TITLE

Generation of a neutralizing antibody against RD114-pseudotyped viral vectors

AUTHORS

Mekkaoui L[1], Ferrari M[2], Mattiuzzo G[4], Ma B[2], Nannini F[1], Onuoha S[2], Kotsopoulou E[2], Takeuchi Y[3,4] and Pule M[1]

[1] UCL Cancer Institute, University College London; Paul O'Gorman Building, 72 Huntley street, WC1E 6BT

[2] Autolus Limited; Forest House, 58 Wood Lane, W12 7RZ

[3] Division of Infection and Immunity, University College London; Cruciform building, Gower street, WC1E 6BT

[4] National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, EN6 3QC

ABSTRACT

The feline endogenous RD114 glycoprotein has proved an attractive envelope to pseudotype both retroviral and lentiviral vectors. As a surface protein, its detection on packaging cells as well as viral particles would be useful in different fields of its use. To address this, we generated a monoclonal antibody against RD114 by immunization of rats, termed 22F10. Once seroconversion was confirmed, purified 22F10 was cloned into murine Fc and characterised with a binding affinity of 10nM. The antibody was used to detect RD114 and its variant envelopes on different stable viral packaging cell lines (FLYRD18 and WinPac-RD). 22F10 was also shown to prevent the infections of different strains of RD-pseudotyped vectors but not related envelope glycoproteins by blocking cell surface receptor binding. We are the first to report the neutralization of viral particles by a monoclonal αRD114 antibody.

KEYWORDS

RD114 – monoclonal antibody – detection – neutralization

INTRODUCTION

Envelope glycoproteins are composed of mushroom-shaped trimer of heterodimers with each dimer consisting of an extracellular surface unit (SU) attached to a transmembrane (TM) subunit by non-covalent interactions and a labile disulphide bond (1). After virion budding, certain envelopes such as RD114 and MLV-ampho are processed by viral protease at the R peptide site in their endodomains resulting in viral particle maturation (2).

Viral glycoproteins that utilise the same cell surface receptors for infection are classified into interference groups. The largest group identified the "RD114 interference group" consists of type C γ-retroviruses (RD114 feline endogenous virus, Baboon Endogenous Retrovirus (BaEV) and avian reticuloendotheliosis virus (REV)), some type D simian β-retroviruses (simian retrovirus-1 (SRV1) to SRV5 including Mason-Pfizer monkey virus MPMV, also known at SRV-3) and the human endogenous retrovirus-W (HERV-W); which all use the same cell surface receptor for host cell infection, the human sodium-dependent neutral amino acid transporter type 2 (ASCT-2) (3,4).

The RD114 envelope glycoprotein is increasingly utilized to pseudotype γ-retroviral vectors (RV) for gene therapy applications, due to its lack of inactivation by serum (5,6), high titer and stability (7) and wide tropism for many human cells due to the broad expression profile of its cell surface receptor ASCT-2 (8–12). Modification of the RD114's endodomain (13) extends its application to lentiviral vector (LV) pseudotyping (14). Furthermore, unlike other envelope proteins widely used for pseudotyping, such as GaLV and VSV-G, stable expression of RD114 on cell lines does not result in cytopathy. Hence, RD114 is an increasingly common choice of pseudotype for RV and LV stable packaging cell lines (15,14,16–19).

Apart from use in vector production for gene therapy application, RD114 is also a consideration during cat and dog vaccine production since endogenous RD114 sequence is present in the cat genome (20,21). As vaccines for both dogs and cats are usually produced by using feline cell lines, infectious RD114 related viruses have been detected in preparations of live attenuated vaccines (22,23). Although no diseases in both cats and dogs have been associated with RD114 (24), both species challenged with replication competent RD114 virus were not able to seroconvert against the envelope (25,26). RD114 contamination is hence a regulatory consideration (EMA/CVMP/IWP/592652/2014).

A monoclonal antibody (mAb) which binds to the RD114 SU would be useful to allow characterization of packaging cell lines and viral particles for gene therapy applications. The practical utility of such a mAb would be even greater if the mAb blocked infectivity. We therefore set about to generate an antibody capable of both detection of surface expression of RD114 and blocking of RD114 pseudotyped viral particles.

RESULTS

αRD114 hybridoma generation by rat immunization

A genetic immunization approach was used to generate a mAb against RD114. To that end, RDpro (an RD114 variant with increased lentiviral vector pseudotyping efficiency, achieved by mutating its R peptide cleavage site from VHAMVLAQ to SQNYPIVQ (14)) was cloned into the pVAC2 expression plasmid. Three rats were immunized using gene-gun delivery of golden nanoparticles coated with the pVAC2 plasmid by Aldevron (Germany). The construct used for vaccination included the endogenous transmembrane domain to allow cell surface expression of the target protein. Seroconversion was validated by staining HEK-293T cells transiently transfected with an RDpro expression plasmid with rat serum pre and post-vaccination (Fig. 1). Subsequently, monoclonal hybridomas were generated by the fusion of splenocytes from the seroconverted rats with the murine myeloma SP2/0 cell line by Genscript (USA). Growing clones were then subjected to an indirect ELISA primary screening using recombinant soluble RD114 receptor binding domain (RBD) fragment as antigen (which consists of N-terminal 222 amino acids of RD114 protein fused to mouse IgG1 (termed "ASCT2-RBD-mouse-IgG1" by its supplier, Metafora Biosytems, France) and pre- and post-immunization serums were used as negative and positive controls, respectively. Supernatants from two hybridoma clones, termed 22F10 and 30F3, resulted in increased absorbances over background (Fig 2a). Functional antibody production from these clones was further validated by flow cytometry analysis of their supernatants by staining HEK-293T cells transiently transfected with an RDpro expression plasmid, with post-immunization serum as positive control (Fig. 2b). Although the supernatants were not quantified, these results served as a functional screen and thus indicated the production of αRD114 antibody from these two isolated clones. As the supernatant from clone 22F10 resulted in the brightest staining of HEK-293T cells expressing RDpro, this clone was selected for further study.

Production and purification of recombinant monoclonal 22F10 antibody

For the widespread application of the generated antibody, first the heavy and light variable sequences (V_H and V_L, respectively) of the 22F10 hybridoma clone were determined using 5' RACE PCR and analysed using the abYsis software (Fig. 3a). Subsequently, in order to purify the antibody using the commonly used HiTrap^{TM} Protein A HP (GE healthcare Bioscience, USA), the constant regions were altered to murine IgG2A which, unlike rat constant chains, confers a strong affinity to Protein A. To that end, 22F10-V_H DNA sequence was cloned into Invivogen's pFUSE2ss-CHIg-mG2a plasmid directly upstream of murine IgG2A heavy by EcoRI/AfeI double digest; and 22F10-V_L sequence was cloned into pFUSE2ss-CLIg-mK upstream Kappa light constant region by EcoRI/BstAPI double digest. Full-length recombinant monoclonal antibody (mAb) was then produced and purified by large scale transfection of CHO cell line by Evitria (Switzerland). Antigen binding of full-length 22F10-mIgG2A was then validated by staining HEK-293T cells transiently transfected to express RDpro, with post-vaccination serum serving as positive control (Fig. 3b).

Kinetics characterisation of 22F10 mAb

Next, we sought to characterize the binding kinetics of our mAb by surface plasmon resonance analysis. 22F10-mIgG2A produced in CHO cells was digested by papain and processed to generate a Fab fragment to enable the measurement of 1:1 binding kinetics without avidity effects of a bivalent antibody. ASCT2-RBD-mIgG1 recombinant protein was covalently attached to a CM5 chip using amine coupling. 22F10-Fab was injected onto the chip at decreasing concentration from 1 μM with 2-fold serial dilutions to obtain a 100-fold concentration range around the measured kinetic affinity. Kinetic binding of 22F10-Fab was then measured and a binding affinity of 10 nM ±0.1 nM was determined with k_a of 3.4 x10^4 M^{-1} s^{-1} and k_d of 3.5 x10^{-4} s^{-1} (Fig. 4), whereas no binding was observed with a control, non-specific Fab fragment (data not shown).

Antibody-mediated neutralization of RD-pseudotyped viral vectors

First, the ability of 22F10-mIgG2a to detect RD114 expression on two stable viral vector packaging cell lines: lentiviral WinPac-RD expressing RDpro envelopes (18) and retroviral FLYRD18 expressing RD114 envelopes (6), was determined. Staining by the mAb resulted in clear detection of RD114 positive populations for both packaging cell lines, with 99.8% and 98.4% RD-positive cells for WinPac-RD and FLYRD18 cells, respectively (Fig. 5a). These results demonstrate the application of the 22F10-mIgG2A as a specific detection reagent for packaging cell lines expressing RD114 variant envelope glycoproteins.

We next determined the potential of 22F10-mIgG2a to block the infectivity of RD114 pseudotyped vectors i.e. serve as a neutralizing antibody. To that end, 22F10-mIgG2A was evaluated for its ability to neutralize three viral vectors: RV and LV pseudotyped with wild-type RD114 and RDpro, respectively; along with VSV-G pseudotyped LV as negative control (Fig. 5b). Unlike VSV-G-LV, the infectivity of both RD114-RV and RDpro-LV were blocked by 22F10-mIgG2A, with a 50% neutralization of infections achieved by mAb concentrations of 0.134 ±0.012 μg/mL (R^2=0.985) and 0.042 ±0.006 μg/mL (R^2=0.970), respectively. These results indicate the ability of the generated mAb to neutralize viral vectors pseudotyped with both wild-type RD114 and its variant RDpro glycoprotein.

Antigen specificity of 22F10 mAb

The specificity of our mAb was investigated by analysing its cross reactivity to related viruses belonging to the same interference group as RD114: BaEV, SRV-1 and SRV-2 and MPMV. MLV-packaging cells lines, TECeB6 (6) expressing a GFP vector, were infected with replication competent stocks of RD114, SRV-1, SRV-2 and MPMV. After 6 weeks in culture the viral titres of the pseudotyped viruses reached a plateau suggesting that all the cells were infected. For BaEV, RV pseudotyped with BaEV envelope was instead produced by transient triple transfection of 293T cells. Producer cells for all glycoproteins were then stained using 22F10-mIgG2A and analysed by flow cytometry (Fig 6a). Only RD114-RV producer cells showed a distinctive shift in fluorescence in comparison with the secondary antibody only control, suggesting that 22F10-mIgG2A does not cross react with other viruses sharing the same receptor. To further confirm these results, 22F10-mIgG2A-mediated

neutralization was tested on RD114-RV, SRV-1-RV, SRV-2-RV, MPMV-RV and BaEV-RV (Fig 6b). Only RD114-RV produced a dose response inhibition curve with an 50% neutralisation titre of 0.089 ±0.024 μg/mL (R^2=0.973), while no effect was observed for the remaining pseudotyped viruses. These results suggest that 22F10-mIgG2A is specific for RD114 and its derivative envelope glycoproteins.

Binding interference of RD114 RBD to cell surface receptor by 22F10 antibody

To further characterise the specificity of the mAb, its ability to block its antigen RD114 from binding its cell surface receptor ASCT2 was investigated by a fluorescence-based receptor blocking assay. First, an optimal concentration of 204 nM of recombinant RD114 (ASCT2-RBD-mIgG1 Fc protein) was determined to ensure antibody saturation of antigen (Fig. 1S). Since, both ASCT2-RDB-mIgG1 and 22F10-mIgG2A comprise of the same murine IgG constant region, the Fab fragment of 22F10-mIgG1A was used. [The Fab fragment showed negligible recognition by an Fc specific polyclonal secondary antibody (Fig 7a)].Antibody-mediated receptor binding interference was then tested by first incubating 204 nM of ASCT2-RBD-mIgG1 with serially diluted 22F10-Fab, with concentrations ranging from 1.05 μM to 512 pM (equivalent to 5:1 to 0.003:1 molar ratio of 22F10:ASCT2-RBD). Antigen:antibody mixtures were then assayed for ASCT2 binding on HEK-293T cells (Fig 7b) using the anti-Fc secondary. Inhibiton of ASCT2-RBD binding to cell surface ASCT2 was obeserved at Fab concentration over 131 nM (corresponding to 0.64:1 molar ratio) with an IC_{50} of 277 ±76 nM (R^2=0.901). These results suggest that 22F10 binding to the RD114's SU domain interferes with the latter ability to bind its receptor ASCT-2.

DISCUSSION

Here we report the generation of a mAb against the feline endogenous retrovirus RD114 glycoprotein, which may have several useful applications for varying fields utilizing this protein.

The antibody could be of use for the detection and quantification of RD114 on both viral vectors and cells. Vector packaging cells using RD114 variants to date have used antibiotic resistance to select cells expressing the glycoproteins (6,14,17–19,27). The use of our reagent can aid in the characterization of these cells as well as the demonstration of envelope expression stability. In addition, antibiotic selection can be either circumvented or aided by the ability to select and sort glycoprotein-expressing cells by flow-cytometry using the 22F10 mAb.

The developed mAb has been demonstrated to neutralise viral vectors pseudotyped with RD114 variants. Antibodies can neutralize the infectivity of viruses by interfering with different steps of the viral entry pathway such as blocking viral attachment, fusion or internalization into target cells. However, antibody-mediated neutralization of several viruses has been reported to predominantly occur by blocking viral attachment to cells and early entry function such as cell surface receptor recognition (reviewed in (28,29)). In line with these results, the binding of 22F10 to the N-terminal 222 amino acids of RD114 blocked the latter's ability to bind its cell surface receptor ASCT-2 and in turn leading to viral neutralization. Interestingly, the mAb was not able to bind viral envelopes belonging to the same interference group as RD114; BaEV, SRV-1, SRV-2 and MPMV which utilise the same cell surface receptor for target cell entry. Moreover, 22F10 mAb did not inhibit the infection mediated by these envelope glycoproteins. Therefore, the generated mAb appears to recognise an RD114-specific structure in its RBD and specifically neutralise viruses containing RD114 RBD in their envelope protein.

22F10 may serve as a valuable tool for the gene therapy field in the characterisation of packaging cell lines and viral vectors. Additionally, it could be of use for the field of canine and feline vaccines, where endogenous RD114 mobilization is frequently detected posing a theoretical hazard according to the EMA and a current hindrance in vaccine production (30). We have placed the V_H and V_L sequences into the public domain (GenBank, MH383329 - MH383330) with no restrictions on their use. Similarly, pFUSEss expression plasmids of 22F10-mIgG2A have been deposited with addgene.org (#110555 and #110556) for use by the vecterology / virology community.

MATERIALS AND METHODS

Cell culture and viruses

HEK-293T (ATCC, CRL-11268), WinPac-RD and FLYRD18 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Lonza; 12-726F) supplemented with 10% Foetal Calf Serum (FCS, Biosera, FB 1001/500) and 2mM GlutaMAX™ (Gibco; 35050061) at 37°C with 5% CO_2. TE671 cells stably infected with RD114, SRV-1, SRV-2 and MPMV, and TECeB6 cells (3,6,4) were cultured in Dulbecco's modified essential medium (DMEM) (Gibco; 11960069) supplemented with 10% FCS and 2mM GlutaMAX™ at 37°C with 5% CO_2. Cells were passaged 1:4 – 1:10 when cell density reached 75% - 85% confluence using trypsin-EDTA solution (Sigma; T4049).

Transient transfection

Single plasmid transient transfections were performed in multiwell TC-treated 6-well plates. HEK-293T cells were seeded the day prior to transfection at a density of 2.1 x 10^5 cell/cm^2. Genejuice® reagent (Merck Millipore; 70967) was used as transfection reagent and 2µg was transfected into cells according to manufacturer's protocol. Large-scale antibody expression was performed via transient transfection in CHO cell lines and purified via MabSelect SuRe protein A affinity chromatography by Evitria.

Fab fragment generation

8 mg of purified antibody was processed using Fab fragmentation kit (ThermoFisher Scientific; 44985). Antibody was dialysed in Fab digestion buffer using 5 ml Zeba spin column (MWCO 2 kDa) and incubated with 0.5 ml of papain immobilised on Agarose beads at 37°C for 8h with 250 rpm agitation. Digested antibody was recovered by gravity flow using a disposable 2 ml column. Beads were washed with 2 ml PBS to recover remaining digested antibody. Fab fragment was purified from Fc and undigested antibody by affinity chromatography using protein A MabSelect SuRe column on Akta pure system. Antibody mixture was loaded on the column and flow-through containing the Fab fragment was collected following in-line desalting in PBS using two 5 ml HiTrap desalting columns. Protein A captured Fc and undigested antibodies were recovered by elution using 0.1M glycine pH 2.8 and buffer exchanged in PBS using in-line HiTrap desalting columns.

Flow cytometric analysis of cells and reagents

Flow cytometry was performed using BD LSRFORTESSA X-20 cell analyser. Harvested cells were washed once with PBS followed by primary and secondary staining, each for 30 minutes at room temperature with PBS washes in between. A fixable viability dye (eBioscience™ fixable viability Dye eFluor™ 780 (1:1000; Invitrogen, 65-0865-14) was included in the secondary staining step and used for the acquisition of 10,000 events live cells in all the flow cytometry assays performed.

The following reagents and their respective dilutions were used: pre- and post-vaccination rat sera (1:1000); purified 22F10-mIgG2A (1:100); PE-conjugated α-rat IgG (1:100; Biolegend,

405406); FITC-conjugated α-mouse IgGFc specific (1:100; JIR, 115-095-071); AF488-conjugated α-mouse IgG (H+L) (1:100; Invitrogen A11029); PE-conjugated α-mouse IgG (1:100; Sigma, P9670) and eBioscience™ fixable viability Dye eFluor™ 780 (1:1000; Invitrogen, 65-0865-14).

Indirect enzyme linked immunosorbent assay (ELISA)

The antigen ASCT2-RBD was coated at 0.2 μg/mL in 100 μL PBS per well and incubated overnight at 4°C followed by a single wash with 0.05% PBS-Tween (washing buffer). Next, non-specific binding was blocked by adding 100 μL of 0.05% PBS-Tween with 1% w/w BSA (blocking buffer) was added for 1hr at 37°C followed by a single wash with washing buffer. Then, 100 μL of both non-diluted hybridoma supernatant and post-immunization serum diluted 1:1000 in blocking buffer, were added into respective wells and incubated for 1hr at 37°C. Wells were then washed four times with washing buffer, followed by the incubation of 100 μL of secondary antibody HRP-conjugated goat α-rat IgG (JIR; 112-035-071) diluted 1:1000 in blocking buffer for 30 minutes at 37°C. Subsequently, wells were washed four time in washing buffer and then 100 μL of TMB reagent (Genscript; M00078) was added and wells were incubated at room temperature for 15-20 minutes. Reactions were then terminated by the addition of 100 μL stop solution containing 8.3% v/v 12 M HCL. The absorbance was read immediately at 450nm using an absorbance microplate reader.

5' Rapid amplification of cDNA ends (5' RACE)

Total RNA free of genomic DNA was extracted from 1 – 3 × 10^6 hybridoma cells using illustra RNAspin Mini Kit (GE Healthcare; 25050071). Template-switched cDNA was synthesized using 1 – 2 μg total RNA as the template in a reaction containing 2.5 μM of an equal molar mix of Oligo$(dT)_{19}$A, Oligo$(dT)_{19}$G and Oligo$(dT)_{19}$C, 1 μM of template switch oligo [5'-AAGCAGTGGTATCAACGCAGAGTACGC(rGrGrG)-3'], and 200 unit of Superscript II reverse transcriptase (ThermoFisher; 18064014) following the manufacturer's instructions. Rat IgG heavy chain transcript was amplified using 1 μl template-switched cDNA as the template, a mix of universal forward primers [5'-CGACGTGGACTATCCATGAACGCAAAGCAGTGGTATCAACGCAGAGT-3' (final concentration 0.06 μM) and 5'-CGACGTGGACTATCCATGAACGCA-3' (final concentration 0.3 μM)], Rat IgG heavy chain-specific reverse primer (5'-ccagactgcaggacagctgg-3', final concentration 0.3 μM) and KOD Hot start DNA polymerase (Merck; 71086) following the manufacturer's instructions. Rat IgG kappa chain transcript was amplified in the same fashion as the heavy chain but with kappa specific reverse primer (5'-cttggtcaacgagagggtgctg-3'). Specific PCR products were cloned into pJet1.2 vector using CloneJET PCR cloning kit (ThermoFisher; K1231) for further sequencing analysis.

Surface plasmon resonance (SPR)

Recombinant ASCT2-RBD-mIgG1 Fc protein (Metafora) was covalently captured on a Series S CM5 chip using Amine capture method in 10 mM acetate buffer pH 5.5 on a Biacore T200 instrument. Immobilisation was achieved by 10 μl/minute injections of 6.8 μg/mL of ASCT2-RBD-mIgG1 for 400 seconds. Various concentrations of Fab antibody fragment were

then injected over the flow chambers in HBS-P^+ buffer at 30 µl/minute in a Biacore T200 instrument, starting at 1 µM with 2-fold serial dilutions to 3.91 nM, for a total of 150s of contact time and 500s dissociation time. Regeneration buffer 10 mM Glycine pH 1.5 was selected as the optimal regeneration buffer as it showed unaltered capture capacity and low baseline levels following repeated cycles. Response Unit (RU) determination was performed following double reference subtraction with buffer. Kinetic rates were calculated using BIAevaluation v3.0 (GE Healthcare) by curve fitting according to a 1:1 Langmuir binding model.

Viral vectors production

Viral vectors productions were achieved by triple transiently transfecting HEK-293Ts in 100mm plates using GeneJuice (Merck; 70967) with a total of 12.5 µg of DNA. γ-retroviral vectors were produced by triple transient transfection of 4.69 µg Peq-Pam plasmid (encoding Moloney GagPol), 3.13 µg of RDF plasmid (encoding RD114 envelope) and 4.69 µg retroviral backbone SFG (31) expressing gene of interest. Similarly, γ-retroviral vector carrying BaEV Env was produced by triple transfection using FuGene®-6 (Promega; E2691) transfection reagent with 1 µg pCMV (encoding MLV *gagpol* (6)), 1.5 µg retroviral vector pCNCG expressing GFP (32) and 1 µg of BaEVTR plasmid for BaEV envelope expression (a kind gift from F-L Cosset and Els Verhoeyen). Supernatants were collected 48h and 72h post-transfection. Lentiviral vectors were generated by GeneJuice transfection of cells with 5.42 µg of pCMV-dR8.74 (encoding lentiviral GagPol), 2.92 µg of envelope plasmid (pMD2.G, or RDpro (14) expressing plasmid; both kind gifts from Dr. Yasu Takeuchi) and 4.17 µg of lentiviral backbone pCCL encoding eGFP as transgene. Supernatants were collected 48h after transfection and processed by centrifugation at 1000g for 10 mins at 4°C to remove cellular debris followed by microfiltration using Millex-HV 0.45 µm syringe filter units (Merck; SLHV033RB). Viral supernatants were either kept on ice for 2hrs for further use or frozen down at -80°C for storage.

For the D-type retroviruses, TECeB6 cells stably expressing eGFP vector pCNCG were generated by transduction with a VSV-G RV produced by transient transfection of 293T cells as described above. TECeB6 GFP cells were then infected with viral stocks of SRV-1, SRV-2, MPMV and RD114. Cells were cultured for 6 weeks and titres were monitored by eGFP expression via flow cytometry analysis. Viral vectors containing supernatant were harvested and filtered using Minisart® NML 0.45 µm filters and kept at 4°C before using them in the neutralisation assay.

Determination of lentiviral vector titer

Functional infectious viral titres were determined by flow cytometry analysis (using BD LSRFORTESSA X-20 cell analyser) of transgene expression in transduced HEK-293T cells at different dilutions. Experiments were performed in 24-well plates (50,000 cells per well). Serially diluted viral supernatants were added onto seeded cells in the presence of 8 µg/mL polybrene. Transduction efficiencies were determined 72hrs later using BD LSRFORTESSA X-20 cell analyser and eGFP expression between 0.5% - 20% were used in the following equation to determine viral titer:

$$\text{Titer}\left(\text{infectious units}\frac{\text{IU}}{\text{mL}}\right) = \left(\frac{\left(\frac{\%\ \text{transduction efficiency}}{100}\right) \text{x no.of cell at transduction}}{\text{vector volume}}\right) \text{x dilution factor}$$

Antibody neutralization assay

22F10-mIgG2A was serially diluted in plain OptiMEM to 13 decreasing concentrations ranging from 200 μg/mL to 91.81 fg/mL (6-fold serial dilution). Each antibody dilution was then mixed 1:1 with RD114-RV, RDpro-LV and VSV-G-LV at 1.0 $x10^5$ IU/mL titer to a final volume of 200 μL, incubated at 37°C for 1hr. Antibody:virus mixtures were then cultured with HEK-293T cells at MOI 0.2 in the presence of 8 μg/mL of polybrene for 48hrs. Similarly, RD114-MLV (3.63±1.3 10^5 IU/mL), SRV-1-MLV (7.36±1.35 $x10^3$ IU/mL), SRV-2 MLV (2.28±0.94 $x10^4$ IU/mL), MPMV-RV (8.48±1.59 $x10^2$ IU/mL) and BaEV-MLV (3.51±0.19 $x10^2$ IU/mL) viral supernatants were incubated with 5-fold serially diluted 22F10-mIgG2A with an initial concentration of 8 μg/mL at 37°C for 1hr. Mixtures were then added to HEK-293T cells in the presence of 8 μg/mL of polybrene for 72hrs. SRV-1-MLV and MPM-MLV were also spin-inoculated for 2 hrs at 2500rpm. Viral titers were then quantified by eGFP expression in target cells and infectivity of all fractions was determined as a percentage of viral titers in the absence of the mAb.

Fluorescence-based receptor blocking assay

22F10 Fab was reconstituted at 1.05 μM to a final concentration of 513 pM by a 2-fold serial dilution. Decreased concentrations of 22F10 Fab were then incubated with recombinant ASCT2-RBD-mIgG1 (Metafora) at a constant concentration of 204 nM for 30 minutes at 37°C. Mixtures were then added to HEK-293T cells and incubated for 30 minutes at 37°C. After a PBS wash, cells were stained with FITC-conjugated α-mouse Fc specific for 30 minutes at room temperature. Subsequently, cells were washed to remove any unbound antibody and analysed by flow cytometry.

FIGURE LEGENDS

Figure 1: Rat seroconversion and binding of purified mAb αRD114. (a) HEK-293T cells transiently transfected with RDpro (293T RDpro) were stained with pre- and post-immunisation serums 48hrs post-transfection, and non-transfected (293T) cells are used as negative control. PE-conjugated α-rat IgG was used as secondary antibody (2°). Data presented as overlapping histograms.

Figure 2: Screening of monoclonal hybridomas for productive clone. Antigen binding of hybridoma supernatants was assessed first by **(a)** indirect ELISA; non-transfected cells supernatant and post-immunisation serum (diluted 1:1000) used as negative and positive controls, respectively. **(b)** Supernatants of hybridoma clones, along with positive control post-vaccination serum, were then used to stain HEK-293T cells transfected with RDpro (293T RDpro) 48hrs post-transfection and non-transfected (293T) cells as negative control. PE-conjugated α-rat IgG was used as secondary antibody (2°). Data presented as overlapping histograms.

Figure 3: Sequence and binding analysis of 22F10. (a) Protein sequence analysis of heavy (V_H) and light (V_L) variable chains using the abYsis software. First row of the tables represents the residues of both sequences with the second row indicating residues' numbers and the third row highlighting the CDR regions in red and framework regions in green. Annotations also include symbols indicating sites of potential post-translational modification such as N-linked glycosylation and phosphorylation sites, as well as unusual residues which occur in less than 1% of sequences. **(b)** Non-transfected HEK-293T (grey population) and RDpro transiently transfected HEK-293T (red population) cells were stained with either full-length 22F10-mIgG2A mAb (Purified) or post-vaccination α-RD114-rIgG sera (Serum). PE-conjugated to α-mouse IgG and α-rat IgG were used as secondary antibodies (2°). Data presented as overlapping histograms.

Figure 4: Kinetics analysis of 22F10. BIAcore sensogram of 22F10 Fab fragment binding to ASCT2-RBD-mIgG1 immobilised on a CM5 chip. Binding measurements were performed using serial dilutions of the Fab fragment from 1 μM to 3.91 nM and ligand interactions for each dilution are measured by an increase in the refractive index which is presented as response units (RU) over time (s). Kinetic rates were calculated using BIAevaluation v3.0 by curve fitting according to a 1:1 Langmuir binding model.

Figure 5: Antigen detection and neutralization of RD-pseudotyped viral vectors. (a) Flow cytometric analysis of stable viral producing cells WinPac-RD and FLYRD18 for envelope glycoprotein surface expression by 22F10-mIgG2A staining. FITC-conjugated α-mouse IgG was used as secondary antibody (red population). Background binding controls were included in which cells were stained with the secondary antibody only (blue population), along with unstained controls (grey population). Data presented as overlapping

histograms **(b)** RD114-RV, RDpro-LV and VSV-G-LV infectivity post-incubation with decreasing concentrations of 22F10-mIgG2A. Infectivity of the pseudotyped vectors was calculated by GFP expression in target cells and data presented as mean percentage of infectivity relative to the no antibody control ± standard deviation of three independent experiments. The curves were fitted by nonlinear regression analysis using the GraphPad Prism 8 modelled as an [inhibitor] vs. normalised response curve with IC_{50} values calculated.

Figure 6: Specificity of 22F10 mAb. (a) RD114, SRV-1, SRV-2 and MPMV infected TECeB6 cells expressing GFP and transiently transfected 293T cells producing BaEV-RV, were stained with 22F10-mIgG2A followed by secondary α-mouse IgG (2°) conjugated to phycoerythrin (red population). Background binding controls were included by staining cells with secondary only (grey population). Data presented as overlapping histograms. **(b)** MLV vectors pseudotyped with RD114, SRV-1, SRV-2, MPMV and BaEV were incubated with decreasing concentration of 22F10-mIgG2A. Infectivity of the pseudotyped RV was calculated by GFP expression in target cells and presented as mean percentage of infectivity relative to the no antibody control ± standard deviation of two independent experiments. The curves were fitted by nonlinear regression analysis using the GraphPad Prism 8 modelled as an [inhibitor] vs. normalised response curve with IC_{50} value calculated.

Figure 7: Binding interference of 22F10 to RD114's cell surface receptor. (a) Non-transfected HEK-293T (grey population) and RDpro transiently transfected HEK-293T (red population) cells were stained with either 22F10-mIgG2A (Full Length) or the Fab fragment (Fab) followed by either AF488-conjugated α-mouse IgG (H+L) or FITC-conjugated α-mouse IgG Fc specific secondary antibodies (2°). Data presented as overlapping histograms. **(b)** ASCT2-RBD binding to ASCT-2 in the presence of increasing Fab fragment. Binding curve representing the Median Fluorescence Intensity (MedFI) of ASCT2-RBD ± standard deviation of three independent experiments and was fitted by nonlinear regression analysis using the GraphPad Prism 8 modelled as an [inhibitor] vs. response curve with IC_{50} value calculated.

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CONFLICT OF INTEREST

We have no conflict of interest to declare.

REFERENCES

1. Blumenthal R, Durell S, Viard M. HIV Entry and Envelope Glycoprotein-mediated Fusion. J Biol Chem. 2012 Nov 30;287(49):40841–9.

2. Green N, Shinnick TM, Witte O, Ponticelli A, Sutcliffe JG, Lerner RA. Sequence-specific antibodies show that maturation of Moloney leukemia virus envelope polyprotein involves removal of a COOH-terminal peptide. Proc Natl Acad Sci. 1981 Oct 1;78(10):6023–7.

3. Sommerfelt MA, Weiss RA. Receptor interference groups of 20 retroviruses plating on human cells. Virology. 1990 May;176(1):58–69.

4. Tailor CS, Nouri A, Zhao Y, Takeuchi Y, Kabat D. A Sodium-Dependent Neutral-Amino-Acid Transporter Mediates Infections of Feline and Baboon Endogenous Retroviruses and Simian Type D Retroviruses. J Virol. 1999 May;73(5):4470–4.

5. Takeuchi Y, Cosset FL, Lachmann PJ, Okada H, Weiss RA, Collins MK. Type C retrovirus inactivation by human complement is determined by both the viral genome and the producer cell. J Virol. 1994 Dec;68(12):8001–7.

6. Cosset FL, Takeuchi Y, Battini JL, Weiss RA, Collins MK. High-titer packaging cells producing recombinant retroviruses resistant to human serum. J Virol. 1995 Dec;69(12):7430–6.

7. Bell AJ, Fegen D, Ward M, Bank A. RD114 envelope proteins provide an effective and versatile approach to pseudotype lentiviral vectors. Exp Biol Med. 2010 Oct 1;235(10):1269–76.

8. Rasko JE, Battini JL, Gottschalk RJ, Mazo I, Miller AD. The RD114/simian type D retrovirus receptor is a neutral amino acid transporter. Proc Natl Acad Sci U S A. 1999 Mar 2;96(5):2129–34.

9. Kelly PF, Vandergriff J, Nathwani A, Nienhuis AW, Vanin EF. Highly efficient gene transfer into cord blood nonobese diabetic/severe combined immunodeficiency repopulating cells by oncoretroviral vector particles pseudotyped with the feline endogenous retrovirus (RD114) envelope protein. Blood. 2000 Aug 15;96(4):1206–14.

10. Hanawa H, Kelly PF, Nathwani AC, Persons DA, Vandergriff JA, Hargrove P, et al. Comparison of various envelope proteins for their ability to pseudotype lentiviral vectors and transduce primitive hematopoietic cells from human blood. Mol Ther J Am Soc Gene Ther. 2002 Mar;5(3):242–51.

11. Goerner M, Horn PA, Peterson L, Kurre P, Storb R, Rasko JE, et al. Sustained multilineage gene persistence and expression in dogs transplanted with CD34(+) marrow cells transduced by RD114-pseudotype oncoretrovirus vectors. Blood. 2001 Oct 1;98(7):2065–70.

12. Green BJ, Lee CS, Rasko JEJ. Biodistribution of the RD114/mammalian type D retrovirus receptor, RDR. J Gene Med. 2004 Mar;6(3):249–59.

13. Sandrin V. Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary

lymphocytes and CD34+ cells derived from human and nonhuman primates. Blood. 2002 Jul 18;100(3):823–32.

14. Ikeda Y, Takeuchi Y, Martin F, Cosset F-L, Mitrophanous K, Collins M. Continuous high-titer HIV-1 vector production. Nat Biotechnol. 2003 May;21(5):569–72.

15. Ward M, Sattler R, Grossman IR, Bell AJ, Skerrett D, Baxi L, et al. A stable murine-based RD114 retroviral packaging line efficiently transduces human hematopoietic cells. Mol Ther J Am Soc Gene Ther. 2003 Nov;8(5):804–12.

16. Relander T, Johansson M, Olsson K, Ikeda Y, Takeuchi Y, Collins M, et al. Gene Transfer to Repopulating Human CD34+ Cells Using Amphotropic-, GALV-, or RD114-Pseudotyped HIV-1-Based Vectors from Stable Producer Cells. Mol Ther. 2005 Mar;11(3):452–9.

17. Stornaiuolo A, Piovani BM, Bossi S, Zucchelli E, Corna S, Salvatori F, et al. RD2-MolPack-Chim3, a Packaging Cell Line for Stable Production of Lentiviral Vectors for Anti-HIV Gene Therapy. Hum Gene Ther Methods. 2013 Jun 17;24(4):228–40.

18. Sanber KS, Knight SB, Stephen SL, Bailey R, Escors D, Minshull J, et al. Construction of stable packaging cell lines for clinical lentiviral vector production. Sci Rep [Internet]. 2015 Mar 12 [cited 2015 Jun 3];5. Available from: http://www.nature.com/srep/2015/150312/srep09021/full/srep09021.html

19. Marin V, Stornaiuolo A, Piovan C, Corna S, Bossi S, Pema M, et al. RD-MolPack technology for the constitutive production of self-inactivating lentiviral vectors pseudotyped with the nontoxic RD114-TR envelope. Mol Ther Methods Clin Dev. 2016 May 11;3:16033.

20. Okabe H, Gilden RV, Hatanaka M. RD 114 virus-specific sequences in feline cellular RNA: detection and characterization. J Virol. 1973 Nov;12(5):984–94.

21. Shimode S, Nakaoka R, Shogen H, Miyazawa T. Characterization of feline ASCT1 and ASCT2 as RD-114 virus receptor. J Gen Virol. 2013 Jul;94(Pt 7):1608–12.

22. Okada M, Yoshikawa R, Shojima T, Baba K, Miyazawa T. Susceptibility and production of a feline endogenous retrovirus (RD-114 virus) in various feline cell lines. Virus Res. 2011 Jan;155(1):268–73.

23. Yoshikawa R, Shimode S, Sakaguchi S, Miyazawa T. Contamination of live attenuated vaccines with an infectious feline endogenous retrovirus (RD-114 virus). Arch Virol. 2014 Mar 1;159(3):399–404.

24. Narushima R, Horiuchi N, Usui T, Ogawa T, Takahashi T, Shimazaki T. Experimental infection of dogs with a feline endogenous retrovirus RD-114. Acta Vet Scand. 2011 Jan 27;53(1):3.

25. Mandel MP, Stephenson JR, Hardy WD, Essex M. Endogenous RD-114 virus of cats: absence of antibodies to RD-114 envelope antigens in cats naturally exposed to feline leukemia virus. Infect Immun. 1979 Apr;24(1):282–5.

26. Shimode S, Nakagawa S, Miyazawa T. Multiple invasions of an infectious retrovirus in cat genomes. Sci Rep. 2015 Feb 2;5:8164.

27. Ward M, Sattler R, Grossman IR, Bell AJ, Skerrett D, Baxi L, et al. A stable murine-based RD114 retroviral packaging line efficiently transduces human hematopoietic cells. Mol Ther J Am Soc Gene Ther. 2003 Nov;8(5):804–12.

28. Klasse PJ, Sattentau QJ. Occupancy and mechanism in antibody-mediated neutralization of animal viruses. J Gen Virol. 2002 Sep;83(Pt 9):2091–108.

29. Pantophlet R, Burton DR. GP120: target for neutralizing HIV-1 antibodies. Annu Rev Immunol. 2006;24:739–69.

30. Miyazawa T, Yoshikawa R, Golder M, Okada M, Stewart H, Palmarini M. Isolation of an Infectious Endogenous Retrovirus in a Proportion of Live Attenuated Vaccines for Pets. J Virol. 2010 Apr;84(7):3690–4.

31. Büeler H, Mulligan RC. Induction of antigen-specific tumor immunity by genetic and cellular vaccines against MAGE: enhanced tumor protection by coexpression of granulocyte-macrophage colony-stimulating factor and B7-1. Mol Med Camb Mass. 1996 Sep;2(5):545–55.

32. Towers G, Bock M, Martin S, Takeuchi Y, Stoye JP, Danos O. A conserved mechanism of retrovirus restriction in mammals. Proc Natl Acad Sci. 2000 Oct 24;97(22):12295–9.

Fig 1

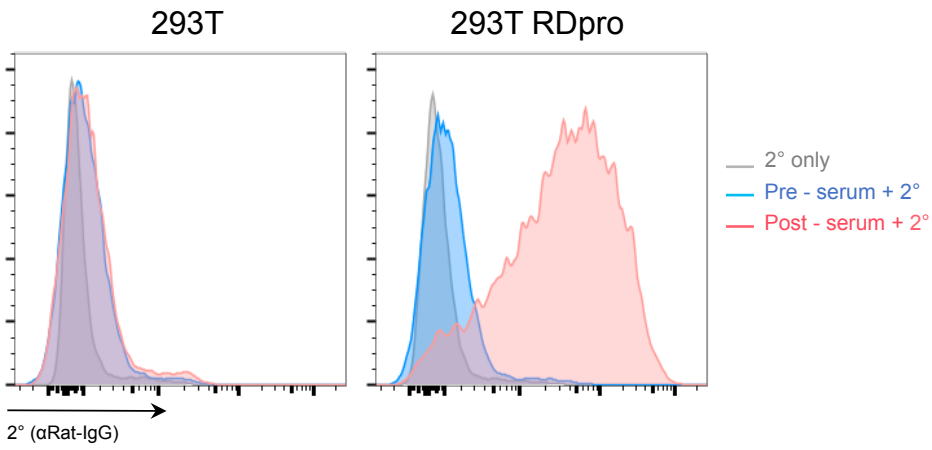


Fig 2

a

Sample supernatant		Abs (450nm)
Negative control		0.151
Positive control		1.145
Hybridoma	22F10	1.724
	30F3	1.714

b

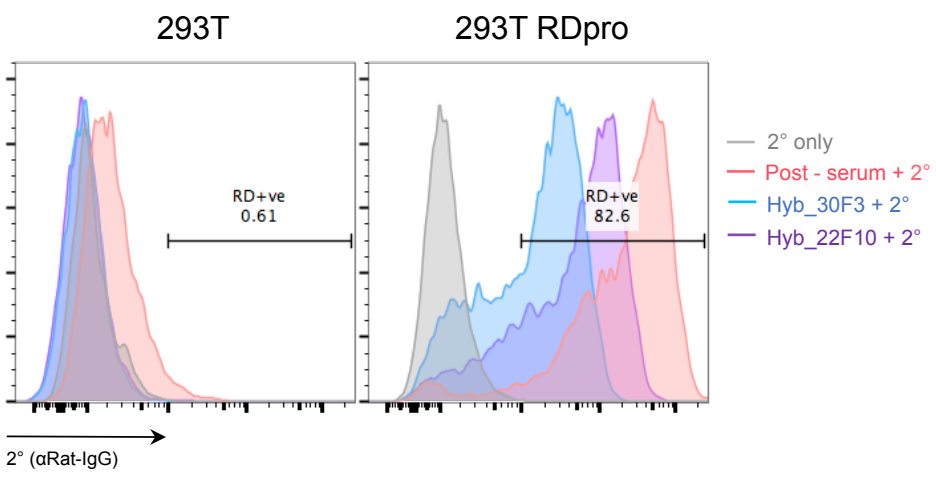
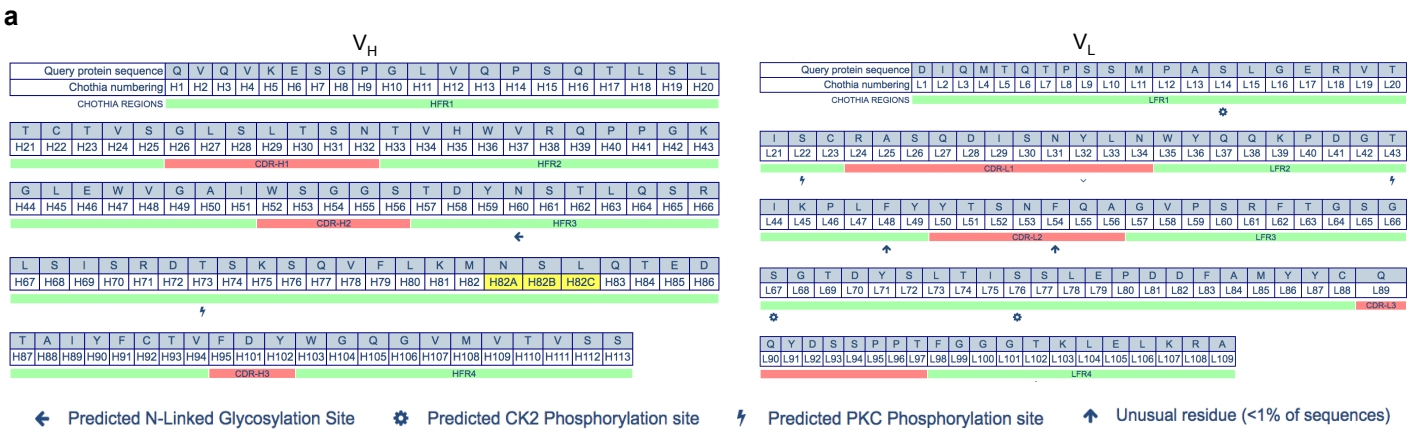


Fig 3


a
V_H
Query protein sequence
Chothia numbering
CHOTHIA REGIONS
HFR1
CDR-H1
HFR2
CDR-H2
HFR3
CDR-H3
HFR4
V_L
CDR-L1
LFR2
CDR-L2
LFR3
CDR-L3
LFR4
Predicted N-Linked Glycosylation Site
Predicted CK2 Phosphorylation site
Predicted PKC Phosphorylation site
Unusual residue (<1% of sequences)

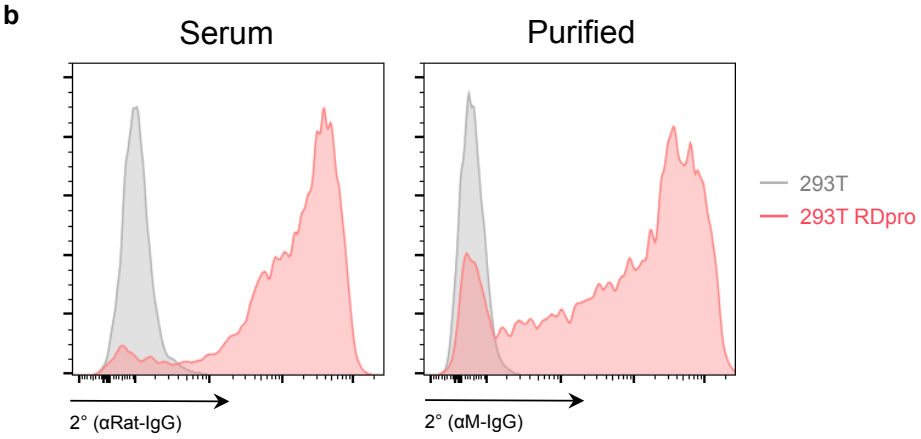

b
Serum
Purified
293T
293T RDpro
2° (αRat-IgG)
2° (αM-IgG)

Fig 4

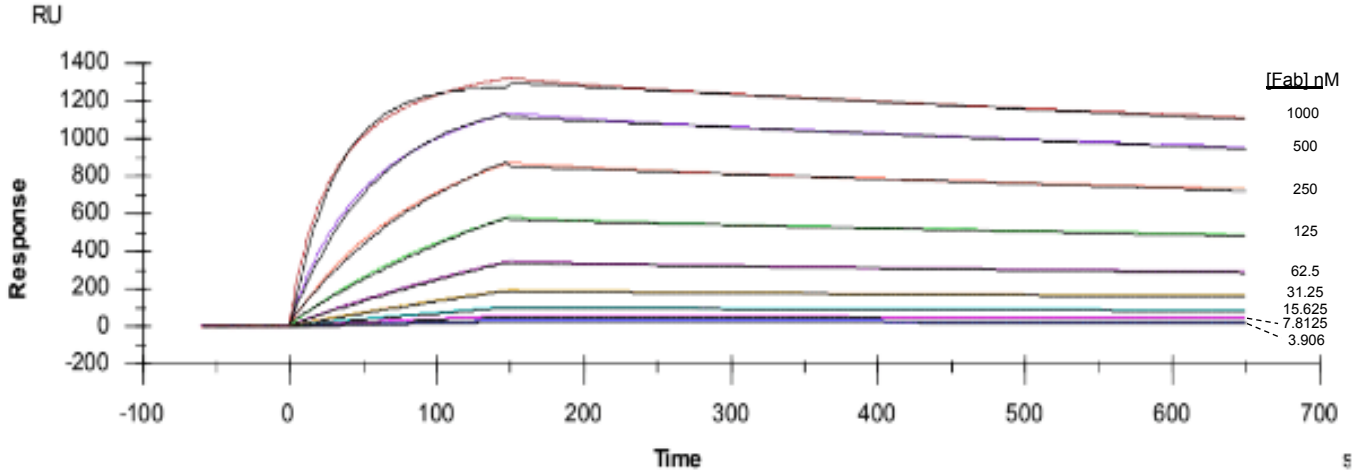

RU
1400
1200
1000
800
600
400
200
0
-200
Response
-100
0
100
200
300
400
500
600
700
Time
[Fab] nM
1000
500
250
125
62.5
31.25
15.625
7.8125
3.906

Fig 5

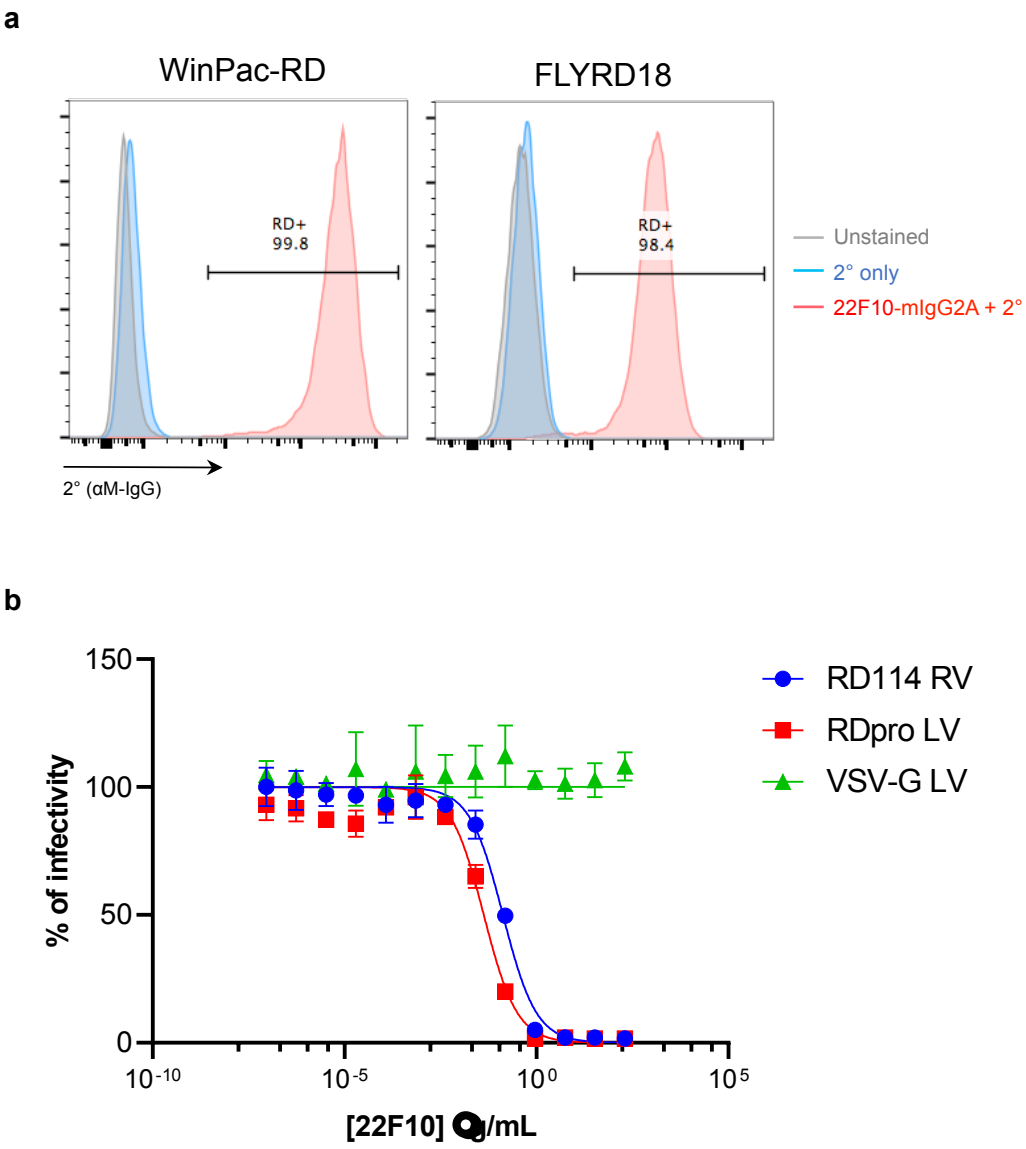

a
WinPac-RD
FLYRD18
RD+
99.8
RD+
98.4
Unstained
2° only
22F10-mIgG2A + 2°
2° (αM-IgG)
b
150
100
50
0
% of infectivity
10-10
10-5
100
105
[22F10] µg/mL
RD114 RV
RDpro LV
VSV-G LV

Fig 6

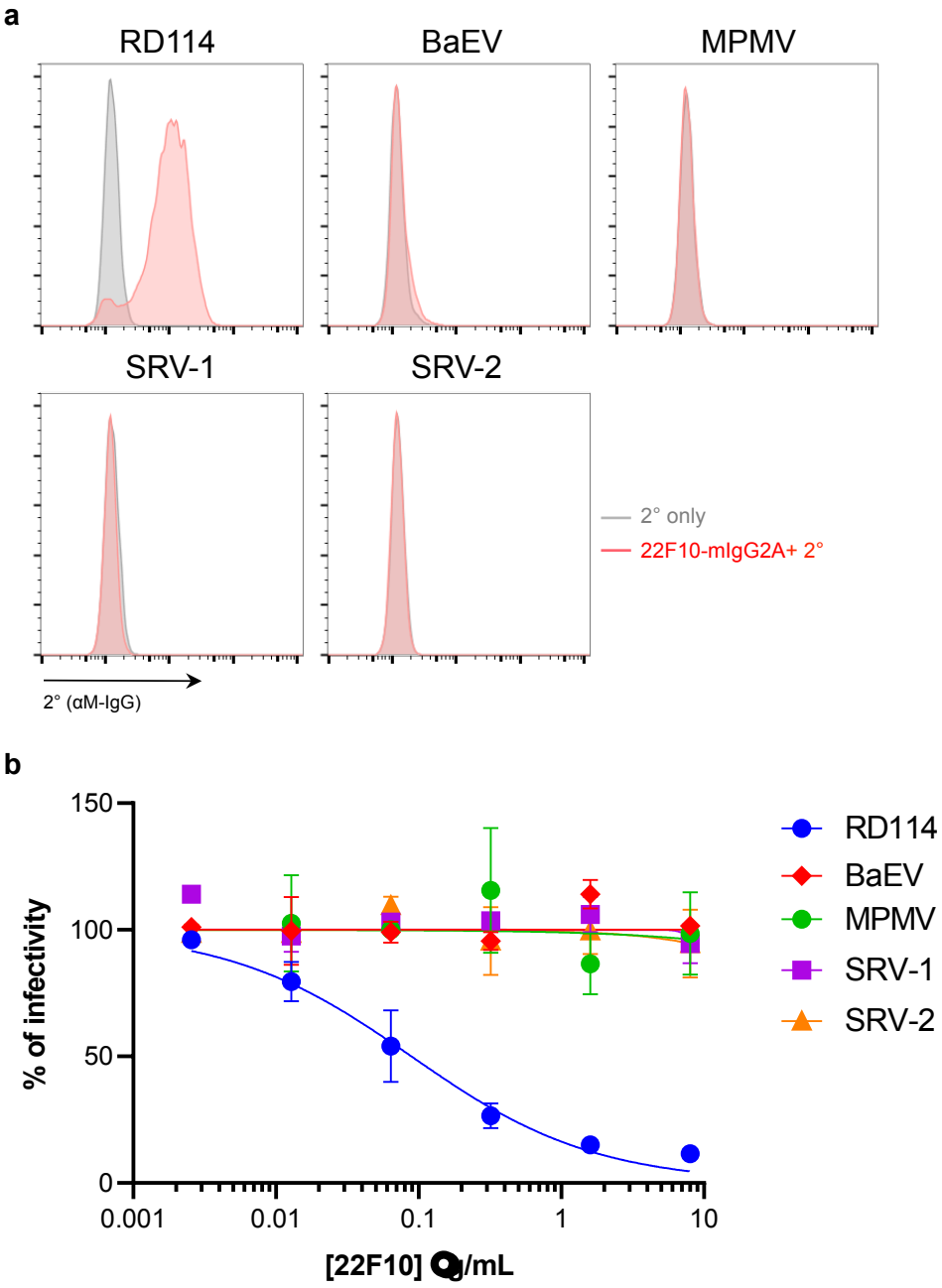

a
RD114
BaEV
MPMV
SRV-1
SRV-2
2° only
22F10-mIgG2A+ 2°
2° (αM-IgG)
b
150
100
50
0
% of infectivity
0.001
0.01
0.1
1
10
[22F10] µg/mL
RD114
BaEV
MPMV
SRV-1
SRV-2

Fig 7

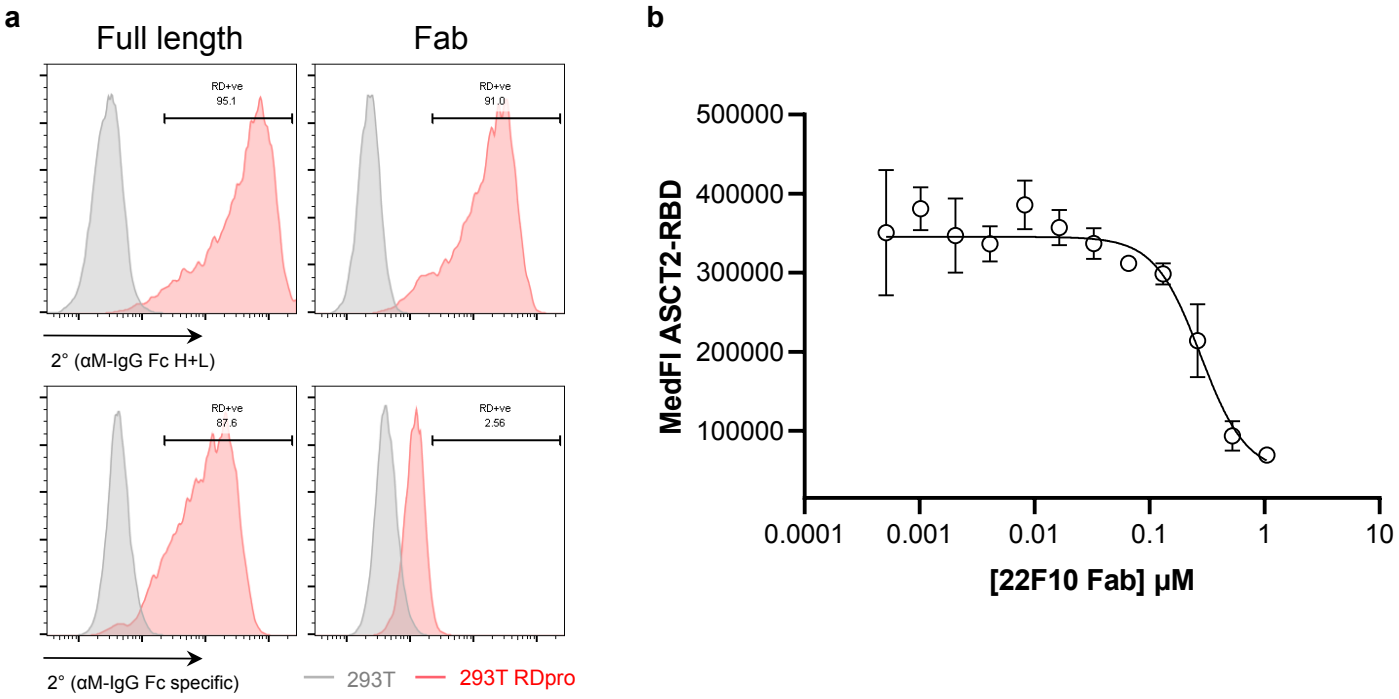
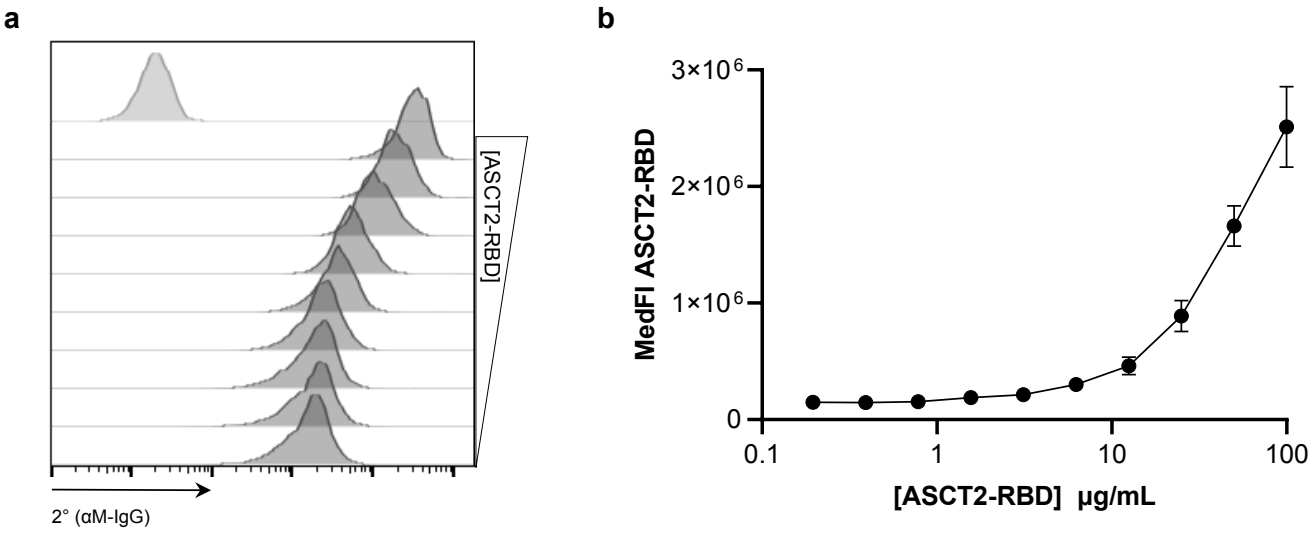

a
Full length
Fab
RD+ve
95.1
RD+ve
91.0
2° (αM-IgG Fc H+L)
RD+ve
87.6
RD+ve
2.56
2° (αM-IgG Fc specific)
293T
293T RDpro
b
MedFI ASCT2-RBD
500000
400000
300000
200000
100000
0.0001
0.001
0.01
0.1
1
10
[22F10 Fab] μM

Fig S1


a
[ASCT2-RBD]
2° (αM-IgG)
b
3×10^6
2×10^6
1×10^6
0
MedFI ASCT2-RBD
0.1
1
10
100
[ASCT2-RBD] µg/mL