasmids encoding HIV Env were co-transfected into HEK 293T or 293S cells with pSG3ΔEnv, an Env-deficient genomic backbone plasmid, in a 1:2 ratio using X-tremeGENE HP (Roche) as transfection reagent. Cell culture supernatants were harvested 3 days post transfection and sterile filtered through a 0.22μm filter. Neutralizing activity was measured by incubating monoclonal antibodies or sera with replication incompetent pseudovirus for 1h at 37C before transferring onto TZM-bl target cells as described previously (Sok et al., 2014a; Walker et al., 2011). Pseudoviruses produced in the presence of glycosylation inhibitors were generated by treating 293T cells with either 25 μM kifunensine, 20 μM swainsonine or 2 mM N-butyldeoxynojirimycin (NB-DNJ) (Cayman Chemical Co.) on the day of transfection (Doores and Burton, 2010).

Protein Production

HIV gp120 proteins were truncated in the C5 region for increased stability and N-terminally fused to an Avi-tag for subsequent in vitro biotinylation. Gp120 plasmids were transfected into HEK 293F cells (Invitrogen) using 293fectin as transfection reagent (Invitrogen). Cell culture supernatants were harvested 4 days after transfection, 0.22μm sterile filtered and passed over Galanthus nivalis lectin (GNL) columns (Vector Laboratories) before separating monomeric gp120 by size exclusion chromatography (SEC) using a HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare) as previously described (Sok et al., 2013a). Gp120s used in flow cytometry were in vitro biotinylated using the BirA enzyme according to the manufacturer protocol (Avidity), in-between GNL and SEC purification steps.

Antibody plasmids containing heavy chain and light chain genes were co-transfected (1:1 ratio) in either HEK 293T or 293F cells using X-tremeGENE (Roche) or 293fectin (Invitrogen) as transfection reagents, respectively. Antibody containing supernatants were harvested 4 days after transfection and 0.22μm sterile filtered. Antibodies produced in 293T cells were quantified by anti-Fc ELISA and used directly in neutralization assays for screening purposes. Antibody supernatants produced in 293F cells were purified over Protein A Sepharose 4 Fast Flow (GE healthcare) columns as described previously (Sok et al., 2013a).

Serum NAb depletion assay

To test whether gp120 proteins were suitable sorting probes, we conjugated 1mg of C5 truncated gp120-Avi protein or BSA to 250 μl of Tosyl-activated MyOne DynaBeads (Invitrogen) according to the manufacturers protocol. We tested WT and N332A mutated gp120 variants of the 92BR020, IAVI C22 and JR-CSF HIV isolates and BSA as a control, in combination with N332 reactive donor sera. In brief, gp120 coated DynaBeads were washed 3 times in complete DMEM cell culture medium (Gibco, 10% FBS, PenStrep and L-glutamine added) and incubated for 30min at room temperature (RT) before isolating the beads using a strong magnet. Donor serum was pre-diluted 1:12.5 in cell culture medium and added to beads for 30 min at 37 °C while gently shaking. Beads were then removed by magnetic force, washed thrice with 1ml washing buffer (PBS containing 0.5M NaCl) and bound antibodies were eluted with 3x 500 μl 100mM Glycine HCl pH2.2 into tubes containing 45 μl 1M Tris pH9.0 buffer. Beads were immediately regenerated by flushing three times with washing buffer before transferring them into cell culture media and incubating for 30min at RT to neutralize the pH. Following this procedure, donor sera were depleted for a total of three rounds and directly tested in pseudovirus neutralization assays for reduced neutralizing activity. Eluted antibodies were pooled, buffer exchanged into PBS and concentrated before testing in ELISA.

Single-Cell Sorting of Donor PBMCs using Flow Cytometry

Sorting of donor PBMCs was performed as described previously (Sok et al., 2014b; Tiller et al., 2008; Wu et al., 2010). Donor PBMCs were stained with primary fluorophore-conjugated antibodies binding human CD3, CD8, CD14, CD19, CD20, CD27, IgG, and IgM (BD Pharmingen) and 50 nM of both WT and N332A mutated biotinylated gp120-Avi protein coupled to streptavidin-APC or PE (Life Technologies) in equimolar ratios. Cells were stained for 1 h at 4 °C in PBS containing 1 mM EDTA and 1% FBS. We selected epitope specific gp120 reactive memory B cells by first excluding T cells and monocytes ($CD3^-/CD8^-/CD14^-$) before gating on $CD19^+$/ $CD20^+$/ $CD27^+$/ IgG^+/ IgM^-/ gp120 WT^+/ gp120 $N332A^-$ cells. Target cells were single-cell sorted into 96-well plates containing lysis buffer on a BD FACSAria III sorter and were frozen on dry ice immediately (Tiller et al., 2008; Wu et al., 2010).

Single Cell PCR Amplification and Cloning of Antibody Variable Genes

C-DNA synthesis of frozen RNA and subsequent rounds of PCR amplification of antibody variable genes were performed as

previously described (Tiller et al., 2008; Wu et al., 2010). PCR reactions were set up in 25 µl volume with 2.5 µl c-DNA or PCR1 product using HotStarTaq Master Mix (Qiagen). Amplified IgG variable regions were sequenced and analyzed using the international ImMunoGeneTics information system (IMGT) High V-quest webserver (www.IMGT.org) (Lefranc et al., 2009) and IMGT results were entered into SQL databases for parametric analysis of antibody features (e.g. CDRH3 length). Wells for which productively rearranged heavy and light chains were retrieved (based on IMGT analysis), respective variable genes were cloned into corresponding Igγ1, Igκ, and Igλ expression vectors as previously described (Sok et al., 2014b; Wu et al., 2010).

Glycan microarray Analysis

All monovalent glycans were prepared in 10 mM concentration individually and served as stock solutions that need to be diluted with printing buffer to prepare a working solution. Structural confirmation of oligosaccharides was obtained by NMR analysis, ESI mass spectrometry, and MALDI-TOF mass spectrometry. Microarrays were printed (BioDot; Cartesian Technologies) by robotic pin (SMP3; TeleChem International) deposition of ~ 0.6 nl of various concentrations of amine-containing glycans in printing buffer onto Nexterion H NHS-coated glass slides (SCHOTT North America).

Amine-functional glycans were printed in replicates of three onto NHS-activated glass slides at a 100 mM concentration. Printing efficiency and quality was examined by Con A (Concanavalin A, Vector Laboratories), SNA (Sambucus Nigra Lectin, Vector Laboratories), RCA (Ricinus Communis Agglutinin, Vector Laboratories), WGA (Wheat Germ Agglutinin, Vector Laboratories), LEL (Lycopersicon Esculentum, Vector Laboratories), and ECA (Erythrina Cristagalli Lectin, Vector Laboratories). Unless otherwise stated, reagents were obtained from commercial suppliers and used without purification. All aqueous solutions were prepared from distilled deionized water filtered with a Milli-Q purification system and sterile filtered through a 0.2 µm filter. Buffers used in the experiment include the printing buffer (pH 8.5, 300 mM phosphate buffer containing 0.005% (v/v) Tween-20), the blocking buffer (superblock blocking buffer in PBS, Pierce), and the washing buffer (PBST buffer; PBS and 0.05% Tween 20). Printing buffer and blocking buffer were prepared freshly before use.

i) Antibody binding assay

Antibodies (10 μg in 50μl PBS) were pre-complexed with AlexaFluor 647 AffiniPure mouse anti-human IgG-Fc (5 μg, Jackson) for 2 h at 4 C. The sample was added to the glycan array and incubated at 4 °C for 1 h. The slides were washed sequentially in PBST (0.05% Tween-20), PBS and water.

ii) Human Serum Profiling on Carbohydrate Microarray

Slides were fitted with 16-well slide holders (Grace Bio-Labs) and blocked with block solution with 3% BSA/PBS overnight at 4 °C, then washed with PBST. Serum samples were diluted 1:100 in 3% BSA/PBST, added to arrays, and allowed to incubate with gentle shaking for 4 h at 37 °C. After washing with PBST, detection of bound IgG was carried out by incubating with AlexaFluor 647 - AffiniPure mouse anti-human IgG-Fc (Jackson) in 1% BSA/PBS at 37 °C. After 1 h, slides were washed sequentially in PBST (0.05% Tween-20), PBS and water before being centrifuged at 453g for 30 second.

iii) Image processing and data analysis.

The slide was scanned with a microarray fluorescence chip reader (ArrayWorx microarray reader). Image analysis was carried out with Genepix Pro 6.0 analysis software (Molecular Devices Corporation, Union City, CA). The image resolution was set to 5 µm per pixel. Spots were defined as circular features with maximum diameter of 100 µm. Local background subtraction was performed. PMT voltage was balancing according to the supplier's instructions.

Cell Surface-Binding Assays

Titrating amounts of mAbs were added to HIV-1 Env-transfected 293T cells and were incubated for 1 h at 4 °C in 1× PBS. After washing, cells were fixed with 2% para-formaldehyde (PolySciences) for 20 min at room temperature. The cells then were washed and stained with a 1:200 dilution of PE-conjugated goat anti-human IgG $F(ab')_2$ (Jackson) for 1 h at room temperature. Binding was analyzed using flow cytometry. Binding competitions were performed by titrating amounts of competitor mAbs before adding biotinylated antibody at the concentration required to achieve IC_{70} and then measuring binding with PE-labeled streptavidin (Invitrogen). FlowJo soft- ware was used for data interpretation.

CCR5-Fc mimic peptide ELISA

ELISA plates were first coated with an anti-C5 gp120 antibody at 4 °C in 1x PBS overnight. Plates were then washed and blocked with 3% BSA in 1x PBS at room temperature for 1 hour. Mutant pseudovirus supernatants, lysed with 1% NP40, were then captured at 37 °C for 2 hours. Following this, biotinylated CCR5-Fc mimic peptide was preconjugated with streptavidin-alkaline phosphatase in a 1:1 molar ratio and added at a final concentration of 10 μg/ml. Following washing, plates were measured at OD405.

Electron microscopy data collection and processing

BG505 SOSIP.664 trimers (Sanders et al., 2013) were incubated with 6x molar excess Fab fragments at RT for 30 minutes, following which the EM grids were prepared as previously described, using 2% w/v uranyl formate (UF) (Kong et al., 2013). Data were collected on a Tecnai T12 electron microscope coupled with a 4k x 4k Tietz TemCam-F416 CMOS CCD camera, using an exposure dose of ~25 e-/Å2 via the Leginon interface (Suloway et al., 2005), at a magnification was 52,000x for which the pixel size was 2.05

Å/pix at the specimen plane.

Particles were picked, stacked, and sorted by reference-free 2D class averaging, and refined as previously described (Scharf et al., 2014). Each Fab-trimer complex was refined with C3 symmetry enforced, for the following number of iterations: PGDM14-BG505 SOSIP.664: 80 iterations and PGDM21-BG505 SOSIP.664: 60 iteration. The resolutions of the models were determined at a Fourier shell correlation (FSC) cut-off of 0.5 and were ~21 Å for both PGDM14- and PGDM21-BG505 SOSIP.664 complexes.

ADDITIONAL REFERENCES:

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