Anti-HIV IgM protects against mucosal SHIV transmission

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Methods

Preparation of 33C6 mAbs

We previously described the production of 33C6-IgG1 mAb [1]; 33C6-IgM mAb was prepared as follows. Human μ chain constant region and human λ chain constant region were PCR amplified from pFUSEss-CHIg-hM and pFUSE2-CLIg-hl2 (InvivoGen) using Q5 High-Fidelity 2X Master Mix (NEB) following the manufacturer's recommendation. Following gel purification, PCR products were assembled with a Kozak-murine Ig leader sequence and cloned downstream of a CMV promoter in a pcDNA3.4 plasmid (ThermoFisher Scientific) using NEBuilder HiFi DNA Assembly Master Mix (NEB). The resulting plasmids were designated pTBRI-hM and pTBRI-hl, respectively. 33C6 heavy chain and light chain variable gene fragments were PCR amplified from the 33C6-IgG1 expression plasmids [2] and cloned into pTBRI-hM and pTBRI-hl to yield plasmids pTBRI-33C6-hM and pTBRI-33C6-hl, respectively. Full-length 33C6-IgM mAb was expressed in Expi293F cells (ThermoFisher Scientific) transiently co-transfected with pTBRI-33C6-hM, pTBRI-33C6-hl and human J chain precursor expression plasmids [2] using ExpiFectamine 293 Transfection Kit (ThermoFisher Scientific). Cells were maintained in Expi293 expression medium (ThermoFisher Scientific) for 4 days at 37°C, 8% CO_2 with continuous shaking at 135 rpm. Antibody was purified from filtered supernatants using Thiophilic Resin (G-Biosciences) followed by HiTrap SP HP column separation (GE Healthcare Life Sciences). Purity and polymeric state of 33C6-IgM was verified under denaturing and non-reducing polyacrylamide gel electrophoresis (NuPAGE™ 3-8% Tris-Acetate Protein Gel, ThermoFisher Scientific). The presence of human μ and J chains was verified by western blot with horse-radish peroxidase (HRP)-conjugated goat anti-human IgM, Fc5μ fragment-specific antibody (Jackson

ImmunoResearch) and J-chain antiserum (InvivoGen) in conjunction with HRP-conjugated donkey anti-rabbit IgG(H+L) (Jackson ImmunoResearch), respectively.

Dynamic light scattering studies

The particle sizes of the purified 33C6-IgM and 33C6-IgG1 were measured by Dynamic Light Scattering in PBS at 24°C using a Protein Solutions DynaPro (Wyatt Technology) and analyzed with Dynamics software (Wyatt Technology). The predicted molecular weight of 33C6-IgM and 33C6-IgG1 was calculated based on the amino acid sequences using compute pI/Mw tool at ExPASy Bioinformatics Resource Portal (SIB Swiss Institute of Bioinformatics; https://web.expasy.org/compute_pi/).

Surface plasmon resonance (SPR) studies

SPR studies were carried out utilizing a Biacore T200 instrument on a CM5 chip in HBS-EP+ running buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.05% surfactant P20) at 25ºC. An anti-human IgM Fc5µ fragment-specific capture antibody (Jackson ImmunoResearch) was immobilized on FC1 and FC2 utilizing an amine coupling kit (GE Healthcare). An anti-human IgG Fc capture antibody from a human IgG capture kit (GE Healthcare) was immobilized on FC3 and FC4 utilizing an amine coupling kit (GE Healthcare). 33C6-IgM was captured on FC2, and 33C6-IgG1 was captured on FC4. A concentration series of SHIV-1157ip gp120 ranging from 4.6 pM to 10 nM was flowed over all 4 surfaces at 100 µl/min, interspersed with buffer blanks for double referencing. During each cycle, gp120 was injected for 220 s, followed by a change to running buffer for 3000 s. FC1 and FC2 were regenerated by an injection of 10 mM glycine pH 1.7 for 180 s at 20 µl/min, while FC3 and FC4 were regenerated by an injection of 3 M $MgCl_2$ for

30 s at 20 µl/min. Data were double referenced, first by subtracting the reference cell data from the experimental cell data (i.e., FC2-FC1) and (FC4-FC3), and then by subtracting the buffer blanks. Overlay plots were made for all curves from 41 pM - 10 nM, and data were globally fit to a 1:1 binding model utilizing Biacore T200 evaluation software v. 2.0 (GE Healthcare), from which the association rate constant k_a, the dissociation rate constant k_d, and the equilibrium dissociation constant K_D were derived. Average values for each quantity were determined from 3 replicates, and average values and standard error were calculated utilizing OriginPro 2017 Software (OriginLab Corp.), and are indicated directly on Fig. 1e and Fig. 1f. The fit parameters for the IgM data set shown in Fig. 1e are $k_a = 4.0 \times 10^6\ M^{-1}s^{-1}$, $k_d = 1.2 \times 10^{-5}\ s^{-1}$, $K_D = 2.9$ pM, $chi^2 = 0.030\ RU^2$. The fit parameters for the IgG1 data set shown in Fig. 1f are $k_a = 3.0 \times 10^5\ M^{-1}s^{-1}$, $k_d = 4.3 \times 10^{-5}\ s^{-1}$, $K_D = 146$ pM, $chi^2 = 0.042\ RU^2$.

Animals

Eighteen adult, outbred, naïve, male, Indian-origin rhesus monkeys (RMs) (*Macaca mulatta*) were housed at the Southwest National Primate Research Center (SNPRC), San Antonio, Texas. SNPRC, a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, adheres to the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Animal Care and Use Committee of Texas Biomedical Research Institute, SNPRC's parent institution. All RMs were negative for Mamu-B*17 allele associated with spontaneous virologic control; one RM 32547 (randomized to the control group) was positive for the Mamu-B*08 allele also associated with spontaneous virologic control. Similarly, RMs with the Mamu-A*01 allele that contributes to spontaneous virologic control were evenly distributed in each group. RMs were randomized into groups (n = 6 per group).

Plasma viral RNA (vRNA) loads

RNA was isolated by QIAamp Viral RNA Mini-Kits (Qiagen), and vRNA levels were measured by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for SIV *gag* sequences [3]. Assay sensitivity was 50 vRNA copies/ml. Time to first detection of viremia was analyzed by Kaplan-Meier analysis.

ELISAs

Plasma mAb binding to SHIV-1157ip gp120 was evaluated by ELISA as described [1]. Briefly, plates were coated with monomeric SHIV-1157ip gp120 (1 µg/ml) in 100 µl carbonate buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6, Sigma) overnight at 4°C, washed 3x with 0.05% Tween 20 in PBS (0.05% PBS/T), and blocked with 4% non-fat milk in PBS for 1 h at 37°C. One hundred µl of heat-inactivated plasma diluted serially in dilution buffer (1% non-fat milk in PBS) were added to duplicate wells and incubated for 2 h at 37°C. Plates were washed 3x in 0.05% PBS/T, and binding was detected with HRP-conjugated rabbit anti-monkey IgG (whole molecule) (Sigma) antibody. After 1 h of incubation at 37°C, 3,3',5,5'-Tetramethylbenzidine (TMB) single solution (ThermoFisher Scientific) was added, and the reaction was terminated by the addition of 1 N H_2SO_4. Plates were read at 450 nm by a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies). Antibody titers were calculated as the reciprocal sample dilution giving optical density (OD) > mean + 5x standard deviation of background (pre-immune samples) at the same dilution.

Epitope binding specificity for 33C6-IgM mAbs was determined by ELISA with consensus clade C peptides (NIH AIDS Research and Reference Reagent Program) performed as described above. Briefly, plates were coated with corresponding peptides (5 µg/ml) in triplicates, blocked

and probed with various concentration of 33C6-IgM or human serum IgM (Sigma). To detect binding, plates were probed with HRP-conjugated goat anti-human IgM antibody (Jackson ImmunoResearch). The sequences of the various peptides used were as follows: peptide 9258, VEIVCTRPNNNTRKS; peptide 9259, CTRPNNNTRKSIRIG; peptide 9260, NNNTRKSIRIGPGQT; peptide 9261, RKSIRIGPGQTFYAT and peptide 9262, RIGPGQTFYATGDII.

Avidity assay

Analysis of antibody avidity was performed as described [4]. Briefly, ELISA plates were coated with 50 μg/ml concanavalin A (Sigma) for 1 h at room temperature. After overnight incubation with 2 μg/ml monomeric SHIV-1157ip gp120 at 4°C, plates were blocked with 3% N-Z-Case plus (Sigma) 0.5% PBS/T for 1 h at 37°C, then incubated with 50 μl of serial dilutions of mAbs in two sets of triplicate wells for 1 h at 37°C. One hundred μl of 8 M urea solution (Sigma) was added to one set of the triplicate wells, and 100 μl of PBS was added to the other set of triplicate wells and incubated 3x (5 min each with wash steps in between) at 37°C. Then wells were washed thoroughly with 0.05% PBS/T and incubated with HRP-conjugated goat anti-human IgG1 or HRP-conjugated goat anti-human IgM (Jackson ImmunoResearch). Finally, the colorimetric reaction was developed with TMB single solution (ThermoFisher Scientific) and terminated with 1 N H_2SO_4. Avidity index (AI) was calculated as follows:

A1 (%) = (OD450 nm of samples washed with 8 M urea / OD450 nm of samples washed with PBS) x 100.

Neutralization assay

Neutralization of SHIV-1157ipEL-p by 33C6 mAbs or antibodies in RM plasma was determined using the TZM-bl assay as described [5]. Briefly, virus was incubated with serially diluted mAbs or plasma for 1 h at 37°C. TZM-bl cells (5×10^3/well) and DEAE-dextran (Sigma) were added to the virus without antibody (baseline) or with antibodies. After incubation for 48 h at 37°C, luciferase activity was quantified in a CentroPRO LB 962 Budget Microplate Luminometer (Berthold Technologies) upon addition of Bright-Glo luciferase assay substrate (Promega). VRC01-IgG1 was used as positive control; IgM isotype control (ThermoFisher Scientific) and Fm-6-IgG1 were used as negative controls. Percentage neutralization was calculated relative to baseline luciferase activity or luciferase activity level of pre-immune samples for 33C6 mAb or RM plasma sample neutralization, respectively. Neutralizing antibody titers were estimated as the reciprocal serum dilution giving 50% inhibition of virus replication.

Virion capture assay (VCA)

We performed the VCA as follows. Briefly, 10 μg/ml of 33C6 mAbs were coincubated with 10^8 vRNA copies/ml of SHIV-1157ipEL-p (in 200 μl reaction) for 1 h at 37°C to form antibody-virion immune complexes (ICs). To enable capture of IgM-virus complexes by the Protein G micro beads (Miltenyi Biotec), the ICs were incubated for additional 1 h at 37°C in the presence of 20 μg/ml goat anti-human IgM antibody (Jackson ImmunoResearch). After ICs were mixed with Protein G micro beads for 30 min at 37°C, the mixture was loaded to a μ Column (Miltenyi Biotec) under a magnetic field and washed. Unbound free virions were collected in the flow-through and measured by Gag p27 ELISA (Advanced BioScience Laboratories). The infectivity of the flow-through virus was evaluated by TZM-bl assay. A virus-only control (no antibody) was used to set maximum

limit of infectivity; VRC01-IgG1 was used as positive control; IgM isotype control (ThermoFisher Scientific) and Fm-6-IgG1 were used as negative controls.

The percentage of total physical virus particles captured (pVirion) was calculated as: pVirion = [1 – (p27 concentration of virus with mAbs in flow-through) / (p27 concentration of virus in flow-through of virus-only control)] x 100%.

The percentage of captured infectious virions (iVirion) was calculated as: iVirion = [1 – (flow-through infectivity of virus with mAbs) / (flow-through infectivity of virus-only control infectivity)] x 100%.

Results

Dynamic light scattering to determine size of 33C6 mAbs

The particle sizes of the purified 33C6-IgM and 33C6-IgG1 were measured by Dynamic Light Scattering as described above. The experiment was repeated 4 times on each sample, and representative data are shown. In the experiment shown, 100% of the mass of the IgM antibody was found in a particle with a diffusion coefficient of 1.98×10^{-7} cm^2/s, a radius of 11.4 nm, 15.3% polydispersity, and a mass of 994,000 Da. The average mass from the 4 independent measurements was calculated as 994,000 ± 23,000 Da. This is consistent with the MW expected for the pentameric particle, since the masses of 1 J chain, 10 heavy chains, and 10 light chains as calculated from the amino acid sequence is 874,707 Da, which is expected to be further increased by glycosylation. In the experiment shown, 100% of the mass of the IgG1 antibody was found in a particle with a diffusion coefficient of 4.47×10^{-7} cm^2/s, a radius of 5.0 nm, 24.9% polydispersity, and a mass of 148,000 Da. The average mass from the 4 independent measurements was calculated as 148,000 ± 3,600 Da. This is consistent with the MW expected for the monomeric particle, since

the masses of 2 heavy chains and 2 light chains is 145,291 Da, which is expected to be further increased by glycosylation.

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