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Supplementary Materials for

Neutralizing antibodies to HIV-1 envelope protect more effectively in vivo than those to the CD4 receptor

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The PDF file includes:

SUPPLEMENTARY MATERIALS AND METHODS

Generation of an anti-CD4 antibody

Naïve BALB/c mice were primed with DNA encoding the rhesus macaque CD4 gene three times at two week intervals, followed by a boost with recombinant adenovirus carrying the same rhesus CD4 (Fig. S1A). Three days after the final boost, the spleens of immunized mice were fused with myeloma cells to generate hybridomas that were cultured in 96-well cell culture plates in selection media. The supernatants from each well of the hybridoma cultures were then screened for binding to human CD4 expressed on the surface of MAGI-CCR5 cells (Fig. S1B). The positive clones were then tested for inhibition of viral entry as described below. The clone 2D5 was selected for further study due to its potent inhibition of HIV-1 env-mediated entry into CD4 target cells (Fig. S2).

2D5/CD4 complex preparation

2D5 was cleaved into Fabs by incubating with papain (Roche, 0.5 μg papain/mg 2D5) for 135 minutes at 37°C and quenching with 50 mM iodoacetamide for 2 hours. Fab was purified by loading onto a Protein G column (GE Healthcare HiTrap) and eluting with glycine pH 2.7. 2D5 Fab was added to an equimolar amount of human CD4 extracellular domains 1 and 2 to make a 1 mg/ml solution of 2D5/CD4 and dialyzed into 100 mM NaCl, 50 mM HEPES pH 7.5. The 2D5/CD4 complex was methylated by adding 20 μl of 1 M dimethylamine borane (DMAB, Fluka 15584) per ml of solution, and 40 μl of 1 M formaldehyde (Sigma 33220) per ml of solution to the 1 mg/ml 2D5/CD4 complex and incubating for 2 hours at 4°C in the dark with gentle mixing. This step was repeated by adding DMAB and formaldehyde again and incubating another 2.5 hours, after which another 10 μl of DMAB was added per ml of solution and

incubated overnight at 4°C. After 0.22 μm filtering, the methylated 2D5/CD4 was further purified by gel filtration on a Superdex 200 26/60 column (GE Healthcare) in 10 mM Tris pH 8.0, 150 mM NaCl.

Crystallization and 2D5/CD4 structure determination

Methylated 2D5/CD4 was crystallized at 10 mg/ml in 1.5 M $(NH_4)_2SO_4$, 200 mM $MgCl_2$, 150 mM NaCl, 100 mM NaAc pH 4.5. Crystals were cryofrozen in 2.0 M Li_2SO_4, 500 mM LiCl, 200 mM $MgCl_2$, 100 mM NaAc pH 4.5 and X-ray data was collected to 3.65 Å resolution at the Southeast Regional Collaborative Access Team (SER-CAT) 22-BM beamline at the Advanced Photon Source, Argonne National Laboratory. X-ray data was processed using HKL2000 (*57*), and the structure was solved by molecular replacement using PHASER (*58*) using the antibacterial antibody 19D9D6 Fab (PDB 1MHH) and the human CD4 extracellular domains 1 and 2 (PDB 3CD4) as search models. The four complexes in the asymmetric unit were refined by iterative use of COOT (*59*) and PHENIX (*60*) to an R = 22.5% and Rfree = 25.7% (Table S1). Later stages included TLS groups and optimized weights. Analysis of the interaction surface was performed using PISA (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver) and structural figures were created with PyMol (The Pymol Molecular Graphics System, Version 1.5, Schrödinger, LLC, http://www.pymol.org).

Antibodies

#19 is an anti-CD4 antibody that is known to block HIV-1 binding to CD4 (*38*). Leu3a is an anti-CD4 clone that was obtained from BD Biosciences. The human monoclonal antibodies VRC01, 10E8 and PG9 were produced as IgG1s by recombinant expression in mammalian cells

and purified using protein A column chromatography. Normal human IgG (Sigma) was used as the control IgG in all challenge experiments. Endotoxin was removed, if needed, from the antibody preparations using an endotrap column (Hyglos) to obtain low endotoxin material (<1 EU/mg) for use in animals.

Binding of anti-HIV-1 antibodies to the cell surface

293T cells were transfected with plasmids expressing gp145 constructs using the ProFection® Mammalian Calcium Phosphate Transfection System (Promega). GnTI(-) 293S cells were transfected by the 293fectin reagent (Invitrogen) according to the manufacturer's instructions. 24 hours later, the transfected cells were collected for staining using 2G12, PG9 and PG16 antibodies (5 μg/ml) for 30 minutes on ice (Fig. S4A). GnTI(-) 293S cells were also treated with 5 μg/ml Kifunensine (Enzo Life Sciences) for 48 hours, followed by staining using PG9 and PG16 antibodies (5 μg/ml) for 30 minutes on ice (Fig. S4B). For the lectin blocking assay, GnTI(-) 293S cells were incubated with 2.5 μg/ml Galanthus Nivalis Lectin (GNL: α-1,3 mannose) or Sambucus Nigra Lectin (SNA: α-2,6 sialic acid attached terminal galactose) for 30 minutes on ice, followed by incubation with PG9 and PG16 (5 μg/ml) for 30 minutes on ice (Fig. S4C). Cells were then washed twice with PBS and incubated with PE-conjugated Affinipure goat anti-human IgG(H+L) (5 μg/ml) for 30 minutes on ice, then washed with PBS and resuspended in PBS with 0.5% paraformaldehyde. Samples were assayed on the BD™ LSR-II Flow Cytometer using FACSDiva software (BD Biosciences). The data were analyzed with FlowJo 9.5.2 software (Tree Star).

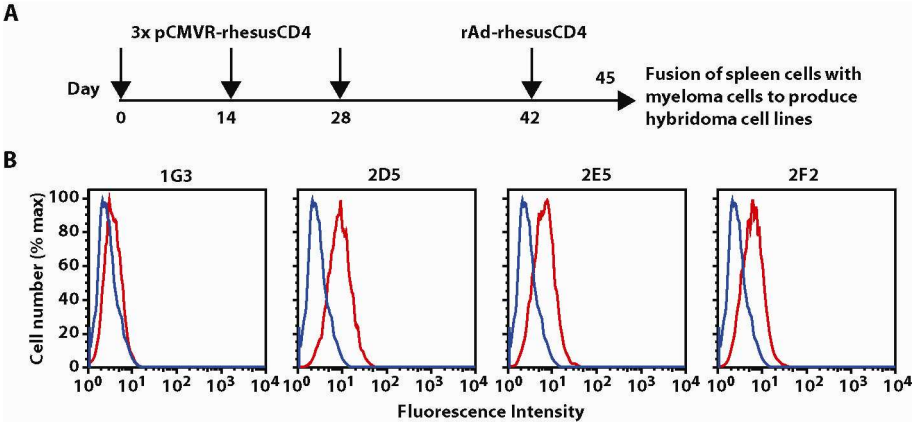
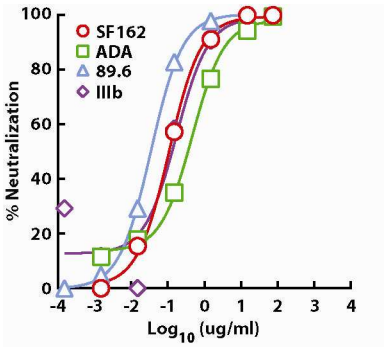


Figure S1. Generation of mAbs specific for primate CD4 in mice after prime-boost immunization. A, Schema for generation of anti-CD4 antibodies. Mice were immunized in a prime-boost strategy using plasmid DNA carrying the gene for rhesus macaque CD4 (pCMVR-rhesusCD4) followed by a boosting immunization with recombinant adenovirus expressing rhesus macaque CD4 (rAd-rhesusCD4). Three days after the final boost, mouse spleens were fused with myeloma cells to produce hybridoma cell lines. **B,** Flow cytometry-based screening of hybridoma supernatants for CD4 binding antibodies. Representative positive hybridoma clones (1G3, 2D5, 2E5, 2F2) were identified by screening the hybridoma culture supernatants for antibodies binding to human CD4 expressed on the surface of MAGI-CCR5 cells by flow cytometry.



Virus Strain	IC50	IC80
SF162	0.11	0.54
ADA	0.47	2.4
89.6	0.03	0.13
IIIb	0.17	0.2

Figure S2. Neutralization of HIV-1 by 2D5. Neutralization of the indicated HIV-1 isolates by 2D5, measured using an Env-pseudotyped lentiviral reporter assay and MAGI-CCR5 target cells. The table lists the IC50 and IC80 values (μg/ml) for 2D5 against each HIV-1 strain.

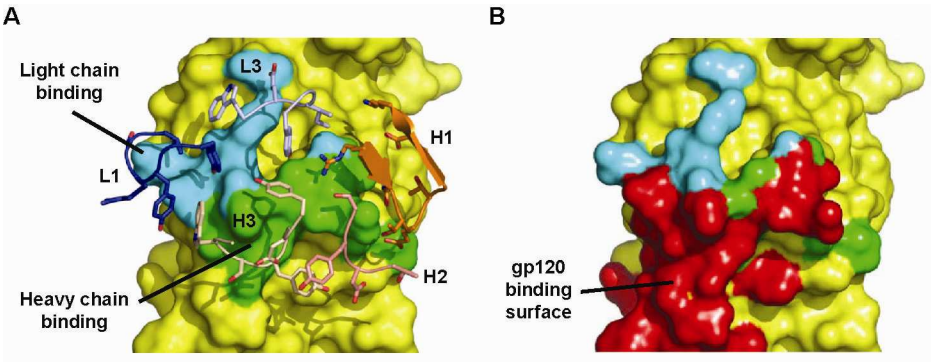


Figure S3. The 2D5 epitope on domain 1 of CD4. **A,** Surface representation of CD4 (yellow) with the interacting 2D5 CDR loops overlaid using ribbon and stick renderings. The CD4 regions buried by 2D5 light and heavy chains are colored cyan and green respectively. **B,** The same view as in **A** shows the CD4 surface buried by gp120 binding in red overlapping with the mAb2D5 epitope.

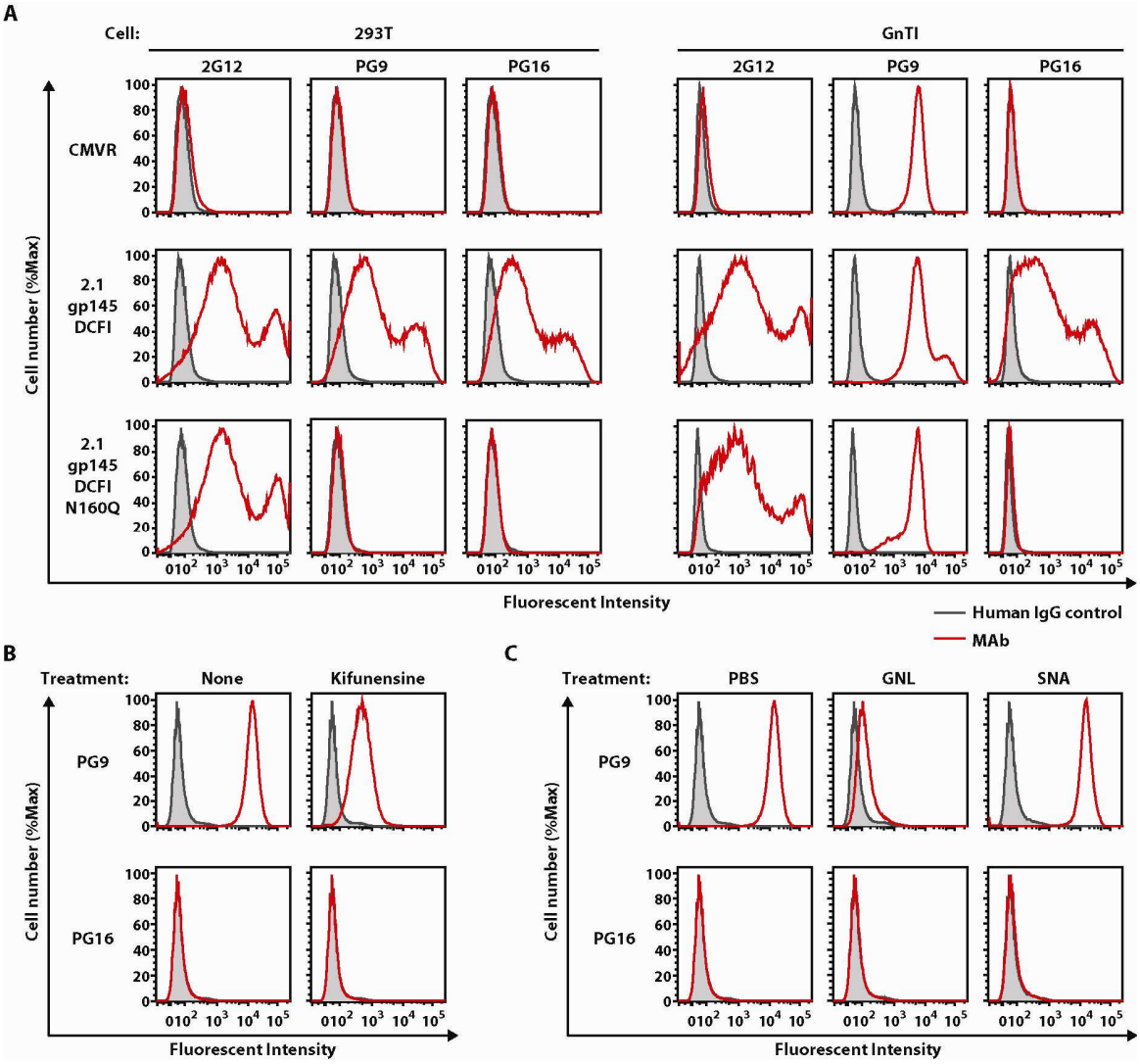


Figure S4. Binding of PG9 to GnTI⁻ 293S cells. A, The binding of indicated monoclonal antibodies (Red) or human control IgG (Grey) to the 293T or GnTI⁻ 293S cells transfected with CMVR backbone, gp145ΔCFI or its N160Q mutations was analyzed by flow cytometry. **B,** Binding of PG9 to GnTI(-) 293S cells after treatment with 5 μg/ml Kifunensine for 48 hours in culture. **C,** Binding of PG9 and PG16 to GnTI⁻ 293S preincubated with 2.5 μg/ml Galanthus Nivalis Lectin (GNL: α-1,3 mannose) or Sambucus Nigra Lectin (SNA: α-2,6 sialic acid attached terminal galactose).

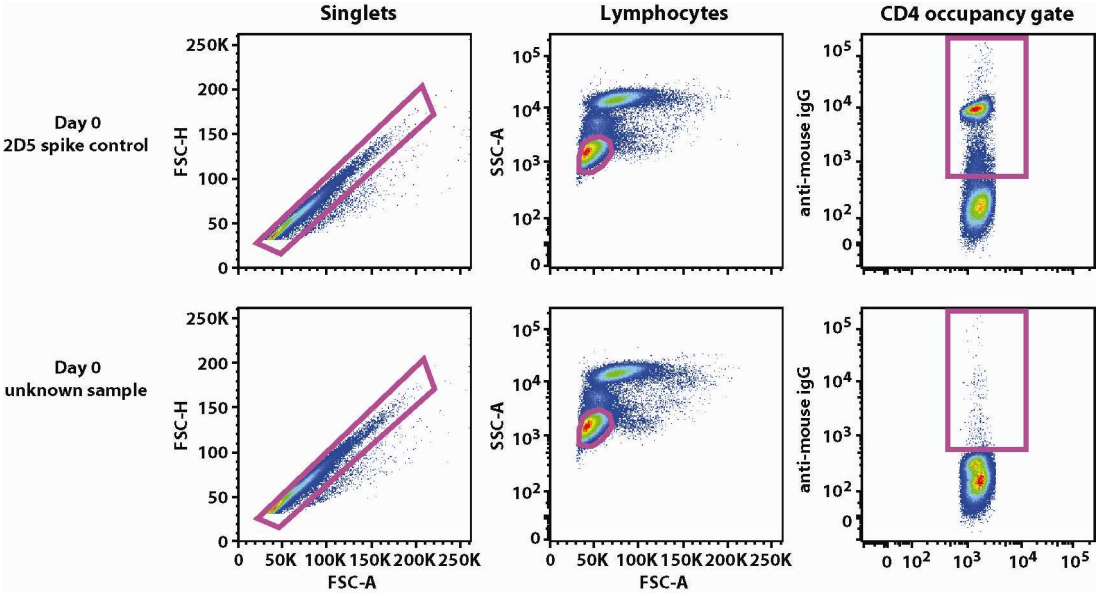


Figure S5. Gating strategy for determining percent CD4 occupancy by 2D5. Whole monkey blood was stained with anti-mouse IgG FITC conjugate and run on a flow cytometer using the above gating strategy. The cells were first gated for singlets based on forward scatter height and area. Then these singlets were gated for lymphocytes based on forward and side scatter. A gate was then drawn for the cells that were positive for FITC to denote binding to the anti-CD4 antibody, 2D5 (CD4 occupancy gate). A day 0 2D5 spike control (100 % occupancy) is shown in which 2D5 (100 μg/ml) was spiked into the sample before staining alongside the corresponding unknown sample. Percent CD4 occupancy was then calculated based on the ratio of mean fluorescence in the unknown sample compared to that in the 2D5 spike control.

Supplementary Table 1. Crystallographic data collection and refinement statistics.

Data

Space group	P4
Complexes/asu	4
Cell dimensions	
a, *b*, *c* (Å)	149.6, 149.6, 183.9
α, β, γ (°)	90.0, 90.0, 90.0
Wavelength (Å)	1.00
Resolution (Å)	30-3.65 (3.78-3.65)*
Unique reflections	46157
Redundancy	3.7 (2.6)
I/σI	19.4 (1.9)
Completeness (%)	96.8 (75.4)
R_{sym} (%)[†]	6.2 (40.4)

Refinement

R_{work} / R_{free} (%)[‡§]	22.6/25.7
r.m.s. deviations	
bonds (Å)	0.003
angles (°)	0.784
Avg. B-factor (Å^2)	146.7
No. of atoms	
Non-hydrogen	19200
Ramachandran statistics	
Favored (%)	90.4
Allowed (%)	8.5
Outliers (%)	1.1

*Parentheses indicate the highest resolution shell

[†]$R_{sym} = \Sigma|I-\langle I\rangle|/\Sigma\langle I\rangle$, where I is the observed intensity and <I> is the average intensity of multiple observations of symmetry related reflections.

[‡]$R=\Sigma_{hkl}||F_{obs}|-|F_{calc}||/\Sigma_{hkl}|F_{obs}|$

[§]R_{free} was calculated from 5% of the reflections that were excluded from refinement.

Supplementary Table 2. mAb2D5/CD4 interactions.

mAb2D5		CD4	
Residue	**Buried surface area (Å^2)***	**Residue**	**Buried surface area (Å^2)***
CDR H1		His27	13.8
Thr30	4.7	Lys29	15.3
Asp31	12.0	Ser31	29.3
Tyr32	5.6	Asn32	75.6
Ser33	24.2	Gln33	143.0
		Ile34	38.2
CDR H2		Lys35	42.3
Arg50	46.6	Lys46	13.3
Asn52	37.1	Gly47	7.8
Thr52A	1.7	Pro48	118.2
Glu53	29.9	Ser49	5.2
Thr54	2.84	Lys50	111.1
Glu56	9.3	Asn52	73.0
		Asp53	65.7
CDR H3		Arg54	0.7
Tyr95	28.3	Glu77	10.0
Asp96	4.17	Ile83	1.7
Gly97	64.7	Lys90	16.6
Tyr98	63.6		
Ser99	10.4		
Trp100	87.8		
Asp100A	1.0		
CDR L1			
Thr27D	40.3		
Ser28	18.0		
Arg29	26.4		
Tyr30	26.5		
Tyr32	49.4		
CDR L2			
Leu50	21.7		
CDR L3			
Trp92	43.0		
Glu93	39.2		
Ile94	18.9		
Tyr96	13.9		

*Calculated from chains C, H and L with hydrogens removed using the PISA web server (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver)

Supplementary Table 3. Hydrogen bonds and salt bridges between mAb2D5 and CD4.

Hydrogen bonds*

mAb2D5 atom	Dist. (Å)	CD4 atom
H:Thr 30 [O]	3.3	C:Gln 33 [NE2]
H:Asn 52 [ND2]	3.0	C:Ser 31 [O]
H:Thr 52A [N]	3.0	C:Gln 33 [OE2]
H:Thr 52A [OG1]	3.0	C:Gln 33 [OE2]
H:Thr 52A [OG1]	3.3	C:Gln 33 [NE2]
H:Gly 97 [O]	3.0	C:Lys 35 [N]
H:Trp 100 [NE1]	3.7	C:Lys 46 [O]
L:Tyr 32 [OH]	2.9	C:Pro 48 [O]
L:Tyr 32 [OH]	2.5	C:Ser 49 [O]

Salt bridges*

mAb2D5 atom	Dist. (Å)	CD4 atom
L:Glu 93 [OE2]	2.6	C:Lys 50 [NZ]

*Calculated from chains C, H and L with hydrogens removed using the PISA web server (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver)