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2 Evidence of antigenic imprinting in sequential Sarbecovirus immunization

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SUMMARY (150 words max.)

Antigenic imprinting, which describes the bias of antibody response due to previous immune history, can influence vaccine effectiveness and has been reported in different viruses. Given that COVID-19 vaccine development is currently a major focus of the world, there is a lack of understanding of how background immunity influence antibody response to SARS-CoV-2. This study provides evidence for antigenic imprinting in *Sarbecovirus*, which is the subgenus that SARS-CoV-2 belongs to. Specifically, we sequentially immunized mice with two antigenically distinct *Sarbecovirus* strains, namely SARS-CoV and SARS-CoV-2. We found that the neutralizing antibodies triggered by the sequentially immunization are dominantly against the one that is used for priming. Given that the impact of the background immunity on COVID-19 is still unclear, our results will provide important insights into the pathogenesis of this disease as well as COVID-19 vaccination strategy.

INTRODUCTION

Coronavirus disease 2019 (COVID-2019) pandemic is an ongoing global public health crisis and has imposed a huge burden on the world economy. The causative agent of COVID-2019, SARS-CoV-2, belongs to the subgenus *Sarbecovirus* of the genus *Betacoronavirus* and has nearly 80% sequence identity with another *Sarbecovirus*, SARS-CoV, which caused a global epidemic in 2003 (Peiris et al., 2003). While many SARS-CoV-2 vaccine candidates are being actively developed (Funk et al., 2020), the influence of immune history on vaccine effectiveness remains elusive. In fact, it is now well recognized that the effectiveness of a given influenza vaccine varies among people with different influenza immunization or infection histories (Henry et al., 2018). Such phenomenon is known as antigenic imprinting, which describes immunological memory induced by primary innate and adaptive immune responses to the first encounter with a microbial pathogen or vaccination that can be retained over a person's life time. Antigenic imprinting has also been reported in dengue virus (Mongkolsapaya et al., 2003), HIV (Klenerman and Zinkernagel, 1998) and influenza virus (Gouma et al., 2020). Since SARS-CoV-2 may become a seasonal human coronavirus (Kellam and Barclay, 2020) and other zoonotic *Sarbecovirus* strains continue to pose a pandemic threat (Menachery et al., 2015), it is important to understand how antigenic imprinting may affect the antibody response to *Sarbecovirus*. In this study, we explored the antigenic imprinting effect of *Sarbecovirus* by characterizing the antibody response from mice that were sequentially immunized two antigenically distinct *Sarbecovirus* strains, namely SARS-CoV and SARS-CoV-2.

RESULTS

Balb/c mice (6-8 weeks of age) were intraperitoneally (i.p.) immunized twice, with viruses plus adjuvant Addavax (Wu et al., 2019). Four immunization schemes were explored: 1)

two rounds of SARS-CoV (SARS-CoV homologous prime-boost); 2) first round with SARS-CoV and second round with SARS-CoV-2 (heterologous SARS-CoV-prime, SARS-CoV-2-boost); 3) two rounds of SARS-CoV-2 (SARS-CoV-2 homologous prime-boost); and 4) first round with SARS-CoV-2 and second round with SARS-CoV (heterologous SARS-CoV-2-prime, SARS-CoV-boost). Plasma samples were collected and antibody immune responses were measured at day 14 after the second round of immunization. Compared to round one of immunization (Lv et al., 2020), the second round homologous boost induced higher homologous binding and neutralizing antibody titers ($p < 0.05$, two tailed t test; Figure S1a-d). These results suggest that SARS-CoV or SARS-CoV-2 specific memory B cells can be recalled and produce neutralizing antibodies during the second round of immunization.

While all our four prime-boost vaccination schemes resulted in cross-reactive RBD-binding antibodies (Figure 1a-d), the virus that was used for priming seemed to dictate the neutralizing antibody response after boosting, regardless of the virus that was used for the boost (Figure 1e-h). For example, no matter whether SARS-CoV or SARS-CoV-2 was used for boosting, the neutralizing antibody response was much stronger against SARS-CoV if SARS-CoV was used for priming (Figure 1e-f), and stronger against SARS-CoV-2 if SARS-CoV-2 was used for priming (Figure 1g-h). Interestingly, while one round of SARS-CoV immunization was sufficient to elicit a detectable SARS-CoV neutralization response (Figure S1c), a SARS-CoV neutralization response was undetectable when SARS-CoV was used as a heterologous boost after priming with SARS-CoV-2 (Figure 1h). A similar observation was made for SARS-CoV-2 (Figure 1f and S1d).

Angiotensin-converting enzyme 2 (ACE2) is the host receptor for SARS-CoV and SARS-CoV-2 entry. Previous study has shown that neutralizing activity of sera correlates with the ACE2-competition activity (Tan et al., 2020). ACE2-competition assay was then performed for four groups of plasma samples. Briefly, the binding of plasma samples were tested against RBD and RBD/ACE2 complex. Plasma samples with a stronger ACE2-competition activity should show a greater reduction in binding to RBD/ACE2 complex compared to RBD alone. When binding to SARS-CoV RBD was tested, plasma samples from mice that were primed with SARS-CoV had stronger ACE2-competition activity than did plasma samples from mice that were primed with SARS-CoV-2 (Figure 2a). Similarly, when binding to SARS-CoV-2 RBD was tested, plasma samples from mice that were primed with SARS-CoV-2 had stronger ACE2-competition activity than did plasma samples from mice that were primed with SARS-CoV (Figure 2b). These results indicate that the heterologous boost predominantly induces antibodies to conserved regions outside of the ACE2-binding site that have minimum neutralizing activity. One such example is CR3022, which has strong cross-reactive binding activity to a conserved epitope on RBD, but has weak neutralization activity to SARS-CoV and undetectable neutralization activity to SARS-CoV-2 (Yuan et al., 2020b).

Overall, our results suggest that antigenic imprinting can impact the antibody response against *Sarbecovirus*. Specifically, the antibody response to a *Sarbecovirus* strain can be suboptimal if there exists a prior immune history against an antigenically distinct or drifted *Sarbecovirus* strain. This study has important implications for vaccine development against the ongoing COVID-19 pandemic as well as for *Sarbecovirus* and other coronaviruses in general.

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AUTHOR CONTRIBUTION

H.L., N.C.W., and C.K.P.M. conceived and designed the study. N.C.W., M.Y., H.L. and D.C.L. expressed and purified the proteins. H.L., R.T.Y.S., G.K.Y., W.W.N., and C.R.W., performed the experiments. H.L., R.T.Y.S., N.C.W., and C.K.P.M. analyzed the data. H.L., R.T.Y.S., N.C.W., J.S.M.P., I.A.W., and C.K.P.M. wrote the paper, and all authors reviewed and edited the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure. 1. ELISA binding and neutralizing titers of homologous and heterologous sequential immunization with SARS-CoV and SARS-CoV-2.

a-d RBD (receptor binding domain) proteins from SARS-CoV and SARS-CoV-2 were used as the antigen coating on the ELISA plates. Binding of RBD to 1:100 diluted plasma sample was analyzed from 5 mice immunized using **(a)** SARS-CoV homologous prime-boost, **(b)** heterologous SARS-CoV-prime, SARS-CoV-2-boost, **(c)** SARS-CoV-2 homologous prime-boost, and **(d)** heterologous SARS-CoV-2-prime, SARS-CoV-boost. The mean OD₄₅₀ value of two replicates are shown. **e-h** Neutralizing titers of plasma

samples from mice immunized with **(e)** SARS-CoV homologous prime-boost, **(f)** heterologous SARS-CoV-prime, SARS-CoV-2-boost, **(g)** SARS-CoV-2 homologous prime-boost, and **(h)** heterologous SARS-CoV-2-prime, SARS-CoV-boost, were analyzed by a PRNT (plaque reduction neutralization test) assay. Each data point in the figure represents the mean of two replicates. Error bars represent standard deviation.

Figure. 2. Epitope mapping of the neutralizing and non-neutralizing group

SARS-CoV RBD **(a)** or SARS-CoV-2 RBD **(b)** protein was used as antigen to coat on the 96 well ELISA plate. Four groups of mice plasma samples were added into the plate as primary antibody after with or without 100 ng hACE2 protein blocking. The ΔOD_{450} values were calculated as WT OD_{450} value minus hACE2 blocking OD_{450} value. Each data point in the figure represents the mean of two replicates. P-values were calculated using two-tailed t-test (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$). Error bars represent standard deviation. Of note, while SARS-CoV-2-SARS-CoV has a stronger ACE2-competition activity to SARS-CoV-2 RBD than SARS-CoV-SARS-CoV, the large standard deviation in SARS-CoV-SARS-CoV makes the difference statistically insignificant.

Figure. S1. Neutralization titers of one or two rounds of homologous SARS-CoV or SARS-CoV-2 immunization

a-b Neutralizing titers of plasma samples from mice immunized by one or two rounds of homologous virus against **(a)** SARS-CoV or **(b)** SARS-CoV-2 were measured by a PRNT assay. Each data point in the figure represents the mean of two replicates. P-values were calculated using two-tailed t-test (* $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$). Error bars represent standard deviation

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170 **STAR METHODS**

171 **RESOURCE AVAILABILITY**

172 **Lead Contact**

173 Information and requests for resources and reagents should be directed to and will be
174 fulfilled by the Lead Contact, Chris K. P. Mok (ch02mkp@hku.hk).

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176 **Materials Availability**

177 This study did not generate new unique reagents.

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179 **Data and Code Availability**

180 NA.

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182 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

183 **METHOD DETAILS**

184 **RBD protein expression and purification**

185 The receptor-binding domain (RBD) (residues 319-541) of the SARS-CoV-2 spike (S)
186 protein (GenBank: QHD43416.1) and RBD (residues: 306-527) of the SARS-CoV spike
187 (S) protein (GenBank: ABF65836.1) were cloned into a customized pFastBac vector
188 (Ekiert et al., 2011) and fused with an N-terminal gp67 signal peptide and C-terminal
189 6xHis-tag(Yuan et al., 2020b). A recombinant bacmid DNA was generated using the
190 Bac-to-Bac system (Life Technologies). Baculovirus was generated by transfecting
191 purified bacmid DNA into Sf9 cells using FuGENE HD (Promega), and subsequently
192 used to infect suspension cultures of High Five cells (Life Technologies) at an MOI of 5
193 to 10. For protein expression, the infected High Five cells were incubated at 28°C for
194 72h with shaking at 110r.p.m. The supernatant was then concentrated using a 10

kDa MW cutoff Centrimate cassette (Pall Corporation). The RBD protein was purified by Ni-NTA, followed by size exclusion chromatography, and buffer exchanged into 20 mM Tris-HCl pH 7.4 and 150 mM NaCl.

ACE2 protein expression and purification

The expression of human ACE2 was as previously reported (Yuan et al., 2020a). Briefly, the human ACE2 (residues 19 to 615, GenBank: BAB40370.1) was codon optimized and cloned into phCMV3 vector (Yuan et al., 2020a). The construct was fused with a C-terminal 6xHis tag. The plasmid was transiently transfected into Expi293F cells using ExpiFectamine 293 Reagent (Thermo Fisher Scientific) according to the manufacturer's manual. At 6 days post-transfection, the supernatant was harvested and then was then washed and eluted with 10 mM and 300 mM Imidazole containing PBS, respectively. The ACE2 eluent was purified by size exclusion chromatography.

Mouse immunization

6-8 weeks Balb/c mice were immunized with two rounds 10^5 pfu of viruses in 150 μ L PBS mixing with 50 μ L Addavax, including: 1) two rounds of homologous SARS-CoV immunization, 2) two rounds of heterologous immunization with SARS-CoV-prime and SARS-CoV-2-boost, 3) two rounds of homologous SARS-CoV-2 immunization, and 4) two rounds of heterologous immunization with SARS-CoV-2-prime and SARS-CoV-boost, via intraperitoneal (i.p.) route. The plasma samples were collected using heparin tubes on day 35 after the second round of immunization. The experiments were conducted in The University of Hong Kong Biosafety Level 3 (BSL3) facility. This study protocol was carried out in strict accordance with the recommendations and was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong (CULATR 4533-17).

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222 **ELISA binding assay**

223 ELISA plates (96-well, Nunc MaxiSorp, Thermo Fisher Scientific) were coated overnight
224 with 100 µl of purified recombinant protein in PBS buffer at 1 ng/µl. The plates were then
225 blocked with 100 µl Chonblock buffer (Chondrex Inc, Redmon, US) at room temperature
226 for 1 hours. Each mouse plasma sample was 1:100 diluted in Chonblock buffer, added
227 to the coated ELISA plates, and incubated for 2 hours at 37°C. After three extensive
228 washes with PBS containing 0.1% Tween 20, each well was incubated with the HRP
229 goat anti-mouse secondary antibody (1:5000, Beyotime Biotechnology) for 1 hour at
230 37°C. The ELISA plates were then washed five times with PBS containing 0.1% Tween
231 20. Subsequently, 100 µl of TMB buffer (Ncm TMB One; New Cell & Molecular Biotech
232 Co., Ltd) was added into each well. After 15 minutes incubation, 50 µl of 2 M H₂SO₄
233 solution was added to stop the reaction and the plates were analyzed on a Sunrise
234 absorbance microplate reader (Tecan, Männedorf, Switzerland) at 450 nm wavelength.

235

236 **ACE2-competition ELISA assay**

237 ELISA plates (96-well, Nunc MaxiSorp, Thermo Fisher Scientific) were coated overnight
238 with 100 ng of SARS-CoV or SARS-CoV-2 RBD protein in PBS buffer. The plates were
239 then blocked with 100 µl Chonblock buffer (Chondrex Inc, Redmon, US) at room
240 temperature for 1 hours. After washing, 100 ng of ACE2 protein was added into plate
241 and incubated at 37°C for 2 hours, followed by another 2 hours of 1:200 diluted mouse
242 plasma samples incubation. After three extensive washes with PBS containing 0.1%
243 Tween 20, each well was incubated with the HRP goat anti-mouse secondary antibody
244 (1:5000, Beyotime Biotechnology) for 1 hour at 37°C. The ELISA plates were then
245 washed five times with PBS containing 0.1% Tween 20. Subsequently, 100 µL of TMB
246 buffer (Ncm TMB One; New Cell & Molecular Biotech Co., Ltd) was added into each well.

After 15 minutes incubation, 50 µL of 2 M H₂SO₄ solution was added to stop the reaction and the plates were analyzed on a Sunrise absorbance microplate reader (Tecan, Männedorf, Switzerland) at 450 nm wavelength.

Plaque reduction neutralization test (PRNT)

Plasma samples were two-fold diluted starting from a 1:10 dilution and mixed with equal volumes of 120 plaque-forming units (pfu) of SARS-CoV-2 as determined by Vero E6 cells. After 1 hour incubation at 37°C, the plasma-virus mixture were added onto Vero E6 monolayers seated in a 24-well cell culture plate and incubated for 1 hour at 37°C with 5% CO₂. The plasma-virus mixtures were then discarded and infected Vero E6 cells were immediately covered with 1% agarose gel in DMEM medium. After incubation for 3 days at 37°C with 5% CO₂, the plates were formalin fixed and stained by 0.5% crystal violet solution. Neutralization titers were determined by the highest plasma dilution that resulted in >90% reduction in the number of pfus. The test was performed in a BSL3 facility in the University of Hong Kong.

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310

Figure 1

Prime	SARS-CoV	SARS-CoV	SARS-CoV-2	SARS-CoV-2
Boost	SARS-CoV	SARS-CoV-2	SARS-CoV-2	SARS-CoV

Figure 2

Figure S1