Immune Netw. 2023 Apr;23(2):e19
https://doi.org/10.4110/in.2023.23.e19
pISSN 1598-2629·eISSN 2092-6685

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Brief Communication

Pre-existing Immunity to Endemic Human Coronaviruses Does Not Affect the Immune Response to SARS-CoV-2 Spike in a Murine Vaccination Model

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Received: Sep 30, 2022
Revised: Feb 19, 2023
Accepted: Mar 7, 2023
Published online: Mar 31, 2023

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
COVID-19, coronavirus disease 2019; HCoV, human coronavirus; HEK, human embryonic kidney; KRIBB, Korea Research Institute of

ABSTRACT

Endemic human coronaviruses (HCoVs) have been evidenced to be cross-reactive to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Although a correlation exists between the immunological memory to HCoVs and coronavirus disease 2019 (COVID-19) severity, there is little experimental evidence for the effects of HCoV memory on the efficacy of COVID-19 vaccines. Here, we investigated the Ag-specific immune response to COVID-19 vaccines in the presence or absence of immunological memory against HCoV spike Ags in a mouse model. Pre-existing immunity against HCoV did not affect the COVID-19 vaccine-mediated humoral response with regard to Ag-specific total IgG and neutralizing Ab levels. The specific T cell response to the COVID-19 vaccine Ag was also unaltered, regardless of pre-exposure to HCoV spike Ags. Taken together, our data suggest that COVID-19 vaccines elicit comparable immunity regardless of immunological memory to spike of endemic HCoVs in a mouse model.

Keywords: Human coronavirus (HCoV); Pre-existing immunity; SARS-CoV-2; COVID-19 vaccine; Cross-reactivity

INTRODUCTION

The emergence of the novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has posed an unprecedented threat to public health and the economy since its outbreak in 2019. Infection with SARS-CoV-2 causes a wide spectrum of disease, from asymptomatic to fatal, often accompanied by multi-organ failure which may last for several months (1). A SARS-CoV-2-reactive immune response has been detected in pre-pandemic human samples, which raises the possibility of cross-reactivity between endemic human coronaviruses (HCoVs) causing a common cold and SARS-CoV-2 (2-4). While some studies have reported the pathological role of HCoV immunity in coronavirus disease 2019 (COVID-19) severity (5-7), others have concluded that HCoV immunity ameliorates COVID-19 disease (8-10). Although the impact of pre-existing HCoV immunity on disease severity is unclear at this moment due to the inconsistent results from human samples, there is still clear evidence of cross-reactivity between HCoVs and SARS-CoV-2.

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Bioscience and Biotechnology; RBD, receptor binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sVNT, surrogate virus neutralization test; TMB, tetramethylbenzidine.

Author Contributions

Conceptualization: Jeong AY, Kim DJ; Data curation: Jeong AY, Kim DJ; Funding acquisition: Kim DJ; Investigation: Jeong AY, Lee P; Methodology: Jeong AY, Kim DJ; Supervision: Kim DJ; Validation: Jeong AY, Lee P, Lee MS, Kim DJ; Writing - original draft: Jeong AY; Writing - review & editing: Kim DJ.

Cross-reactive immunological memory is an important consideration in vaccine development because it can have both beneficial and detrimental effects on the immune response elicited by vaccination. For instance, pre-existing immunity can be recalled for expansion, boosting the cross-reactive vaccine-induced immune response (11,12). Conversely, secondary expansion of pre-existing memory can limit the magnitude and diversity of the primary immune response to a cross-reactive yet distinct vaccine Ag. This original antigenic sin, or immune imprinting, can impede the development of protective immunity and reduce the efficacy of vaccination (13-15).

Although COVID-19 vaccines have shown significant efficacy in most populations, it is still questionable whether pre-existing immunity to HCoVs influences the efficacy of COVID-19 vaccines. In the present study, we examined the immune response induced by either DNA- or protein-based COVID-19 vaccines in the presence or absence of pre-existing immunity to HCoV spike Ags.

MATERIALS AND METHODS

Construction of plasmid DNA

The full-length coding sequence of the spike genes of HCoV-NL63 (GenBank: KX179496.1, nucleotides 64-4134) and HCoV-OC43 (GenBank: KF572823.1, nucleotides 1-4077) were synthesized *in vitro* (Macrogen, Seoul, Korea) after codon optimization for mice. The codon-optimized SARS-CoV-2 spike gene was purchased from Sino Biological (Beijing, China). Sequences designed for NL63, OC43, and SARS-CoV-2 spike proteins were subcloned into the mammalian expression vector pGX-10 and prepared using the EndoFree Plasmid Maxi Kit (QIAGEN, Hilden, Germany).

Cells

Human embryonic kidney (HEK) 293T cells (ATCC® CLR-3216™) were cultured in DMEM (Corning, Corning, NY, USA) supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific). Cells were maintained at 37°C in a 5% CO_2 atmosphere.

In vitro protein expression and western blotting

HEK293T cells were transiently transfected with 1.5 μg of each construct. After incubation for 48 h, cells were lysed with CETi lysis buffer (TransLab, Daejeon, Korea), and 30 μg of total cell protein was separated by 10% SDS-PAGE. After transferring the proteins to polyvinylidene difluoride membranes (Merck Millipore, Burlington, MA, USA), the blots were subsequently incubated with mouse anti-spike (SARS-CoV-2) (Sino Biological), rabbit anti-spike (NL63) (MyBioSource, San Diego, CA, USA), or rabbit anti-spike (OC43) (Cusabio, Houston, TX, USA) antibodies. The membrane was further incubated with HRP-conjugated secondary anti-mouse or anti-rabbit IgG antibodies (Cell Signaling Technology, Danvers, MA, USA). Bound antibodies were visualized using Immobilon Forte Western HRP substrate (Merck Millipore), and imaged using an EZ-Capture II (ATTO, Tokyo, Japan).

Mice

Seven-week-old female C57BL/6N mice were purchased from KOATECH (Pyeongtaek, Korea) and housed in a specific pathogen-free facility at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). After a week of adaptation, animal experiments were carried out

following the guidelines and approval of the Institutional Animal Use and Care Committee of the KRIBB (KRIBB-AEC-22197).

Immunization

To establish HCoV-specific immunity, mice were immunized with 10 μg of DNA constructs expressing the spike of either NL63 or OC43. Plasmid DNAs were injected into quadriceps muscle followed by *in vivo* electroporation using an ECM 830 electroporator (BTX, Holliston, MA, USA). Five weeks after the final injection of HCoV spike Ags, COVID-19 vaccines were administered twice at 3-wk intervals. The DNA vaccine was injected in the same way as the HCoV injection, and protein vaccine was prepared by mixing 10 μg of spike protein (Sino Biological) with Imject Alum (Thermo Fisher Scientific) at a 1:1 ratio.

ELISA

Serum ELISA was performed as described previously (16). Briefly, plates coated with recombinant proteins (1 μg/ml) were incubated with serum samples, followed by HRP-conjugated anti-mouse IgG secondary antibodies (Cell Signaling Technology). Plates were developed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate (BD Bioscience, Franklin Lakes, NJ, USA), and absorbance was measured at 450 nm using a SpectraMax® 190 (Molecular Device, San Jose, CA, USA). The coating Ags for each assay are as follows: NL63 spike (#40604-V08B; Sino Biological), OC43 spike (#40607-V08B; Sino Biological), SARS-CoV-2 receptor binding domain (RBD) (#40592-V08H; Sino Biological), S1 spike (#40591-V08H; Sino Biological), and S1+S2 extracellular domain spike (#40589-V08H4; Sino Biological).

SARS-CoV-2 surrogate virus neutralization test (sVNT)

Antibodies with potential neutralizing activity against SARS-CoV-2 were assayed using the SARS-CoV-2 sVNT kit (GenScript, Piscataway, NJ, USA) according to the manufacturer's instructions. Serum samples were incubated with HRP-conjugated RBD, which was then added to an ACE2-precoated 96-well plate. Plates were developed using TMB substrate and the absorbance was measured at 450 nm using a SpectraMax®190. Inhibition rates were calculated using the formula: $[1-(OD_{sample}/OD_{negative\ control})]\times100\%$.

ELISPOT

The frequency of spike-specific IFN-γ-producing cells was determined as described previously (16). Briefly, 1×10^6 splenocytes were stimulated for 24 or 48 h with one of the following peptide pools: NL63 spike (JPT, Berlin, Germany), OC43 spike (JPT), SARS-CoV-2 RBD (Miltenyi Biotec, Bergisch Gladbach, Germany), S1 (Miltenyi Biotec), or full-length spike (Miltenyi Biotec). After further incubation with biotin-conjugated IFN-γ detection Ab and HRP-conjugated streptavidin, spots were visualized by adding 3-amino-9-ethyl-carbazole substrates. Spots were counted using a BioSpot analyzer (Immunospot, Cleveland, OH, USA).

Statistical analysis

Statistical analysis was performed using a 2-tailed Student's *t*-test and Mann-Whitney *U* test in Prism software (version 9; GraphPad Software, La Jolla, CA, USA). Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Injection with HCoV spike Ags establishes Ag-specific humoral and cellular immunity in mice

Although the impact of pre-existing HCoV immunity on COVID-19 severity has been actively discussed in recent reports (4,5,9), little information is available regarding its influence on the efficacy of COVID-19 vaccines. To establish pre-existing HCoV immunity in an experimental murine model, we constructed NL63 and OC43 spike protein-expressing plasmids (**Fig. 1A and B**), and injected them into mice (**Fig. 1C**). Then, we observed each spike-specific IgG and IFN-γ-secreting T cell responses (**Fig. 1D-G**), confirming the establishment of HCoV immunity in our murine model.

We established anti-HCoV immunity in mice through vaccination, which elicit immune responses to a defined Ag. It could be difficult to differentiate the influence of a particular HCoV on COVID-19 vaccine efficacy in humans, because most individuals have been exposed to at least one of the 4 common cold HCoVs (7,17). Infection with HCoV is also unsuitable for vaccine studies in mice because, unlike in humans, OC43 infects the murine central nervous system, which is accompanied by severe pathological signs including skeletal muscle atrophy and forelimb paralysis (18). Among the 4 HCoVs, we selected NL63 and OC43, which belong to the alpha- and beta-coronavirus genera, respectively, because these 2 constitute the majority of respiratory infections leading to hospitalization in human (19).

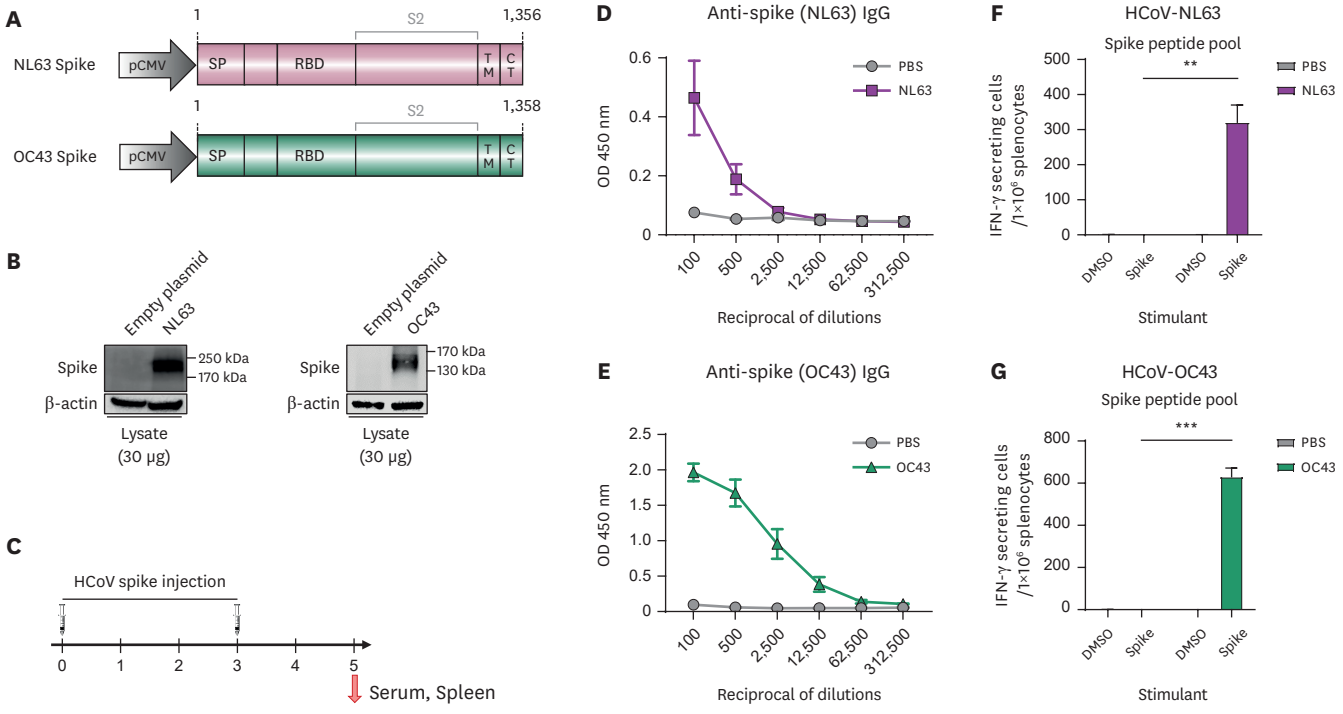

Figure 1. Injection with HCoV spike Ags establishes Ag-specific humoral and cellular immunity in mice. (A) Schematic diagram of the NL63 and OC43 DNA constructs containing a spike protein insert. (B) *In vitro* expression of spike proteins from HEK 293T cells transfected with NL63 spike, OC43 spike, or empty plasmid. Spike protein expression was detected by SDS-PAGE and western blotting, using an Ab specific to each HCoV spike. (C) Schematic representation of vaccination and immunological analysis. C57BL/6 mice were injected with 10 μg of NL63 or OC43 spike-expressing plasmid DNAs (n=6). Injections were administered twice, and samples were isolated 2 wk after the booster injection. (D-E) Serial serum dilutions from boosted mice using ELISA to detect: (D) NL63 or (E) OC43 spike–specific IgG. Data represent the mean OD 450 nm values (mean ± SEM) for each group of 6 mice. (F, G) T cell response was evaluated by the IFN-γ ELISPOT assay. Splenocytes were stimulated for 24 h with overlapping peptide pools spanning the spikes of (F) NL63 or (G) OC43.
pCMV, porcine cytomegalovirus; SP, signal peptide; TM, transmembrane domain; CT, cytoplasmic tail.
**p<0.01, ***p<0.001.

https://doi.org/10.4110/in.2023.23.e19

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Pre-existing immunity to HCoV spike does not affect humoral response to protein-based COVID-19 vaccine

To assess the impact of HCoV immunity on COVID-19 vaccines, we first investigated the humoral and cellular responses to the recombinant spike protein of SARS-CoV-2 in mice previously immunized with HCoV spike Ags, as described previously (**Fig. 2A**). We found that in sera acquired 2 wk after the primary and booster COVID-19 vaccination, pre-existing immunity to NL63 and OC43 spike Ags did not affect the SARS-CoV-2 RBD-, S1-, and spike-specific total IgG responses (**Fig. 2B and C**). Since neutralizing Ab levels are the primary indicator of the protective efficacy of COVID-19 vaccines (20), we performed sVNT with the sera. The neutralizing Ab levels upon priming and boosting with a COVID-19 protein vaccine were not also affected by pre-existing immunity against NL63 or OC43 (**Fig. 2D**).

Given that HCoV-reactive T cells expand upon SARS-CoV-2 infection in humans (9), HCoV immunity may influence the cellular response to COVID-19 vaccines. We therefore determined the frequency of Ag-specific interferon-γ-secreting T cells by stimulating splenocytes with overlapping peptide pools of either RBD-, S1-, or full spike protein from SARS-CoV-2. However, immunization with recombinant spike protein in combination with an alum adjuvant was not efficient for the induction of Ag-specific T cell response; thus, the impact of pre-existing HCoV immunity on the cellular response to the COVID-19 protein vaccine could not be verified (data not shown).

Pre-existing immunity to HCoV spike does not affect humoral and cellular responses to DNA-based COVID-19 vaccine

Along with cross-reactive antibodies, the importance of cross-reactive T cells and their potential contribution to the protection against SARS-CoV-2 has been discussed in detail previously (10,21,22). We used the DNA vaccine platform to evaluate the impact of HCoV immunity on the cellular response to COVID-19 vaccines, based on the ability of DNA vaccines to drive robust cellular immunity (23). We designed a DNA vaccine construct with the spike gene of SARS-CoV-2 and verified the appropriate expression of the spike protein (**Fig. 3A and B**). Again, mice established with HCoV immunity were immunized twice with the COVID-19 DNA vaccine (**Fig. 3C**). Evaluations on the impact of HCoV immunity on the humoral response to the COVID-19 DNA vaccine showed no significant change in RBD-, S1-, or spike-specific IgG levels after priming and boosting (**Fig. 3D and E**). The level of neutralizing Ab was also not affected by prior-injection with NL63 or OC43 spike (**Fig. 3F**). Finally, we evaluated the vaccine-mediated cellular response, and observed that immunization with the COVID-19 DNA vaccine induced a robust T cell response against RBD, S1, and spikes. However, the frequencies of IFN-γ-secreting T cells were comparable among the groups prior-injected with PBS, NL63, and OC43 (**Fig. 3G and H**). Taken together, these data clarify that the COVID-19 DNA vaccine induces humoral and cellular responses at comparable levels irrespective of imprinted HCoV immunity.

Most individuals possess immunity against at least one of the 4 HCoVs (7), which are partially reactive to SARS-CoV-2. Although the influence of pre-existing HCoV immunity on SARS-CoV-2 infection has been appreciated (4,6,10), its potential effects on COVID-19 vaccine efficacy have not yet been addressed (24). In our current study, we evaluated COVID-19 vaccine outcomes in the presence of HCoV immunity and found that pre-existing immunity to HCoV did not have an influence on quantitative or qualitative aspects of the immune response induced by COVID-19 vaccines.

https://doi.org/10.4110/in.2023.23.e19

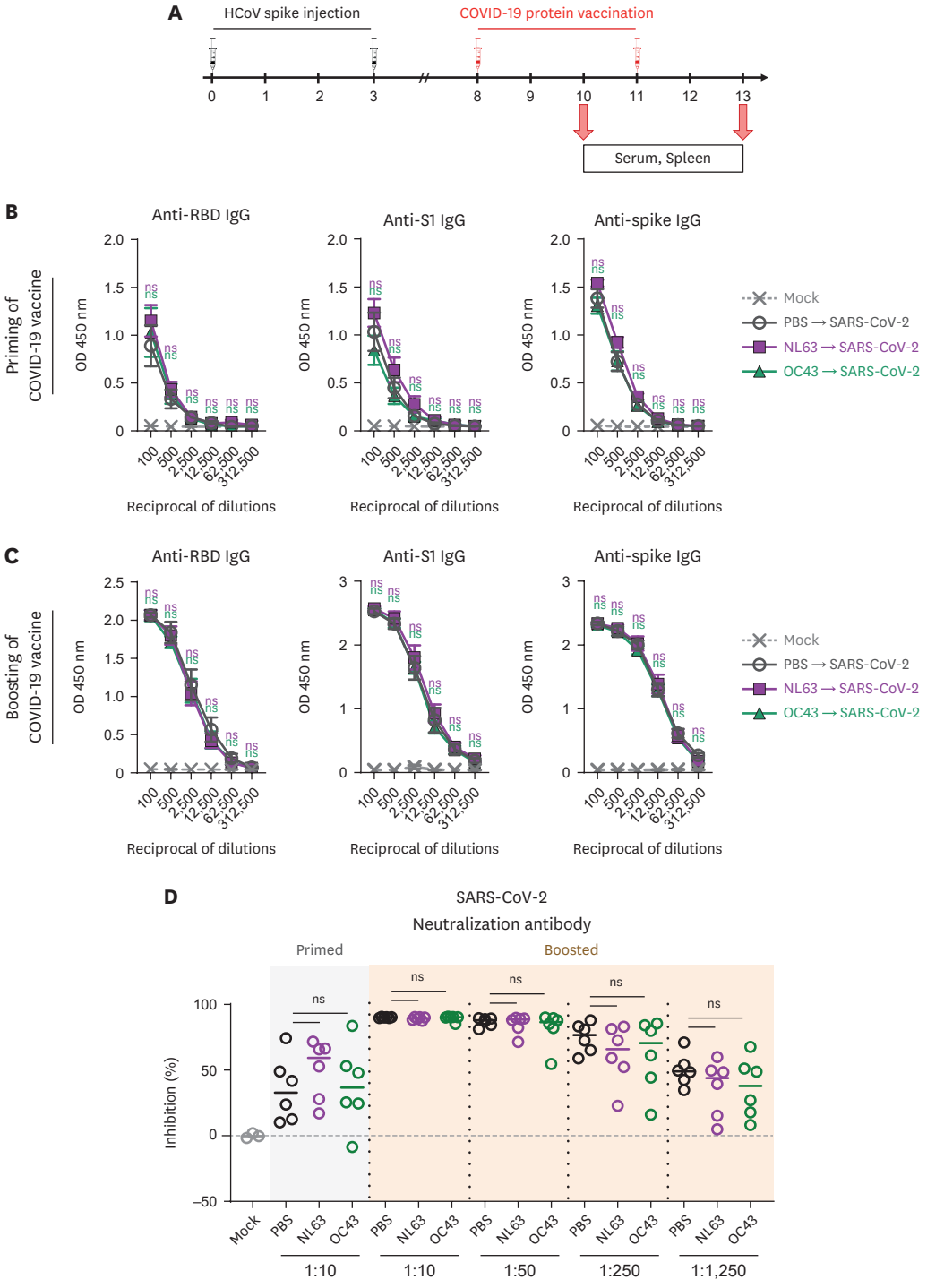

Figure 2. Pre-existing immunity to HCoV spike does not affect humoral response to a protein-based COVID-19 vaccine. C57BL/6N mice pre-established with HCoV were vaccinated with 10 μg alum-adjuvanted COVID-19 protein vaccine. (A) Immunization regimen for COVID-19 protein vaccine (n=6). (B, C) Detection of SARS-CoV-2 RBD-, S1-, or spike-specific IgG by ELISA using sera acquired after priming (B) or boosting (C). Data represent the mean OD 450 nm values (mean ± SEM) for each group of 6 mice. (D) Evaluation of neutralizing antibodies from diluted sera using the SARS-CoV-2 sVNT.
ns, not significant.

Previous studies have reported both the pathological and protective roles of HCoV immunity in the severity of SARS-CoV-2 infection (5,7,8,25-27). Unlike vaccines intended to induce

https://doi.org/10.4110/in.2023.23.e19

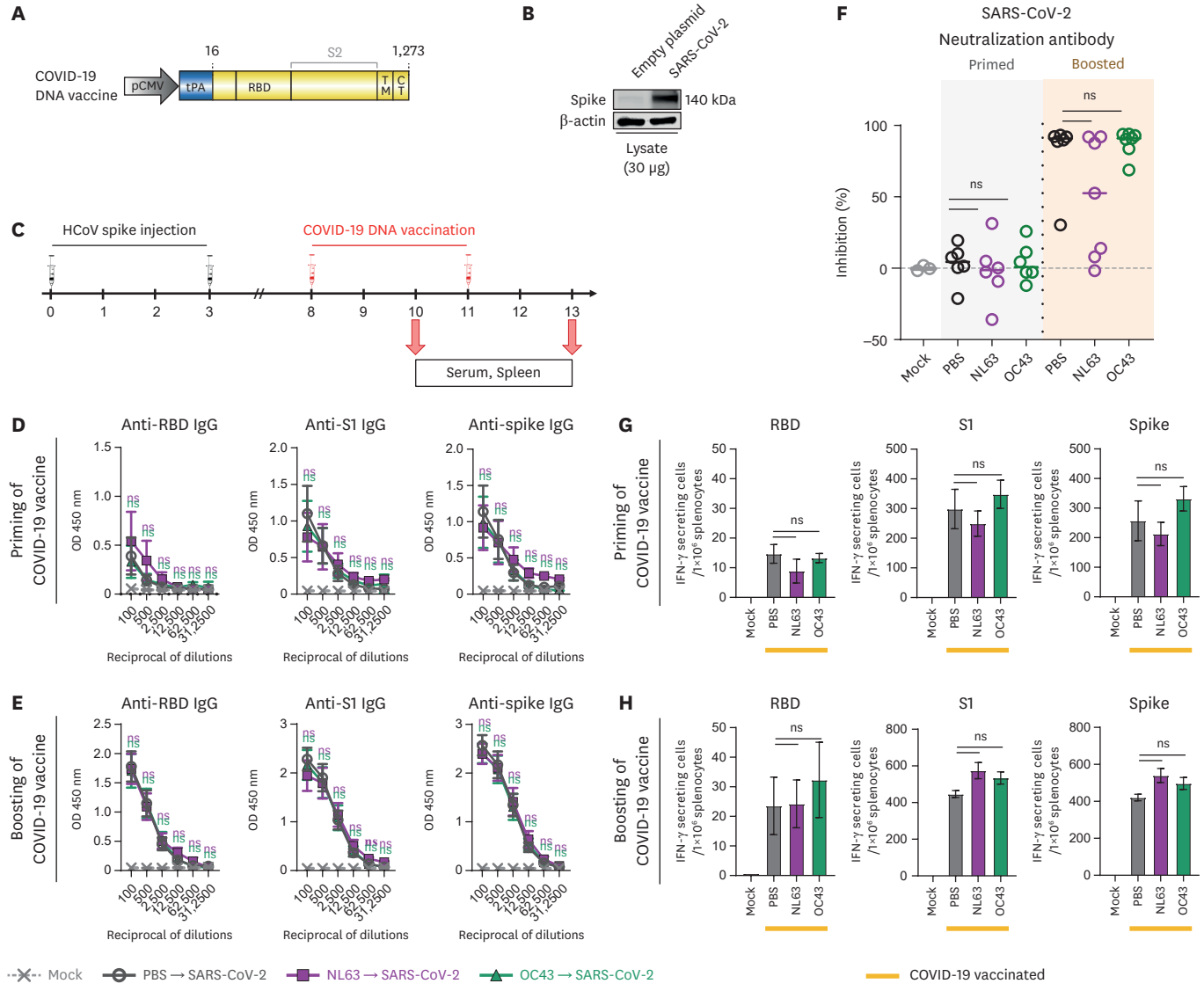

Figure 3. Pre-existing immunity to HCoV spike does not affect humoral and cellular responses to a DNA-based COVID-19 vaccine. C57BL/6N mice pre-established with HCoV were vaccinated with 10 μg of COVID-19 DNA vaccine. (A) Schematic diagram of the synthetic COVID-19 DNA vaccine. (B) *In vitro* expression of spike protein from HEK 293T cells transfected with the COVID-19 DNA construct or empty plasmid. Spike protein expression was detected by SDS-PAGE and western blotting using a SARS-CoV-2-specific Ab. (C) Immunization regimen for COVID-19 DNA vaccine (n=6). (D, E) Detection of SARS-CoV-2 RBD-, S1-, or spike-specific IgG by ELISA using sera acquired after priming (D) or boosting (E). Data represent the mean OD 450 nm values (mean ± SEM) for each group of 6 mice. (F) Evaluation of neutralizing antibodies from diluted sera using the SARS-CoV-2 sVNT. (G, H) Splenocytes isolated after priming (G) or boosting (H) were incubated with overlapping peptide pools spanning SARS-CoV-2 RBD, S1, or spike for 24 h.
pCMV, porcine cytomegalovirus; tPA, tissue plasminogen activator; ns, not significant.

an immune response specifically targeted to a defined Ag, infection with SARS-CoV-2 accompanies responses to other viral proteins, such as the nucleocapsid, which shows even higher sequential homology among coronaviruses than the spike protein (28). Considering this, it is probable that the difference between our observations and those of previous reports stems from the integrated immune response to other viral proteins.

In the midst of constantly emerging SARS-CoV-2 variants, the booster vaccination strategy has been updated to use the bivalent vaccines containing both the ancestral and omicron strains of SARS-CoV-2. One concern regarding this variant-matched boost was the

preferential recall response of the ancestral Wuhan-Hu-1-specific immune memory, rather than the priming of the omicron-specific Ab response. Yet, despite the high conservation among spikes of the Wuhan and variant strains, the booster vaccines have been showing more improved neutralizing efficacy against target strains than the booster of the original monovalent vaccine does (29,30). Likewise, we sought whether immune imprinting by HCoVs interferes with the immunogenicity of COVID-19 vaccines and revealed that vaccination with SARS-CoV-2 spike Ag elicited comparable immunity regardless of the HCoV immune memory. Considering the genetic distance between endemic HCoVs and SARS-CoV-2 spikes (31), it seems improbable that HCoV immunity may influence the development of COVID-19 vaccine-mediated responses.

The major limitation of this study lies in the establishment of anti-HCoV immunity. In general, natural viral infections induce immune responses skewed towards the Th1 response along with cytotoxic T cells as part of antiviral immunity (32,33). To mimic the infection-like immunity as much as possible through vaccination, we adopted a DNA vaccine platform to utilize its Th1-biased systemic immune responses (34-36). However, the characteristics of the systemic immunity produced by natural infection with seasonal HCoVs remain incompletely understood, even in humans, likely leading to differences between respiratory infection and vaccination. In this regard, our results should be interpreted with caution. With the recent establishment of mouse-adapted HCoV-OC43 strain capable of inducing respiratory symptoms (37), we believe further studies could be carried out using the virus and natural infection model to validate our observation in this study.

In conclusion, the present study demonstrates that COVID-19 vaccines elicit comparable immunity regardless of immunological memory to spike of endemic HCoVs, despite the presence of sequence homology between HCoVs and SARS-CoV-2 spike. Our observations provide insights that may have an impact on future vaccine strategies by excluding the potential effects of HCoV immunity on COVID-19 vaccine quality.

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea (NRF) grants funded by the Government of Korea (MSIT: 2018M3A9H4077992) and the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Research Initiative Programs (KGM9942213 and KGM1062211).

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