SUPPLEMENTAL FILE 1

Viruses

HIV-1 IIIB, MN, BaL, YU2, SF162 and JRFL were obtained from the Centralised Facility for AIDS Reagents (CFAR), NIBSC, Potters Bar, Herts, UK. HIV-1 CRF07_BC primary isolate CN54 (7, 8) was obtained from EMPRO. HIV-1 primary isolates 92UG037, 92UG001, 92BR025, 97IN003 and ZA97001 were obtained from the WHO-UNAIDS collection of primary isolates (9). All PBMC isolates were propagated in phytohemagglutinin (PHA)–stimulated peripheral blood mononuclear cells (PBMC). HIV-1 IIIB and MN were propagated in H9 cells. Virus stocks were prepared from replication-competent HIV-1 molecular clones by transfection of 293T cells. Clones 4.10.3, 8.8.8, 23.8.12 and 23.2.E express subtype B gp120 (1), and clones CA6, CB7, C222, C261 subtype C gp120 (W. Koh *et al*., in preparation), held in the pHXB2Δenv vector (6). Viruses C27b, C27d, C37.4.2 and C38.2.2 (W. Koh *et al*., in preparation) are subtype C gp160 clones held in the pNL43 based C2 cassette (11) . To facilitate virus propagation, envelopes from primary isolates 92UG037, 92BR025, 97IN003 and ZA97001 were also cloned into the C2 cassette or the pHXB2Δenv vector.

The subtype B and C HIV-1 Reference Panels of Env Clones (4, 5) were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA. HIV-1 subtype CRF07_BC gp160 clones (CH181.12, CH064.20, CH091.9, CH117.4, CH119.10, CH110.2, CH114.8, CH120.6, CH115.12, CH070.1, CH111.8 and CH038.12), subtype CRF02_AG gp160 clones (T257-31 and T33-7) and the 93MW965.26 and 96ZM651.02 gp160 clones were kindly provided by Dr D. Montefiori (Duke University Medical Center, Durham, USA) through the Comprehensive Antibody Vaccine Immune Monitoring Consortium (CA-

VIMC) as part of the Collaboration for AIDS Vaccine Discovery (CAVD). HIV-1 envelope pseudotyped viruses were produced in 293T cells by co-transfection with the pSG3Δenv plasmid. Virus CVS-11 (10), pseudotyped with rabies virus G protein, was kindly provided by Dr. E. Wright (University College London, UK).

Cloning, expression and purification of recombinant gp120

Genomic DNA was isolated from PBMC infected with HIV-1 primary isolates 92UG037 and 92BR025 obtained from the WHO-UNAIDS collection of primary isolates (9) using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Envelope genes were amplified by PCR from proviral DNA using primers and conditions described previously (2), with the modification that the reverse primer encoded a 6-histidine-tag to enable purification. The resulting PCR products were cloned into expression vector pCDNA3.1 (Invitrogen, Paisley, UK) and sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) or the CEQ DTCS Quick Start Kit (Beckman Coulter, High Wycombe, UK). Recombinant gp120 from the *env* genes were expressed in 293T cells infected with a T7 RNA polymerase recombinant vaccinia virus (vTF7-3, American Tissue Culture Collection number VR-2153) as described previously (2). Envelope protein was harvested 72 h post-transfection and purified using Ni Sepharose™ 6 Fast Flow (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

Selection of anti-CD4bs VHH through panning on gp120 followed by competitive elution with sCD4 and subsequent isolation and screening of individual VHH

Recombinant gp120 (derived from HIV-1 isolates CN54, IIIB, 92BR025 or 92UG037) was immobilized onto 96-well Maxisorp plates (Nalgene, NUNC International, Hereford, UK), either by direct coating at 2.5 and 0.5 μg/ml or by capture by immobilized antibody D7324 (Aalto Bio Reagents, Dublin, Ireland), a sheep polyclonal antibody raised against a conserved motif in the C-terminus of gp120. Phages expressing the cloned VHH repertoire were then incubated with the immobilized gp120 in 0.2% casein in PBS for 1 h, followed by 20 washes with 0.05% Tween in PBS (PBS-T) to remove unbound phage. In order to elute phage targeting the CD4bs, the wells were then incubated with 30 or 200 μg/ml of sCD4 for 0.5-3 h. Recombinant sCD4 (D1-D4) previously prepared (3) was used as was sCD4 (D1-D4) from R&D Systems (Minneapolis, USA, cat. No. 514-CD-050/CF) as well as from the CFAR (NIBSC; cat. No. ARP609). Control wells were incubated with 30 or 200 μg/ml of BSA. Additional control wells were incubated with 0.1 M glycine, for elution of all phage through low pH shock. Eluted phage were titrated onto *E. coli* TG1 cells and phages from selection conditions where enrichment of phage eluted by sCD4 could be observed, were multiplied, precipitated with polyethylene glycol and taken forward to a second round of selection. Individual VHH from selected elutions were amplified using PCR and re-cloned into the pAX051 expression vector followed by transformation into TG1 cells. Expression from the pAX051 vector incorporates a 6-his- and a c-myc-tag to the C-terminus of the VHH. Individual clones were picked and grown in 1 ml of 2×TY medium containing 100 μg/ml ampicillin and 0.1% glucose, followed by induction of VHH production with 0.1 mM isopropyl-ß-D-thiogalactopyranosid (IPTG). Periplasmic extracts containing the expressed VHH were prepared and individual VHH were screened for binding to recombinant gp120 in ELISA

and/or for neutralization of HIV-1. VHH found to be neutralizing or binding were sequenced, expressed in TG1 cells and purified by means of the attached his-tag using TALON Metal Affinity Resin (Clontech, Mountain View, USA). A subset of the isolated VHH was subjected to DNA fingerprint analysis using the restriction enzyme *HinfI*.

VHH binding to recombinant envelope proteins in ELISA

VHH binding to HIV-1 gp120 or gp140 was assayed in ELISA. White 96-well Maxisorp plates (Nalgene) were coated overnight with 10 μg/ml of sheep antibody D7324 (Aalto Bio Reagents). Plates were blocked using 4% milk powder in triethanolamine buffered saline (TBS). Saturating concentrations of gp120 or gp140 were added and the plates were incubated at room temperature for 1 h and subsequently washed four times with 0.05% Tween in TBS (TBS-T). Serial dilutions (in TBS-T containing 4% milk powder and 10% goat serum; TMT/GS) of the VHH to be assayed and of a negative control VHH were then added to the plates in duplicate wells as well as to blank wells (i.e. wells with no gp120). After 1 h of incubation at room temperature the plates were washed four times with TBS-T. The wells were then incubated in two subsequent steps with 0.5 μg/ml of mouse anti-c-myc antibody (cat. No. 1166714900, Roche Diagnostics, Lewes, UK) in TMT/GS for 1 h at room temperature followed by 0.5 μg/ml of alkaline phosphatase-conjugated goat anti-mouse IgG antibody (cat. No. 1030004, Oxford Biotechnology, Kidlington, UK). After six washes with TBS-T, Lumi-Phos Plus substrate (Aureon Biosystems, Vienna, Austria) was added and the plates were incubated at 37°C for 0.5 h. Chemiluminescence was detected and background-subtracted relative light units were plotted against VHH concentration.

Competition ELISAs

The ability of candidate VHH to inhibit binding of sCD4 to gp120 or gp140 was evaluated by ELISA. Serial dilutions of candidate VHH, as well as a negative control VHH, was pre-incubated with 0.5 μg/ml gp120 (or gp140) at room temperature for 1 h and subsequently added to Maxisorp plates (Nalgene) that had been coated overnight with 10 μg/ml of sCD4 and blocked with 4% milk powder. After 1 h at room temperature the plates were washed four times with TBS-T and then incubated with 10 μg/ml sheep polyclonal antibody D7324 (Aalto Bio Reagents) in TBS-T containing 4% milk powder and 10% FCS (TMT/FCS) followed by washing as before and another incubation with 0.5 μg/ml of alkaline phosphatase-conjugated rabbit anti-sheep IgG antibody (cat. No. ab6748-1, Abcam, Cambridge, UK). Alternatively, gp120 or gp140 binding was detected using QC sera 2, 5 and 6 at 1:100 dilution followed by 0.5 μg/ml in TMT/GS of alkaline phosphatase-conjugated goat anti-human immunoglobulin antibody (Harlan SeraLab, Crawley Down, UK). After six washes with TBS-T, Lumi-Phos Plus substrate (Aureon Biosystems) was added and chemiluminescence was detected after 0.5 h incubation at 37°C. Relative light units were plotted against VHH concentration.

VHH competition with anti-CD4bs MAb b12 was assayed by pre-incubating three-fold serial dilutions of VHH with gp120 followed by incubation with 10 μg/ml b12 pre-coated onto Maxisorp plates (Nalgene). HIV-1 IIIB gp120 binding to b12 was detected using sheep antibody D7324 (Aalto Bio Reagents) in TBS-T containing 4% milk powder and 10% bovine serum followed by washing as before and another incubation with 0.5 μg/ml of alkaline phosphatase-conjugated rabbit anti-sheep IgG antibody (Abcam). VHH inhibition of human anti-gp120 MAb b12, 2G12, 447-52D, 17b as well as an irrelevant MAb was assayed by pre-incubating serial dilutions of the MAb with gp120, followed by incubation with immobilized VHH pre-coated

onto Maxisorp plates (Nalgene) and subsequent detection of gp120 binding to immobilized VHH as described above. VHH competition with each other and with fluid-phase sCD4 was assayed by pre-incubating gp120 with serial dilutions of VHH or sCD4, followed by incubation with immobilized VHH pre-coated onto Maxisorp plates (Nalgene) and subsequent detection of gp120 binding to immobilized VHH as described above. VHH inhibition of MAb 654-D and GP68 binding to HIV-1 IIIB gp120 were assayed by incubating four-fold serial dilutions of VHH with gp120 captured onto Maxisorp plates (Nalgene). Wells were washed and incubated with MAb 654-D or GP68. Binding of MAb to gp120 was detected using an alkaline phosphatase-conjugated goat anti-human immunoglobulin antibody (Harlan SeraLab). Chemiluminescence was detected as described above.

Surface plasmon resonance

Biosensor experiments were run on BIAcore 3000 (BIAcore, Uppsala, Sweden). CM5 sensor chips and amine-coupling reagents (N-ethyl-N-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), and ethanolamine HCl) were from BIAcore AB. Using amine-coupling chemistry, recombinant IIIB gp120 was immobilized at 25°C on a CM5 sensor chip surface. The carboxymethyl surface of the CM5 chip was activated for 7 min at a flow rate of 5 µl/min using a 1:1 ratio of 0.4M EDC and 0.1M NHS. IIIB gp120 was diluted in 10 mM sodium acetate, pH 4.0 to a concentration of 15 µg/ml and injected over the surface at a flow rate of 5 µl/min. Excess activated groups were blocked using a 7-min injection of 1 M ethanolamine, pH 8.5, at a flow rate of 5µL/min. Approximately 2700 RU of IIIB gp120 was immobilized using this protocol. Experiments were performed at 25°C. Purified VHH D7 was diluted in HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0,005% P20) to 75, 50, 40, 30 and 20 nM and the samples were injected for 2 min at a flow rate of 45 µl/min over the activated

and reference surface. The reference surface was a flow cell that was also activated with 0.4 M EDC/0.1 M NHS and blocked with 1 M ethanolamine but in between no compound was injected. The samples were allowed to dissociate for 1800 s. To measure the affinity of VHH A12 and C8, biotinylated IIIB gp120 was non-covalently captured on an SA sensor chip which is pre-immobilized with streptavidin. IIIB gp120 was diluted in HBS-EP to a concentration of 15 μg/ml and injected over the surface at a flow rate of 5 μl/min. Approximately 2100 RU of IIIB gp120 were captured using this protocol. Purified VHH A12 and C8 was diluted in HBS-EP buffer to 80, 60, 40, 20 and 10 nM and the samples were injected for 3 min at a flow rate of 45 μl/min over the activated and reference surface (in this case an unmodified flow cell). The samples were allowed to dissociate for 1200 s. For each VHH, the kinetic constants (i.e. the second-order rate constant for the association, k_a, and the first-order rate constant for the dissociation, k_d) were computed from the binding curves using the BIAevaluation software (1:1 interaction) and the equilibrium dissociation constant, KD, was calculated from k_d/k_a.

The ability of the VHH to inhibit binding of sCD4 to gp120 was assayed using a BIAcore X (BIAcore). Mouse monoclonal anti-CD4 antibody L120.3, obtained from the CFAR (NIBSC; original source Becton-Dickinson) was coupled to both flow cells of a CM5 chip (BIAcore) using standard amine-coupling chemistry as described by the manufacturer. Soluble CD4 was cross-linked to the anti-CD4 antibody in flow cell 2 through amine-coupling, while flow cell 1 was kept as a negative control reference cell. Serial dilutions of VHH were pre-incubated with 300 nM of HIV-1 CN54 gp120 and subsequently injected onto the chip at a flow rate of 5 μl/min. Data was analyzed using the BIAevaluation software. Binding of HIV-1 CN54 gp120 to sCD4 was

represented as the difference in response units (RU) observed between flow cell 2 (with sCD4) and flow cell 1 (reference cell) plotted against time.

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