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Selection, identification, and characterization of SARS-CoV-2 monoclonal antibody resistant mutants

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ABSTRACT

The use of monoclonal neutralizing antibodies (mNABs) is being actively pursued as a viable intervention for the treatment of Severe Acute Respiratory Syndrome CoV-2 (SARS-CoV-2) infection and associated coronavirus disease 2019 (COVID-19). While highly potent mNABs have great therapeutic potential, the ability of the virus to mutate and escape recognition and neutralization of mNABs represents a potential problem in their use for the therapeutic management of SARS-CoV-2. Studies investigating natural or mNAB-induced antigenic variability in the receptor binding domain (RBD) of SARS-CoV-2 Spike (S) glycoprotein, and their effects on viral fitness are still rudimentary. In this manuscript we described experimental approaches for the selection, identification, and characterization of SARS-CoV-2 monoclonal antibody resistant mutants (MARMs) in cultured cells. The ability to study SARS-CoV-2 antigenic drift under selective immune pressure by mNABs is important for the optimal implementation of mNABs for the therapeutic management of COVID-19. This will help to identify essential amino acid residues in the viral S glycoprotein required for mNAB-mediated inhibition of viral infection, to predict potential natural drift variants that could emerge upon implementation of therapeutic mNABs, as well as vaccine prophylactic treatments for SARS-CoV-2 infection. Additionally, it will also enable the assessment of MARM viral fitness and its potential to induce severe infection and associated COVID-19 disease.

1. Introduction

The rapid emergence of a novel betacoronavirus, which was later named Severe Acute Respiratory Syndrome CoV-2 (SARS-CoV-2), in December 2019 has led to a major public health threat needing an immediate therapeutic breakthrough. While the process involved in vaccine development might take more than a year before becoming commercially available, the use of monoclonal neutralizing antibodies (mNABs) or plasma from recovered patients become an important part of the conversation to quickly control coronavirus disease 2019 (COVID-19) and COVID-19-associated illnesses. Neutralizing antibodies (NABs) are essential part of the host adaptive immune response that are induced against viral infections following natural infection or administration of vaccine (Plotkin, 2010). A prerequisite for a successful SARS-CoV-2 infection is the interaction between the heterotrimeric Spike (S)

glycoprotein and its functional receptor, the angiotensin converting enzyme 2 (ACE2), found on susceptible host cells (Zhou et al., 2020). The receptor-binding domain (RBD) of SARS-CoV-2 S protein is important for this entry process, and has been identified as a dominant target of many of the currently identified SARS-CoV-2 mNABs (Robbani et al., 2020; Brouwer et al., 2020; Cao et al., 2020; Chen et al., 2020; Chi et al., 2020; Rogers et al., 2020; Ju et al., 2020; Shi et al., 2020; Zost et al., 2020; Wec et al., 2020; Seydoux et al., 2020; Liu et al., 2020; Kreer et al., 2020; Hansen et al., 2020; Weisblum et al., 2020; Baum et al., 2020). During viral entry, SARS-CoV-2 S protein is cleaved at the S1/S2 cleavage site (Fig. 1A) by host cell proteases into S1, containing the RBD, and the S2 subunit, which is responsible for the conformational change that induces the fusion of viral and host cell membranes (Lau et al., 2020). While the receptor binding motif, a sequence within the RBD, dictates the binding activity of SARS-CoV-2 S and also contributes to

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infectivity (Wan et al., 2020), mutations within key residues of the SARS-CoV-2 S which facilitates S1/S2 cleavage may also enhance infectivity (Lau et al., 2020).

The main target of mNABs against SARS-CoV-2 are the epitopes on the S (Zost et al., 2020). While some SARS-CoV-2-induced Abs can preferentially bind to either the S1 or S2 subunit of the S protein, others can bind, with a high affinity, to the RBD located within the S1 subunit (Wang et al., 2020; Wu et al., 2020). A major draw-back for the development of potent antivirals or immunotherapies against RNA viruses is the rapid development of resistant mutants (Baum et al., 2020; Phan, 2020). Being an RNA virus, SARS-CoV-2 is under constant pressure to mutate and evade neutralization of mNABs (Baum et al., 2020; Phan, 2020). It has been reported that a significant proportion of COVID-19 patients induced a low level of NABs (Robbiani et al., 2020) which might have resulted from the inability of natural infection to induce robust B-cell expansion and affinity maturation in such individuals (Robbiani et al., 2020; Weisblum et al., 2020; Luchsinger et al., 2020). Constant exposure of SARS-CoV-2 to suboptimal levels of NABs in infected individuals can promote the selection of monoclonal antibody resistant mutant (MARM) SARS-CoV-2 in COVID-19 patients posing a

great threat to public health. Moreover, the use of mNABs for the therapeutic control of SARS-CoV-2 infection in COVID-19 infected individuals could result in the selection of MARMs (Weisblum et al., 2020). Here we describe in detail molecular approaches that can be employed to select and identify MARMs using an *in vitro* model (Vero E6 cells) of SARS-CoV-2 infection. A comprehensive and detailed characterization of the impact of SARS-CoV-2 S protein mutations on mNAB recognition would increase our current understanding of SARS-CoV-2 evolution and determine the fitness of the resistant mutant, which together can be exploited when designing mNAB-based therapies and vaccines for an efficient control of SARS-CoV-2 infection.

2. Materials

2.1. Supplies and reagents

- 1 Vero E6 cells (American Type Culture Collection, ATCC; catalog # CRL-1586).
- 2 SARS-CoV-2 isolate USA-WA1/2020 (BEI Resources, catalog # NR-52281).

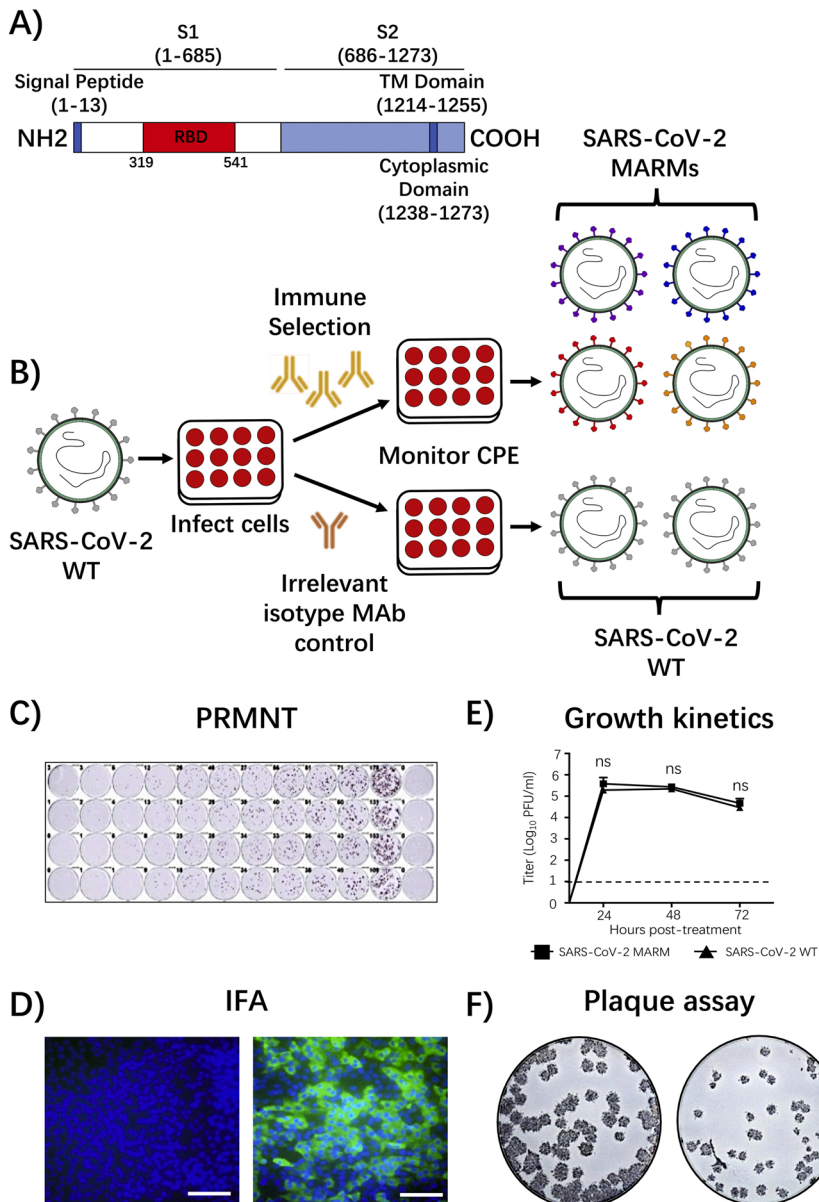


Fig. 1. Schematic representation of SARS-CoV-2 S and experimental approach for selecting and identifying MARM. A) Schematic representation of the SARS-CoV-2 S: TM, transmembrane domain; S1, Spike domain 1; S2, Spike domain 2; RBD, receptor-binding domain. B) Schematic representation for selecting and identifying SARS-CoV-2 MARM: Confluent monolayer of Vero E6 cells (12-well cell-culture plates, 5×10^5 cells/well) are infected (MOI 0.01) with SARS-CoV-2. After viral adsorption, infected cells are incubated with serial dilutions of the mNAB during multiple rounds of replication for selection of MARMs. SARS-CoV-2 MARMs are next characterized by C) plaque reduction micro-neutralization (PRMNT) assay, D) immunofluorescence assay (IFA), E) virus growth kinetics, and F) plaque phenotype. Deep sequencing of the viral genome (not shown) will allow the identification of amino acid residues involved in the lack of neutralization of the MARMs by the used mNABs.

- 3 Dulbecco's modified Eagle medium, DMEM (Corning, catalog # 15-013-CV).
- 4 Penicillin-Streptomycin L-glutamine 100X, PSG (Corning, catalog # 30-009-CI).
- 5 Fetal bovine serum, FBS (Avantor Seradigm, catalog # 1500-500).
- 6 Agar, 2 % (Oxoid, catalog # LP0028).
- 7 Bovine serum albumin, 35 % (BSA; Sigma-Aldrich, catalog # A7409).
- 8 BSA, 2.5 % (Sigma-Aldrich, catalog # A9647).
- 9 DMEM/F-12 powder (Gibco, catalog # 12400-024).
- 10 Cell culture grade water (Corning, catalog # 25-055-CV).
- 11 Sodium bicarbonate, 5 % (Sigma, catalog # S-5761).
- 12 DEAE-Dextran, 1 % (MP Biomedicals, catalog # 195133).
- 13 Avicel PH-101, 1 % (Sigma-Aldrich, catalog # 11365).
- 14 Formalin solution, neutral buffered, 10 % (Sigma-Aldrich, catalog # HT501128).
- 15 Triton X-100, 0.5 % (Sigma-Aldrich, catalog # X100-500ML).
- 16 Human monoclonal antibody (hmAb) 1212C2 (produced in house) (25) was used to select SARS-CoV-2 MARMs. The stock of 1212C2 was 0.31 µg/mL.
- 17 Mouse mAb anti-SARS nucleoprotein (NP) 1C7 generated at the Center for Therapeutic Antibody Development at The Icahn School of Medicine at Mount Sinai, ISMMS (Millipore Sigma, catalog # ZMS1075).
- 18 Mouse mAb anti-SARS spike (S) 3B4E10 generated at the Center for Therapeutic Antibody Development at the ISMMS.
- 19 VECTASTAIN® ABC-HRP Kit, Peroxidase, POD (Mouse IgG) (Vector Laboratory, catalog # PK-4002).
- 20 DAB Substrate Kit, POD (HRP), with Nickel (Vector Laboratory, catalog # SK-4100).
- 21 FITC-conjugated secondary anti-mouse (DAKO, catalog # F026102-2).
- 22 DAPI (4',6-Diamidino-2- phenylindole dihydrochloride (Sigma, catalog # D8417).
- 23 6-well cell culture plate (Greiner Bio-One, catalog # 657160).
- 24 12-well cell culture plate (Greiner Bio-One, catalog # 665165).
- 25 24-well cell culture plate (Greiner Bio-One, catalog # 662165).
- 26 48-well cell culture plate (Greiner Bio-One, catalog # 677180).
- 27 96-well cell culture plate (Greiner Bio-One, catalog # 655180).
- 28 Polystyrene tissue culture flask (Corning, catalog # 431081).
- 29 Polypropylene sterile conical tube, 15 mL (Greiner Bio-One, catalog # 188261).
- 30 Polypropylene sterile conical tube, 50 mL (Greiner Bio-One, catalog # 227270).
- 31 Serological pipette, 5 mL (Greiner Bio-One, catalog # 606180).
- 32 Serological pipette, 10 mL (Greiner Bio-One, catalog # 607180).
- 33 Serological pipette, 25 mL (Greiner Bio-One, catalog # 760180).
- 34 Universal pipette tips, 20 µL (VWR, catalog # 76322-134).
- 35 Universal pipette tips, 200 µL (VWR, catalog # 76322-150).
- 36 Universal pipette tips, 1000 µL (VWR, catalog # 16466-008).
- 37 Microcentrifuge tubes, 1.5 mL (VWR, catalog # 89000-028).
- 38 Sterile basin (VWR, catalog # 89094-680).
- 39 CO₂ incubator (PHCbi, model # MCO-170AICUVDL).
- 40 CTL ImmunoSpot plate reader and counting software (Cellular Technology Limited).
- 41 Fluorescence microscope (Olympus).
- 42 Scanner (Epson, model # Perfection V600 Photo Scanner)
- 43 GraphPad Prism (GraphPad Software Inc., version 8.0).

2.2. Media and recipes

- 44 Cell-maintenance media (DMEM supplemented with 10 % FBS and 1 % PSG).
- 45 Infection media (DMEM supplemented with 1 % PSG).

- 46 Post-infection media (DMEM supplemented with 2 % FBS and 1 % PSG).
- 47 DMEM/F-12/Agar mixture (DMEM-F12 with 1 % DEAE-Dextran, 2 % agar, and 5 % NaHCO₃).
- 48 Plaque reduction microneutralization (PRMNT) media (post-infection media with 1 % Avicel).

2.3. Biosafety recommendations

To date, SARS-CoV-2 has been categorized as a risk group (RG) 3 pathogen according to the National Institute of Health (NIH) guidelines. As such, manipulation of SARS-CoV-2 should follow biosafety precautions and only be carried out in a biosafety level 3 (BSL3) facility with strict adherence to the relevant institutional biosafety committee (IBC) guidelines. Extra care must be taken not to generate aerosols and droplets when handling SARS-CoV-2 samples in a BSL3 containment. All researchers handling SARS-CoV-2 must take proper biosafety training to work in a BSL3 facility and be cleared to carry out procedures requiring working with the virus. All cell culture procedures in this protocol were carried out in BSL2. All works involving SARS-CoV-2 were performed in the BSL3 facility. Proper inactivation procedures of infected plates required full submersion in 10 % formalin solution for 24 h before moving infected plates to a BSL2 laboratory for further processing. Texas Biomedical Research Institute's IBC approved all protocols.

2.4. Cells

An important consideration for selecting an appropriate cell line for the proposed studies is permissibility to SARS-CoV-2 infection. In this study, we used Vero E6 cells, a cell line that has been shown to be permissive to SARS-CoV-2 infection and widely used for the study of SARS-CoV-2 (Chiem et al., 2020; Ye et al., 2020; Oladunni et al., 2020; Park et al., 2021). Other permissive cell types, such as Vero E6 transfected to express transmembrane protease serine 2 (TMPRSS2), a serine protease which activates SARS-CoV-2 infection (Matsuyama et al., 2020); human 293 T (Crawford et al., 2020) or A549 (Chen et al., 2020) cells constitutively expressing the human angiotensin converting enzyme 2 (hACE2), or Calu3 cells (Felgenhauer et al., 2020), could be used instead of Vero E6 cells for the proposed experiments. For all experiments, we seeded cells the day before infection to achieve approximately ~85–95 % confluency. We recommend using fresh Vero E6 cells, between passage 1–10 as we have found that these cells grow better, and are more viable at low passage. It is also important to maintain healthy cells free of any contaminant, including mycoplasma.

2.5. Virus

For the experiments in this manuscript we used SARS-CoV-2 isolate USA-WA1/2020 obtained from BEI Resources (Chiem et al., 2020; Ye et al., 2020; Oladunni et al., 2020; Park et al., 2021). We recommend using a multiplicity of infection (MOI) of 0.001 for the first passage in a 12-well plate. Virus infection with higher MOI may result in rapid replication and cell-to-cell spread even in the presence of a high concentration of mNABs, while virus infection at a low MOI may result in reduced cytopathic effect (CPE). As a result, it is important to optimize the assay to find the right balance for the amount of SARS-CoV-2/mNAB needed. Following the experimental conditions described in this manuscript, it is expected to have ~ 100 % CPE in virus only control cells at 72 h post-infection (h p.i.)

2.6. Antibody

As a proof of concept, we have conducted these studies with a human mNAB which we have previously demonstrated to potently neutralize SARS-CoV-2 both in *in vitro* and *in vivo* settings (Piepenbrink et al., 2020). Monoclonal or polyclonal antibodies from other animal species

or serum samples containing NABs can equally be used for the selection of SARS-CoV-2 MARMs. We recommend heat-inactivation of serum or plasma samples from COVID-19 patients or SARS-CoV-2-infected animals at 56 °C for 1 h to destroy complement proteins or residual SARS-CoV-2 particles.

2.7. Controls

Appropriate controls should be included for the selection of SARS-CoV-2 MARMs. These include the use of virus-only infected cells to rule out the potential generation of SARS-CoV-2 cell-adapted mutations during the selection of MARMs. Likewise, we recommend the selection of SARS-CoV-2 MARMs in the presence of an irrelevant Ig isotype control mAb or serum samples from non-infected humans and/or other animal species. Mock-infected control cells should also be included to accurately evaluate virus-induced CPE.

3. Methods

3.1. Generation of SARS-CoV-2 MARMs

A key factor in the infectivity of SARS-CoV-2 is the ability of the S glycoprotein (Fig. 1A) to bind with high affinity to its receptor, ACE2, to initiate virus infection (Bourgonje et al., 2020). The low proofreading ability of RNA viruses, including SARS-CoV-2, enables them to rapidly mutate under immune pressure by NABs leading to the emergence of new serotypes or quasispecies (Fig. 1B). In an *in vitro* setting, passaging SARS-CoV-2 in the presence of mNABs can alter the antibody binding site of SARS-CoV-2 S leading to subsequent immune escape by disrupting recognition by the mNAB. SARS-CoV-2 population dynamics produced during the selection of such escape mutants allows them to become resistant to neutralization by the mNABs as demonstrated by plaque reduction microneutralization (PRMNT) and immunofluorescence (IFA) assays (Fig. 1C and D, respectively). The key amino acid residues in the identified SARS-CoV-2 MARMs can be identified by comparing the sequence of the SARS-CoV-2 MARMs relative to the parental virus control. We recommend verification of the phenotype of selected SARS-CoV-2 MARMs using virus growth kinetics (Fig. 1E) and plaque assays (Fig. 1F). A schematic representation of the experimental procedure for generating SARS-CoV-2 MARMs is illustrated in Fig. 2 and further described in detail below:

- 1 Seed Vero E6 cells (12-well cell-culture plates, 5×10^5 cells/well) the day before virus infection.

- 2 On the day of infection, check the confluency of the Vero E6 cells under a light microscope. The optimal cell confluency at the time of SARS-CoV-2 infection should be ~85–95 %.
- 3 Using an empty, sterile 12-well cell-culture plate, prepare a 2-fold serial dilution of the mNAB or mNAB-containing samples in post-infection media. **Note:** the starting amount/concentration of the mNAB or mNAB-containing sample to be used in the assay would depend on its neutralizing ability or potency.
- 4 In the BSL3, prepare 1×10^3 PFU/well of SARS-CoV-2 in 100 μ L of infectious media in the biosafety cabinet.
- 5 Remove the media from the cell monolayer and wash the cells with PBS.
- 6 Infect the cell monolayer with 100 μ L of SARS-CoV-2 and incubate for 1 h at 37 °C in a CO₂ incubator. Shake the plate every 10 min to prevent the cells from drying and facilitate virus adsorption. **Note:** control wells with no mNAB (Fig. 2, blue) and with no virus (Fig. 2, white) are included in the assay as internal controls.
- 7 After 1 h of virus adsorption, remove the virus inoculum, wash the cells with PBS, and replace the infectious media with 1 mL of post-infection media containing the serially-diluted mNABs or mNAB-containing samples. As a control, incubate another plate of infected cells with an isotype control mAb or serum samples from non-infected humans and/or other animal species in parallel. **Note:** For the selection of MARMs, we recommend to first infect cells with SARS-CoV-2, to allow viral infection, and then incubate with the different concentrations of the mNAB. Pre-incubation of SARS-CoV-2 with the mNAB could prevent the establishment of a productive infection needed for the selection of MARMs.
- 8 Incubate the cells for 72 h at 37 °C in a CO₂ incubator. **Note:** The duration of incubation is normally determined as the time when the virus-only control well (Fig. 2, blue) produces ~100 % CPE. This time-point is usually influenced by the amount of virus inoculum used to infect the cells. In our experience, infection with 1×10^3 PFU/well of SARS-CoV-2 in a confluent monolayer of Vero E6 cells in 12-well cell-culture plates produced ~100 % CPE at 72 h p.i.
- 9 At 72 h p.i., cells are examined for virus-induced CPE. For the next passage, tissue culture supernatants (TCS) from the well with viral-induced CPE under the greatest mNAB selection similar to that of virus-only infected control well is used (P1). It is important to take images of the CPE using a bright field microscope for a direct comparison with the virus-only control well (Fig. 3).

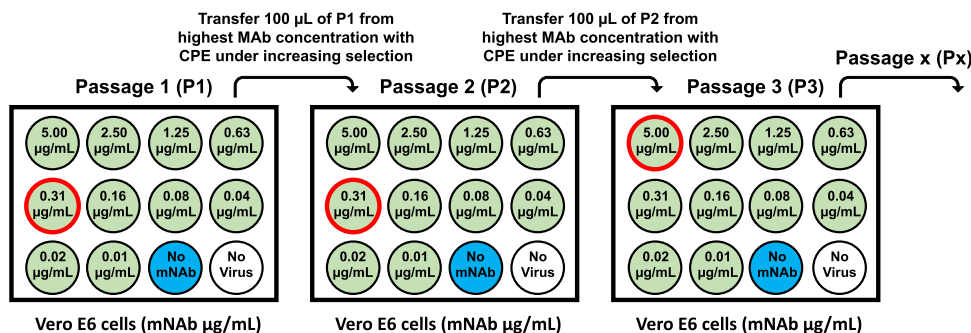


Fig. 2. Selection of SARS-CoV-2 MARMs. Vero E6 cells were infected with SARS-CoV-2 and incubated with decreasing concentration of mNAB (starting concentration of 5.00 μ g/mL). Cells in the absence of mNAB (No mNAB, blue) and mock-infected Vero E6 cells (No Virus, white) were used as internal controls. At 72 h p.i., fresh Vero E6 cells were infected with TCS from wells with the highest concentration of the mNAB (red, P1) with comparable CPE to the well without mNAB in the presence of the same decreasing concentration of mNAB as previously described. The virus was passaged until the CPE observed with the highest concentration of the mNAB (5 μ g/mL) was comparable to that of the virus-only infected cells (No mNAB, blue). A plate of Vero E6 cells with the same concentrations of an irrelevant isotype mAb was also included in the experiment as a control for the selection of SARS-CoV-2 MARMs.

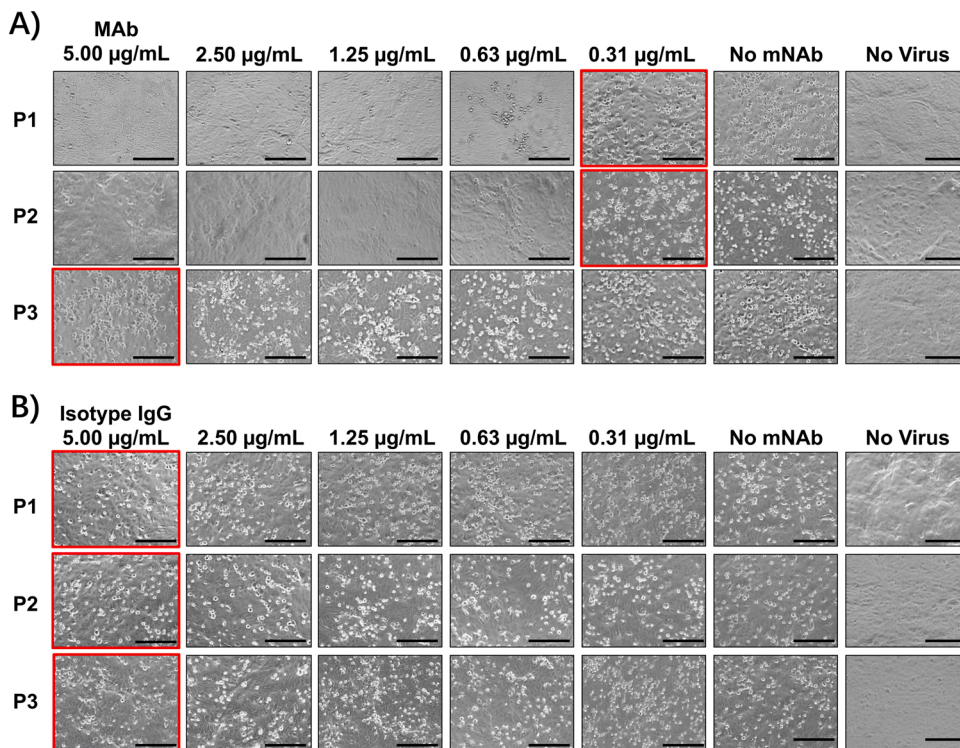


Fig. 3. SARS-CoV-2-induced CPE in the presence of mNAb. A) The ability of SARS-CoV-2 to induce CPE under decreasing concentrations of a mNAb was monitored for each viral passage (P1, P2, and P3) using bright field microscopy. Note that by passage 3 (P3), SARS-CoV-2 induced CPE in all wells containing the mNAb, even at the highest concentration of the mNAb, similar to virus-only infected Vero E6 cells (No mNAb). B) SARS-CoV-2 induced similar CPE with all the concentrations of the isotype control mAb independent of the virus passage. Scale bars, 100 µm.

- 10 Prepare confluent monolayer of Vero E6 cells (12-well cell-culture plates, 5×10^5 cells/well) the day before infection with the TCS from P1.
- 11 Remove the media from the confluent cell monolayer and wash the cells with PBS.
- 12 Repeat steps 6–8, using the TCS from the preceding passage as the inoculum for the next passage, until Px. Px is defined as the passage number when the generated SARS-CoV-2 MARMs overcomes neutralization with the greatest mNAb or NAb-containing sample concentration.

- 13 Harvest the TCS from Px and freeze down at -80°C until further use.

3.2. Evaluation of SARS-CoV-2 MARM neutralization

Before sequencing SARS-CoV-2 MARMs, it is important to first validate the resistance of the generated MARMs to a mNAb (Fig. 4). Our group (Park et al., 2020) and others (Amanat et al., 2020) have previously described a PRMNT assay which can be rapidly used to screen the neutralizing activity of mNAb, or NAb-containing samples. The two variations of this PRMNT assay (pre-treatment and post-treatment) and

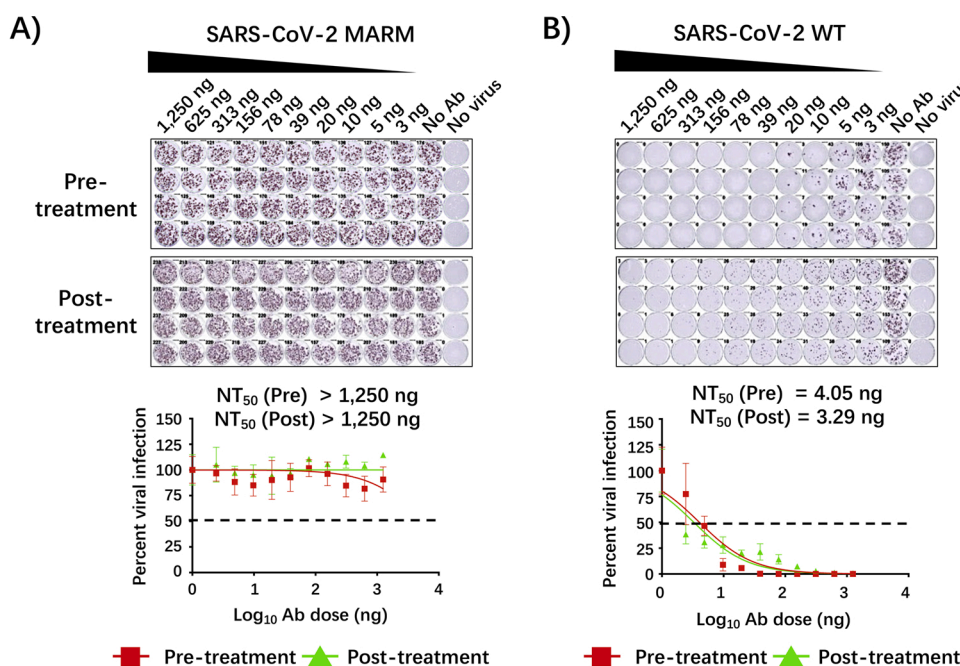


Fig. 4. In vitro neutralizing activity of the mNAb against SARS-CoV-2 MARM. SARS-CoV-2 MARM (A) or WT (B) were incubated with the mNAb 1 h prior (pre-treatment) or after (post-treatment) infection of Vero E6 cells. At 24 h p.i., cells were fixed in 10 % formalin solution and immunostained with a SARS-CoV cross-reactive NP mAb (1C7). Virus neutralization was quantified using ELISPOT, and the percentage of infectivity calculated using sigmoidal dose response curve. Mock-infected cells (No virus) and cells infected with MARM or WT SARS-CoV-2 in the absence of mNAb (No mNAb) were included as internal controls. Dotted line indicates 50 % neutralization. Data were expressed as mean and SD. NT₅₀: 50 % virus neutralization.

the protocol for immunostaining are described below:

3.2.1. Pre-treatment PRMNT assay

- 1 Seed $\sim 1 \times 10^4$ Vero E6 cells/well the day before virus infection in 96-well plates using cell maintenance media. **Note:** The seeding density of Vero E6 in 96-well is $\sim 1 \times 10^4$ cells/well while cells at confluency is $\sim 4 \times 10^4$ cells/well.
- 2 On the day of infection, check the confluency of the Vero E6 cells under a light microscope. The optimal cell confluency should be ~ 85 – 95 %.
- 3 Prepare a 2-fold dilution of the mNAb or NAb-containing samples in an empty, sterile 96-well plate using infection media. Briefly, add 50 μ L of infection media to columns 2–12, and add 100 μ L of the desired starting concentration of each mNAb, or NAb-containing sample, to column 1. Transfer 50 μ L from column 1 to column 2, and mix ~ 10 times using a multi-channel pipette. Repeat this process from column 2 to column 10, changing the tips between dilutions to prevent transfer of residual mNAb or NAb-containing sample. Discard 50 μ L from the solution in column 10 after dilution so that each well of the 96-well plate has 50 μ L. Columns 11 and 12 are included as internal controls, as virus-only and cells-only, respectively. In our study, each mNAb or NAb-containing sample is tested in quadruplicate
- 4 In the BSL3, prepare ~ 100 – 200 PFU/well of SARS-CoV-2 in infectious media in the biosafety cabinet. From the virus stock, calculate ~ 1.0 – 2.0×10^4 PFU and mix with 5 mL of infection media (for one 96-well plate). **Note:** The amount of virus per well can be further optimized based on the virus titer in the stock.
- 5 Add 50 μ L of SARS-CoV-2 to columns 1–11 of the mNAb or NAb-containing plate and incubate the mixture for 1 h at 37 °C. Virus should be added starting at column 11, and sequentially up to column 1 to avoid any carry-over from high to low sample concentration wells.
- 6 After the 1 h incubation, remove cell maintenance media from the 96-well plate of confluent Vero E6 cultured cells. Transfer 50 μ L of the mNAb sample-virus mixture from the 96-well plate to the corresponding Vero E6-96-well plate using a multi-channel pipette. Incubate for 1 h at 37 °C in a 5 % CO₂ incubator to allow for virus adsorption.
- 7 After 1 h of virus adsorption, remove the mNAb sample-virus mixture and overlay with post-infection media containing 1 % Avicel. Incubate the infected Vero E6 cells for 24 h at 37 °C in a 5% CO₂ incubator.
- 8 At 24 h p.i., remove infectious media and fix/inactivate the plate in 10 % formalin solution for 24 h at 4 °C.

3.2.2. Post-treatment PRMNT assay

- 1 Seed $\sim 1 \times 10^4$ Vero E6 cells/well using maintenance media the day before virus infection in 96-well plates, as previously described in the pre-treatment protocol.
- 2 On the day of infection, check the confluency of the Vero E6 cells under a light microscope. The optimal cell confluency is between 85–95 %.
- 3 Prepare a 2-fold serial dilution of the mNAb, or NAb-containing sample, in an empty, sterile 96-well plate using post-infection media as described in step 3 of section 3.2.1. Add 50 μ L of 2 % Avicel in post-infection media to each well containing the diluted mNAb, or NAb-containing sample, or media-only as well as no-virus control wells (columns 11 and 12, respectively) to give a final concentration of 1 % Avicel in each well.
- 4 In the BSL3, prepare ~ 100 – 200 PFU/well of SARS-CoV-2 in infection media in the biosafety cabinet. From the virus stock, calculate ~ 1.0 – 2.0×10^4 PFU and mix with 5 mL of infection media (for one 96-well plate).

- 5 Add 50 μ L of virus inoculum to each well of the cell-cultured Vero E6-96-well plate from column 1–11, and incubate for 1 h at 37 °C in a 5 % CO₂ incubator to allow for virus adsorption.
- 6 After 1 h of virus adsorption, remove the virus inoculum and replace with 100 μ L of post-infection media containing the serially-diluted mNAb or NAb-containing sample containing 1 % Avicel.
- 7 Incubate the Vero E6 cells for 24 h at 37 °C in a 5 % CO₂ incubator.
- 8 At 24 h p.i., remove infectious media and fix/inactivate the plate in 10 % formalin solution for 24 h at 4 °C.

3.2.3. Development of infected cells by peroxidase staining

- 1 Once the plates are in the BSL2 after 24 h inactivation at 4 °C, remove residual formalin solution by gentle wash with double distilled water (DDW).
- 2 Gently wash the cells three times with 100 μ L/well of PBS.
- 3 Permeabilize the cells with 100 μ L/well of 0.5 % Triton X-100 dissolved in PBS, and incubate at room temperature (RT) for 15 min in the biosafety cabinet. **Note:** If you use an Ab against a viral surface protein (e.g. S), you can skip this permeabilization step.
- 4 Wash the cells with 100 μ L/well of PBS, three times, and block with 100 μ L/well of 2.5 % BSA in PBS. Incubate cells at 37 °C for 1 h.
- 5 Prepare primary Ab solution (anti-NP mAb, 1C7, 1 μ g/mL) in 1 % BSA, in PBS. Add 50 μ L/well of primary Ab solution and incubate at 37 °C for 1 h.
- 6 After primary Ab incubation, wash each well three times with 100 μ L/well of PBS.
- 7 Prepare the biotinylated anti-mouse Ab (VECTASTAIN® ABC-HRP Kit, Peroxidase (Mouse IgG); Vector Laboratory) following the manufacturer's instructions. For one 96-well plate, add 75 μ L of normal blocking serum stock and 25 μ L of biotinylated secondary Ab stock to 5 mL of PBS. Add 50 μ L/well of biotinylated Ab solution to each well, and incubate for 30 min at 37 °C.
- 8 Next, wash each well three times with 100 μ L/well of PBS to remove biotinylated Ab solution thoroughly. Prepare VECTASTAIN ABC Reagent by following manufacturer's instructions (VECTASTAIN® ABC-HRP Kit, Peroxidase (Mouse IgG); Vector Laboratory). For one 96-well plate, add 50 μ L of Reagent A (Avidin, ABC) and 50 μ L of Reagent B (Biotinylated HRP, ABC) to 5 mL of PBS. Add 50 μ L/well of VECTASTAIN ABC Reagent and incubate for 30 min at 37 °C.
- 9 Wash cells three times with 100 μ L/well of PBS. Remove PBS, and dry the plate by gently blotting on paper towel. Prepare developing solution by following manufacturer's instructions (DAB Substrate Kit, Peroxidase (HRP), with Nickel; Vector Laboratory).
- 10 Add 50 μ L of developing solution to each well and wait for ~ 3 – 5 min to visualize viral plaques.
- 11 Stop the reaction by removing the developing solution, and wash with PBS. It is important not to wait for too long once the plaques are visible to prevent the entire cells from turning black. Take images and measure the stained positive cells using a CTL ImmunoSpot plate reader and counting software (Cellular Technology Limited, Cleveland, OH, USA). The formula that can be used to calculate percent viral infection for each concentration is given as [(Average # of plaques from each treated wells – average # of plaques from “no virus” wells)/(average # of plaques from “virus only” wells - average # of plaques from “no virus” wells)] x 100.

3.3. Evaluation of SARS-CoV-2 MARM binding

To investigate the resistance of the generated MARMs to binding by the mNAb, we examined Vero E6 cells infected with SARS-CoV-2 MARMs using IFA. We recommend using mock-infected cells as well as cells infected with WT SARS-CoV-2 as internal controls (Fig. 5).

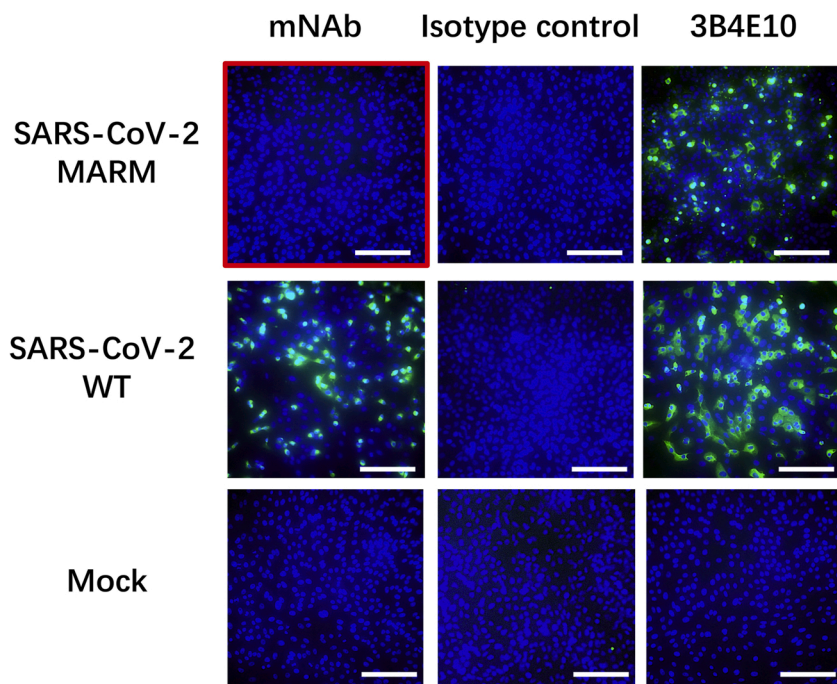


Fig. 5. IFA of SARS-CoV-2 MARM. Vero E6 cells infected with MARM (top) or WT (middle) SARS-CoV-2 were fixed at 48 h p. i. Mock-infected cells (bottom) were included as internal control. Viral detection was carried out with the indicated primary mNAb used for the selection of the MARM. Cellular nuclei were stained with DAPI (blue). As additional internal controls, mock and MARM or WT SARS-CoV-2 infected cells were stained with an isotype control mAb or with the mouse mAb 3B4E10. Images were taken using an inverted fluorescence microscope. Scale bars, 100 μ m.

Likewise, we recommend using isotype control and an antibody that can recognize both the WT and SARS-CoV-2 MARMs, as internal controls in the IFA (Fig. 5). The protocol for performing IFA to evaluate SARS-CoV-2 MARM binding by mNAb is described in details below:

- 1 Prepare Vero E6 cells (48-well plate format, 1.2×10^5 cells/well, triplicates) the day before infection. **Note:** The density of Vero E6 cells in 48-well plates is $\sim 1.2 \times 10^5$ cells/well while cells at confluency is $\sim 2.5 \times 10^5$ cells/well.
- 2 On the day of infection, check the confluency of the Vero E6 cells under a light microscope. The optimal cell confluency should be ~ 85 – 95 %.
- 3 In the BSL3, prepare SARS-CoV-2 (MOI 0.01) in an infection media in the biosafety cabinet.
- 4 Infect the Vero E6 cells with SARS-CoV-2 (250 μ L/well) and incubate for 1 h at 37 °C in a 5 % CO₂ incubator.
- 5 After 1 h of virus adsorption, remove the viral inoculum and add 500 μ L of post-infection media to each well.
- 6 Return the plates back to the 5 % CO₂ incubator and incubate for 24 h at 37 °C.
- 7 At 24 h p.i., remove the infectious TCS and fix/inactivate the plate in 10 % neutral buffered formalin for 24 h at 4 °C.
- 8 After fixation/inactivation, gently rinse the plates with DDW to remove residual 10 % neutral buffered formalin solution.
- 9 Wash the cells with 250 μ L/well PBS, three times.
- 10 Add 250 μ L/well of permeabilization solution (0.5 % Triton X-100 dissolved in PBS) for 15 min at RT in the biosafety cabinet. **Note:** if you are using an Ab against SARS-CoV-2 S glycoprotein as internal control (e.g. 3B4E10), you can skip this permeabilization step. However, if you are using an Ab against an internal viral protein as control (e.g. NP), you need to permeabilize the cells.
- 11 Remove the permeabilization solution and wash the cells, three times, with 250 μ L/well of PBS.
- 12 Add 250 μ L/well of blocking solution (2.5 % BSA in PBS) for 1 h at RT. **Note:** Cells can also be blocked overnight (ON) at 4 °C.
- 13 Remove the blocking solution and incubate the cells with 250 μ L/well of the mNAb used for the isolation of the MARM at 1 μ g/mL dilution in 1 % BSA in PBS. Likewise, incubate the Vero E6 cells

with 250 μ L/well of the isotype control and positive mAb diluted in 1% BSA in PBS. **Note:** In our experiment, we used the anti-SARS S 3B4E10 MAb as internal positive control. Other MAb or polyclonal antibodies for the detection of SARS-CoV-2 may be used in place of 3B4E10 MAb.

- 14 Incubate the cells with the primary Ab at 37 °C for 1 h.
- 15 After 1 h incubation with the primary Ab, remove the solution and wash the cells, three times, with 250 μ L/well PBS.
- 16 Remove the PBS and add 250 μ L/well of anti-mouse IgG-FITC-conjugated antibody (1:200 dilution) and 4',6'-diamidino-2-phenylindole (DAPI; 1 mg/mL) in blocking solution and place the plates in a 37 °C incubator for 1 h. Note that it is important to protect the samples from the photobleaching effect of direct light by covering the plates with an aluminium foil.
- 17 Remove the secondary antibody and wash the cells three times with PBS. Leave ~ 500 μ L of PBS in each well. Samples can be stored at 4 °C while protected from light with an aluminum foil.
- 18 Examine the stained cells under a fluorescence microscope.

3.4. SARS-CoV-2 MARM growth kinetics and plaque phenotype

To determine whether the identified SARS-CoV-2 MARM has similar viral fitness to SARS-CoV-2 WT, we compare the growth kinetics and the plaque phenotype of SARS-CoV-2 MARM and WT in Vero E6 cells. Comparing the growth kinetics and plaque phenotype of SARS-CoV-2 MARM and WT virus will provide information on how the mutations affecting recognition and binding of the mNAb affect viral replication. Likewise, assessing the plaque phenotype of SARS-CoV-2 MARM and WT will provide information on the rate of replication and cell-to-cell spread of the SARS-CoV-2 MARM relative to SARS-CoV-2 WT.

To determine viral growth kinetics (Fig. 6A):

- 1) Seed Vero E6 cells (24-well cell-culture plates, 2.4×10^5 cells/well, triplicates) the day before virus infection.
- 2) Remove the media from the cell monolayer and wash the cells with PBS.
- 3) Infect the confluent monolayers of Vero E6 with SARS-CoV-2 MARM or WT at an MOI of 0.01, and incubate at 37 °C in a 5 % CO₂ incubator for 1 h.

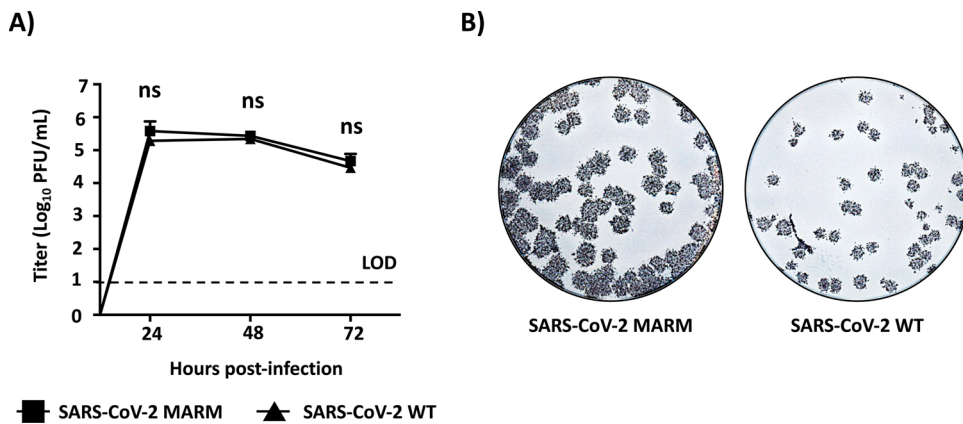


Fig. 6. Replication of SARS-CoV-2 MARM and WT in Vero E6 cells. **A) Growth kinetics:** Vero E6 cells were infected with SARS-CoV-2 MARM or WT, and TCS were collected at 24, 48, and 72 h p.i. to evaluate for the presence of virus using plaque assay. **B) Plaque phenotype:** Vero E6 cells were infected with either SARS-CoV-2 MARM (left) or WT (right). After 72 h of incubation at 37 °C, cells were fixed and immunostained with the 1C7, an anti-NP mAb. Data represents the mean ± SD. ns, not significant.

- After 1 h of virus adsorption, remove the virus inoculum, and wash the cells with PBS.
- Incubate the cells with 500 µL of post-infection media at 37 °C in a 5 % CO₂ incubator for 24, 48, 72, and 96 h p.i., respectively.
- At each time-point, collect the TCS and determine viral titers by plaque assay.

To assess the viral plaque phenotype (Fig. 6B):

- Seed Vero E6 cells (6-well cell-culture plates, 1.0×10^6 cells/well, duplicates) the day before virus infection.
- Prepare a 10-fold serial dilution of SARS-CoV-2 MARM or WT control in infection media. **Note:** We recommend changing of tips between viral dilutions to prevent the transfer of virus particles from lower dilution to a higher dilution.
- Remove the media from the cell monolayer and wash the cells with PBS.
- Infect the confluent Vero E6 cells with the corresponding dilution (-2 to -8) of SARS-CoV-2 MARM or WT virus.
- Incubate for 1 h in a humidified incubator at 37 °C with 5 % CO₂ for virus adsorption.
- To prevent cells from drying and to also facilitate virus adsorption, shake the plates gently every 10 min.
- After 1 h of virus adsorption, remove the virus inoculum and wash the cells before overlaying the cells with DMEM/F-12/Agar mixture.
- Incubate the cells for 72 h in a humidified incubator at 37 °C in the presence of 5 % CO₂.
- At 72 h p.i., fix infected cells with 10 % formalin solution for 24 h.
- Perform immunostaining with the cross-reactive SARS-CoV NP mAb 1C7 as described below:
 - Permeabilize the cells with 1 mL/well of 0.5 % Triton X-100 dissolved in PBS, and incubate at RT for 15 min in the biosafety cabinet.
 - Wash the cells with 1 mL/well of PBS, three times, and block with 1 mL/well of 2.5 % BSA in PBS. Incubate cells at 37 °C for 1 h.
 - Prepare primary Ab (anti-NP mAb 1C7, 1 µg/mL) in 1 % BSA, in PBS. Add 1 mL/well of primary Ab solution and incubate at 37 °C for 1 h.
 - After incubation with the primary 1C7 anti-NP mAb, wash each well three times with 1 mL/well of PBS.
 - Prepare the biotinylated anti-mouse Ab (VECTASTAIN® ABC-HRP Kit, Peroxidase (Mouse IgG); Vector Laboratory) following the manufacturer's instructions.
 - Add 1 mL/well of biotinylated Ab solution, and incubate for 30 min at 37 °C.
 - Wash each well three times with 1 mL/well of PBS to remove biotinylated Ab solution thoroughly. Prepare VECTASTAIN ABC Reagent by following manufacturer's instructions (VECTASTAIN® ABC-HRP Kit, Peroxidase (Mouse IgG); Vector Laboratory). Add 1 mL/well of VECTASTAIN ABC Reagent and incubate for 30 min at 37 °C.
 - Wash cells three times with 1 mL/well of PBS. Remove PBS, and dry the plate by gently blotting on paper towel. Prepare a developing solution by following manufacturer's instructions (DAB Substrate Kit, Peroxidase (HRP), with Nickel; Vector Laboratory).
 - Add 1 mL of developing solution to each well and wait for ~3–5 min to visualize viral plaques.
 - Stop the reaction by removing the developing solution, and wash with 1 mL/well of PBS.
 - Take pictures of the plaque phenotypes using a scanner (Fig. 6B).

3.5. Sequencing of SARS-CoV-2 MARM

To identify the amino acid(s) residue(s) that play a critical role in the antigenic drift of SARS-CoV-2 under immune pressure induced by the presence of the mNAb, it is important to sequence the MARM and compare it to that of the SARS-CoV-2 WT. Comparing the sequence information between SARS-CoV-2 MARM and the WT virus can also help in mapping out the epitope(s) that are critical for the binding activity of the mNAb. Sequence analyses of different SARS-CoV-2 isolates have suggested that the RBD is a highly variable region that is subject to selective pressure by antibody immune responses (Rani et al., 2012). Amino acid substitutions within the RBD have played a major role in the ability of coronaviruses to overcome the species barrier, initially allowing animal-to-human transmission and subsequent adaptation to transmission among humans (Zhang et al., 2006). Similarly, functional mutations in the RBD and the N-terminal domain of SARS-CoV-2 S glycoprotein that conferred immune resistance to mNAb or convalescent plasma have been reported (Weisblum et al., 2020). Importantly, sequence information of these variant pathotypes can help identify amino acid residues that enabled immune evasion and viral fitness.

Since mutations in the SARS-CoV-2 MARM are most likely to be found in the viral S glycoprotein, RT-PCR amplification of the S ORF and Sanger sequencing may help to identify genetic variations in the MARM S protein relative to that of WT S protein. However, deep sequencing analysis of the entire viral genome will reveal detailed genetic alterations spanning the entire viral genome which could possibly be involved in the selection for an escape mutant. Therefore, we

recommend to conduct deep sequencing analysis of the SARS-CoV-2 MARM, as previously described (Ye et al., 2020). Moreover, we also recommend conducting full sequence analysis of the SARS-CoV-2 passaged in the presence of the irrelevant Ig antibody to differentiate mutations related to the passage of the virus in cultured Vero E6 cells from those responsible for the lack of neutralization by the MARMs. Sequences of MARMs are mapped to the SARS-CoV-2 reference genome, in our case, USA-WA1/2020 isolate (GenBank accession no. MN985325). This will help to elucidate the impact of potential variants on the protein-coding regions in the SARS-CoV-2 reference genome and to identify those sequence alterations that are responsible for an escape from neutralization effect of mNABs, or NAB-containing samples.

4. Results

Following the aforementioned protocol (Fig. 1), we selected an escape variant, SARS-CoV-2 MARM, after passaging the virus three consecutive times in the presence of a potent mNAB (Fig. 2). Both P1 and P2 were able to produce similar virally-induced CPE in the presence of 0.31 µg/mL of mNAB (Fig. 3). This indicates that at P2, SARS-CoV-2 can still be neutralized by the mNAB. However, this dynamic of viral inhibition changed by P3 and the virus is now able to produce a CPE similar to that of virus-only infected control wells, even in the well with the highest concentration of the mNAB (Fig. 3). On the other hand, in all the viral passages, SARS-CoV-2 was able to induce CPE even in the highest concentration of the isotype control mAb (Fig. 3), similar to the well with no mAb (Fig. 2, blue).

Our data showed that the MARM generated after serial passage of SARS-CoV-2 now replicates in the presence of the mNAB in both the pre- and post-treatment setting conditions, similar to the control well with no mNAB (Fig. 4). This is an indicator that the virus has now undergone mutation(s) on its epitope binding site enabling it to escape the neutralizing effect of the mNAB. This is in contrast to the parental virus (SARS-CoV-2 WT) which was efficiently neutralized by the same mNAB in both pre- and post-treatment conditions (Fig. 4).

Similarly, the loss of binding activity of the mNAB was also interrogated by IFA (Fig. 5). As expected, the mNAB lost its binding affinity to SARS-CoV-2 MARM-infected Vero E6 cells (Fig. 5). On the other hand, SARS-CoV-2 WT was recognized by the mNAB. Both, cells infected with SARS-CoV-2 WT and MARM were similarly recognized by the 3B4E10 mAb control, while the isotype control mAb was not able to recognize SARS-CoV-2 WT or MARM infected cells (Fig. 5). None of the Abs (mNAB, 3B4E10, or isotype control) were able to detect SARS-CoV-2 S in mock-infected cells (Fig. 5). Altogether, the PRMNT (Fig. 4) and the IFA (Fig. 5) results demonstrate the mutant nature of the SARS-CoV-2 MARM.

We then compared the viral growth kinetics and the plaque phenotypes between the SARS-CoV-2 MARM and the WT virus (Fig. 6). There were no statistically significant differences in the replication kinetics of the selected MARM when compared to the WT virus at 24-, 48- and 72- h p.i., respectively (Fig. 6A). This indicates that the acquisition of mutation within the spike protein of SARS-CoV-2 MARM exerted no effect on viral fitness status. However, the SARS-CoV-2 escape mutant presented a greater average plaque diameter (data not shown) compared to the WT virus at 72 h p.i. (Fig. 6B). It remains unknown how the plaque phenotype of SARS-CoV-2 MARM may affect viral virulence *in vivo*.

5. Discussion

Mutations in the antigenic sites of viruses may occur as a result of positive immune selection or by natural evolution (Borrego et al., 1993; Aragonés et al., 2008). For the newly emergent SARS-CoV-2, increased viral fitness due to positive immune selection or evolution has been reported (Baum et al., 2020; Korber et al., 2020). Owing to the importance of SARS-CoV-2 S during virus entry and its ability to induce NAB responses, SARS-CoV-2 S glycoprotein has been the major site of

antigenic variation and immune escape (Li et al., 2020). In this manuscript, we describe the experimental approaches to select SARS-CoV-2 MARM escape variants while passaging the virus in the presence of a potent mNAB, *in vitro*.

Although our experimental procedures have focused on the selection of SARS-CoV-2 MARMs using a mNAB, similar studies can also be conducted with polyclonal antibodies or with serum/plasma samples containing NABs. Importantly, the selection of SARS-CoV-2 MARMs represent a feasible approach to identify potential viral drift variants that could emerge in human populations when vaccines are used for the treatment of SARS-CoV-2 infection. While we were able to quickly identify and isolate SARS-CoV-2 MARMs after just three passages in the presence of a mNAB, factors such as the potency, or the concentration of the mNAB or mNAB-containing serum/plasma samples may influence the number of passages needed to select for an immune escape SARS-CoV-2 drifted variant.

A limitation of our *in vitro* model for the selection of SARS-CoV-2 MARMs is the direct extrapolation of our results to reflect natural events *in vivo*, including SARS-CoV-2 infections in humans. It has been reported that peak viral burdens in clinical specimens of COVID-19 patients usually commensurate with periods of high virus infectivity and transmission effectiveness, occurring just before seroconversion (Wolfel et al., 2020). Thus, most of the clinical cases of reported SARS-CoV-2 infection in humans may involve viral populations that have not undergone NAB-mediated selective pressure in the infected individual (Weisblum et al., 2020). In contrast, our protocol for the selection of SARS-CoV-2 MARMs involved the incubation of SARS-CoV-2 with a known, potent mNAB, or NAB-containing sample, over consecutive passages.

We recommend to verify the nature of the SARS-CoV-2 MARMs by assessing their ability to be neutralized (PRMNT) or recognized (IFA) by the mNABs used for the selection of escape mutants. Following the observation of an immune escape phenotype by SARS-CoV-2 upon co-incubation of the virus with a NAb, or NAB-containing samples, we also recommend sequencing the entire viral genome of the SARS-CoV-2 MARMs to identify amino acid changes and/or deletions in the viral genome responsible for the emergence of the MARMs. Finally, it will be important to assess how the mutations responsible for the selection for MARMs affect viral fitness using conventional growth kinetics and/or plaque phenotype assays. This will offer useful information on amino acid residues that are critical for the recognition of the mNABs, or mNAB-containing samples, and how these mutations can potentially affect viral fitness to address concerns of MARM pathogenicity.

Declaration of Competing Interest

F.S.O., J.G.P., M.P., M.R.W., J.K., and L.M.S. are listed as inventors on a pending patent application describing SARS-CoV-2 human mNABs.

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