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VACCINES AND ANTIVIRAL AGENTS

Inactivated SARS-CoV-2 Vaccine Shows Cross-Protection against Bat SARS-Related Coronaviruses in Human ACE2 Transgenic Mice

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ABSTRACT Severe acute respiratory syndrome coronavirus (SARS-CoV-1) and SARS-CoV-2 are highly pathogenic to humans and have caused pandemics in 2003 and 2019, respectively. Genetically diverse SARS-related coronaviruses (SARSr-CoVs) have been detected or isolated from bats, and some of these viruses have been demonstrated to utilize human angiotensin-converting enzyme 2 (ACE2) as a receptor and to have the potential to spill over to humans. A pan-sarbecovirus vaccine that provides protection against SARSr-CoV infection is urgently needed. In this study, we evaluated the protective efficacy of an inactivated SARS-CoV-2 vaccine against recombinant SARSr-CoVs carrying two different spike proteins (named rWIV1 and rRsSHC014S, respectively). Although serum neutralizing assays showed limited cross-reactivity between the three viruses, the inactivated SARS-CoV-2 vaccine provided full protection against SARS-CoV-2 and rWIV1 and partial protection against rRsSHC014S infection in human ACE2 transgenic mice. Passive transfer of SARS-CoV-2-vaccinated mouse sera provided low protection for rWIV1 but not for rRsSHC014S infection in human ACE2 mice. A specific cellular immune response induced by WIV1 membrane protein peptides was detected in the vaccinated animals, which may explain the cross-protection of the inactivated vaccine. This study shows the possibility of developing a pan-sarbecovirus vaccine against SARSr-CoVs for future preparedness.

IMPORTANCE The genetic diversity of SARSr-CoVs in wildlife and their potential risk of cross-species infection highlight the necessity of developing wide-spectrum vaccines against infection of various SARSr-CoVs. In this study, we tested the protective efficacy of the SARS-CoV-2 inactivated vaccine (IAV) against two SARSr-CoVs with different spike proteins in human ACE2 transgenic mice. We demonstrate that the SARS-CoV-2 IAV provides full protection against rWIV1 and partial protection against rRsSHC014S. The T-cell response stimulated by the M protein may account for the cross protection against heterogeneous SARSr-CoVs. Our findings suggest the feasibility of the development of pan-sarbecovirus vaccines, which can be a strategy of preparedness for future outbreaks caused by novel SARSr-CoVs from wildlife.

KEYWORDS bat SARS-related coronavirus, SARS-CoV-2, inactivated vaccine, cross-protection

In the past 2 decades, two SARS-related coronaviruses (SARSr-CoVs), SARS-CoV-1 and SARS-CoV-2, have caused severe respiratory diseases in humans, namely, SARS and COVID-19 (1–5). SARS-CoV-1 broke out in southern China in late 2002 and rapidly spread to more than 30 countries and regions within 6 months, resulting in 8,098 human infections and 774

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deaths (6). Epidemiological investigation indicated that palm civet was the major intermediate host of SARS-CoV-1, which led to the massive culling of wild animals in the markets and successful elimination of SARS (7, 8). Seventeen years later, a novel coronavirus named SARS-CoV-2, which was first discovered in the city of Wuhan, China, caused a pandemic of viral pneumonia (3). Similar to SARS-CoV-1, SARS-CoV-2 is transmitted through direct or indirect contact of mucous membranes with infectious respiratory droplets (9, 10). During the pandemic, SARS-CoV-2 rapidly underwent adaptive mutations in humans and became substantially more transmissible (11, 12). It is claimed that, to date, more than 5 million patients have died of COVID-19 (13). Although a number of repurposed drugs exhibit inhibitory activity against SARS-CoV-2 replication *in vivo* or *in vitro*, a clinically specific treatment for severe COVID-19 patients is still not available (14).

Increasing evidence suggests that the ancestor of SARS-CoV-1 and SARS-CoV-2 likely originated from bats; genetically diverse SARSr-CoVs have been identified in *Rhinolophus* bats from China, Southeast Asia, Europe, and Africa (15–22). Our previous studies showed that SARS-CoV-1-related viruses, including bat SARSr-CoV WIV1 and RsSHC014, utilize angiotensin-converting enzyme 2 (ACE2) as a cellular receptor and replicate efficiently in human airway epithelial cells, as well as human ACE2 transgenic mice, indicating their potential to spill over to humans (18, 23–25).

SARS-CoV-2 shares 77 to 79% whole-genome sequence identity and 78% amino acid (aa) identity in the spike (S) protein with WIV1 and RsSHC014. The S protein of WIV1 is highly similar to that of SARS-CoV-1 but differs from that of RsSHC014 in the receptor-binding domain (RBD), sharing 83% aa identity. Previous studies have shown that WIV1, but not rRsSHC014S, can be neutralized by SARS-CoV-1 monoclonal and polyclonal antibodies (26). Hyperimmunized or convalescent-phase sera to SARS-CoV-1 have limited cross-neutralization activity to SARS-CoV-2 and vice versa (27). However, there are few *in vivo* studies on the cross-protection of distantly related SARSr-CoVs by a SARS-CoV-2 vaccine. We previously constructed infectious cDNA clones based on the WIV1 backbone and constructed recombinant viruses that carry the S gene of bat SARSr-CoV WIV1 (rWIV1) and RsSHC014 (rRsSHC014S), respectively (28). In the present study, we tested the cross-protection of a previously developed inactivated vaccine (IAV) against SARS-CoV-2 against these two bat viruses (29–31). We expected our results to pave the way to a strategy for developing pan-sarbecovirus vaccines against SARSr-CoVs.

RESULTS

SARS-CoV-2 IAV provides full protection against rWIV1 infection but partial protection against rRsSHC014S infection in HFH4-hACE2 mice. Six- to eight-week-old HFH4-hACE2 mice were intraperitoneally immunized with 5 μg of IAV and 0.5 mg of aluminum hydroxide (vaccine group) or 0.5 mg of aluminum hydroxide with phosphate-buffered saline (PBS; adjuvant group) according to the D0/D14 program. At 16 days postboost, the mice were challenged with 10^5 PFU of rWIV1, rRsSHC014S, or SARS-CoV-2. Each group comprised six mice. Body weight was monitored daily, and mice were euthanized at 2 or 6 days postinfection (dpi) (Fig. 1A). Upon infection, there were no distinct body weight decreases in the three vaccinated groups, whereas some animals in the adjuvant groups showed rapid body weight loss (Fig. 1B). Viral RNA copies and titers in the lungs were quantified by quantitative reverse-transcription PCR (qRT-PCR) and plaque assays, respectively. The results showed that viral RNA copies and titers in the mouse lungs were substantially lower in the vaccinated groups than in the adjuvant groups. However, in mice in the rRsSHC014S-challenged group, live virus and viral RNA were still detectable at 6 dpi (Fig. 1C and D). Except for one rRsSHC014S-challenged mouse showing brain infection at 2 dpi, all other vaccinated mice were protected from neuronal invasion (Fig. 1E).

To evaluate the protective effect against lung damage further, mouse lungs collected at 2 dpi were sectioned and analyzed by hematoxylin and eosin (H&E) staining or immunofluorescence assay (IFA). Surprisingly, pathological changes in mouse lungs were hardly observed in the vaccinated groups. In contrast, bronchiolar epithelial sloughing and few infiltrations in the lungs were observed in all adjuvant groups. Specifically, alveolar wall thickening and minor fibrin exudation were observed in the adjuvant group challenged with rWIV1 (Fig. 1F). Viral antigen was detected in the lungs of mice in the adjuvant groups but was absent or

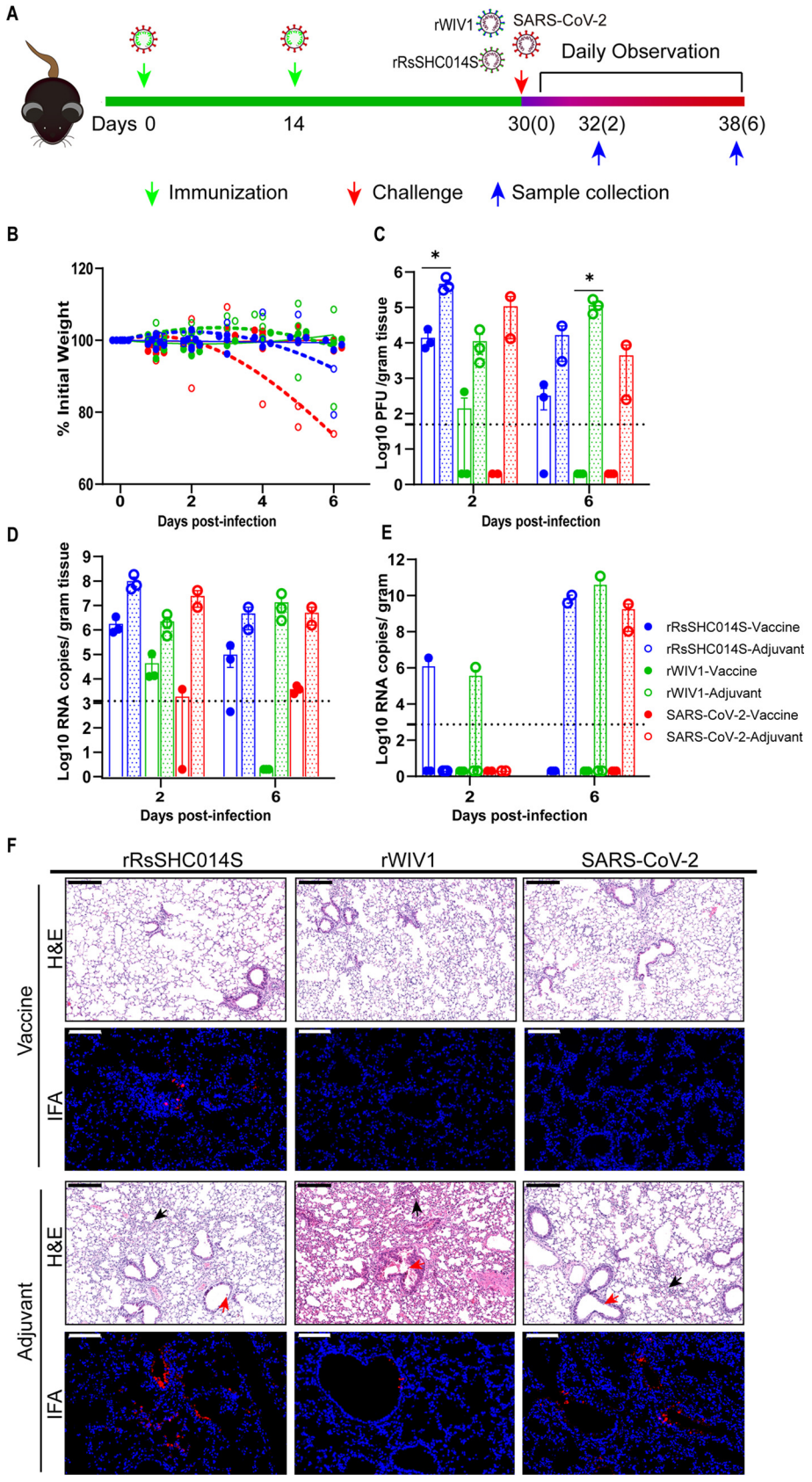

FIG 1 SARS-CoV-2 IAV partially protects mice from bat SARSr-CoV infection. HFH4-hACE2 mice were intraperitoneally immunized with 5 μg of SARS-CoV-2 IAV and 0.5 mg of aluminum hydroxide (vaccine group)

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decreased in mice in the vaccinated groups (Fig. 1F). Despite the differences in viral titer and pathology, the levels of some key cytokines/chemokines in mouse sera at 2 dpi were similar among the vaccinated and adjuvant groups (Fig. 2).

Neutralizing antibody and T-cell response. To better understand the cross-protection mechanism, the virus-specific neutralizing antibody titers and T-cell response were tested. Sera of vaccinated mice were collected after rWIV1 or rRsSHC014S infection, and the cross-neutralization activity titer against SARS-CoV-2 or rWIV1/rRsSHC014S was determined from the 50% plaque reduction/neutralization titer ($PRNT_{50}$). The titer of neutralizing antibody against rWIV1 was significantly lower than that against SARS-CoV-2 (Fig. 3A), whereas there was no detectable $PRNT_{50}$ for neutralizing antibody against rRsSHC014S (Fig. 3B).

We then evaluated the viral structural protein-specific T-cell response in vaccinated mice. To acquire a stronger T-cell response, hACE2 mice were immunized with 5 μg of SARS-CoV-2 IAV following an optimized D0/D21 program. Splenocytes were obtained 5 days post-boost, and a gamma interferon (IFN-γ) intracellular cytokine staining (ICS) assay was performed using peptide pools of viral structural proteins. We found that the peptide pool of membrane (M) protein from SARS-CoV-2 and WIV1 induced a $CD8^+$ IFN-γ^+ T-cell population, suggesting that the cytotoxic effector T cells that specifically recognize SARS-CoV-2 and WIV1 M proteins play an important role in cross-protection (Fig. 3C and D).

Serum immune to SARS-CoV-2 partially protects against rWIV1 but not rRsSHC014S infection. We next assessed passive serum transfer protection in hACE2 mice. Mice in the immunized group were injected with sera collected from BALB/c mice vaccinated with SARS-CoV-2 IAV and pooled. For comparison, sera collected from nonvaccinated BALB/c mice and pooled were used in the control group. The serum $PRNT_{50}$ against SARS-CoV-2 was approximately 1:5,000. Sera from vaccinated (immunized group) or nonvaccinated (control group) mice were intraperitoneally transferred to hACE2 mice 1 day before infection. The mice were infected with 10^5 PFU of SARS-CoV-2, rWIV1, or rRsSHC014S via the intranasal route. All mice were euthanized at 5 dpi, and their lung tissues were harvested and analyzed (Fig. 4A). There were no distinct body weight decreases in any of the immunized groups. In contrast, some animals in the control groups showed rapid body weight loss at 5 dpi (Fig. 4B).

Quantification of viral genome copies in the mouse lungs showed that viral RNA copies in immunized mice infected by rWIV1 and SARS-CoV-2 were significantly lower than those in mice in the control groups. However, there was no significant difference between the immunized and control mice infected by rRsSHC014S (Fig. 4C). The mouse lungs were sectioned and stained with H&E and antiviral antibody. Alveolar inflammatory infiltration and fibrin exudation were observed only in the control mice infected by SARS-CoV-2. In contrast, inflammatory infiltration was observed in immunized and control mice infected by rWIV1 or rRsSHC014S (Fig. 4D). Viral antigen staining indicated the absence of or a decrease in viral replication in the lungs of SARS-CoV-2- and rWIV1-infected mice but not in rRsSHC014S-infected mice (Fig. 4E).

DISCUSSION

COVID-19 has been prevailing in human society for 2 years (13). It is believed that the spread of SARS-CoV-2 will decline in the coming years owing to the global SARS-CoV-2

FIG 1 Legend (Continued)
or 0.5 mg of aluminum hydroxide with PBS (adjuvant group) following a D0/D14 immunization program. At 30 days after the initial injection, the mice were infected with 10^5 PFU of the indicated virus. (A) Experimental scheme. (B) Mouse body weight was monitored for up to 6 dpi. Dotted lines represent the fitted curves for each color-indicated group. Error bars indicate standard errors. (C) Lung viral loads as detected by plaque assays. (D) Lung viral loads as detected by qRT-PCR. (E) Brain viral loads as detected by qRT-PCR. The dotted line indicates the limitation of qRT-PCR detection. (F) Lung pathological changes and viral antigen staining at 2 dpi. Bronchiolar epithelial sloughing (red arrows) and few infiltrations (black arrows) were observed in all mice in the adjuvant groups. Especially, alveolar wall thickening was observed in the rWIV1 adjuvant group. Few pathological changes in the lungs were observed in the vaccinated groups. Viral antigen was detected in all adjuvant groups, whereas no distinct signal was detected in vaccinated mice challenged with SARS-CoV-2 and rWIV1. A weak antigen signal was detected in the lungs of vaccinated mice challenged with rRsSHC014S. Images were acquired using a Pannoramic MIDI system. Black scale bar, 200 μm; white scale bar, 100 μm. Error bars indicate standard errors. Statistical significance was assessed using the Mann-Whitney test (*, $P < 0.05$).

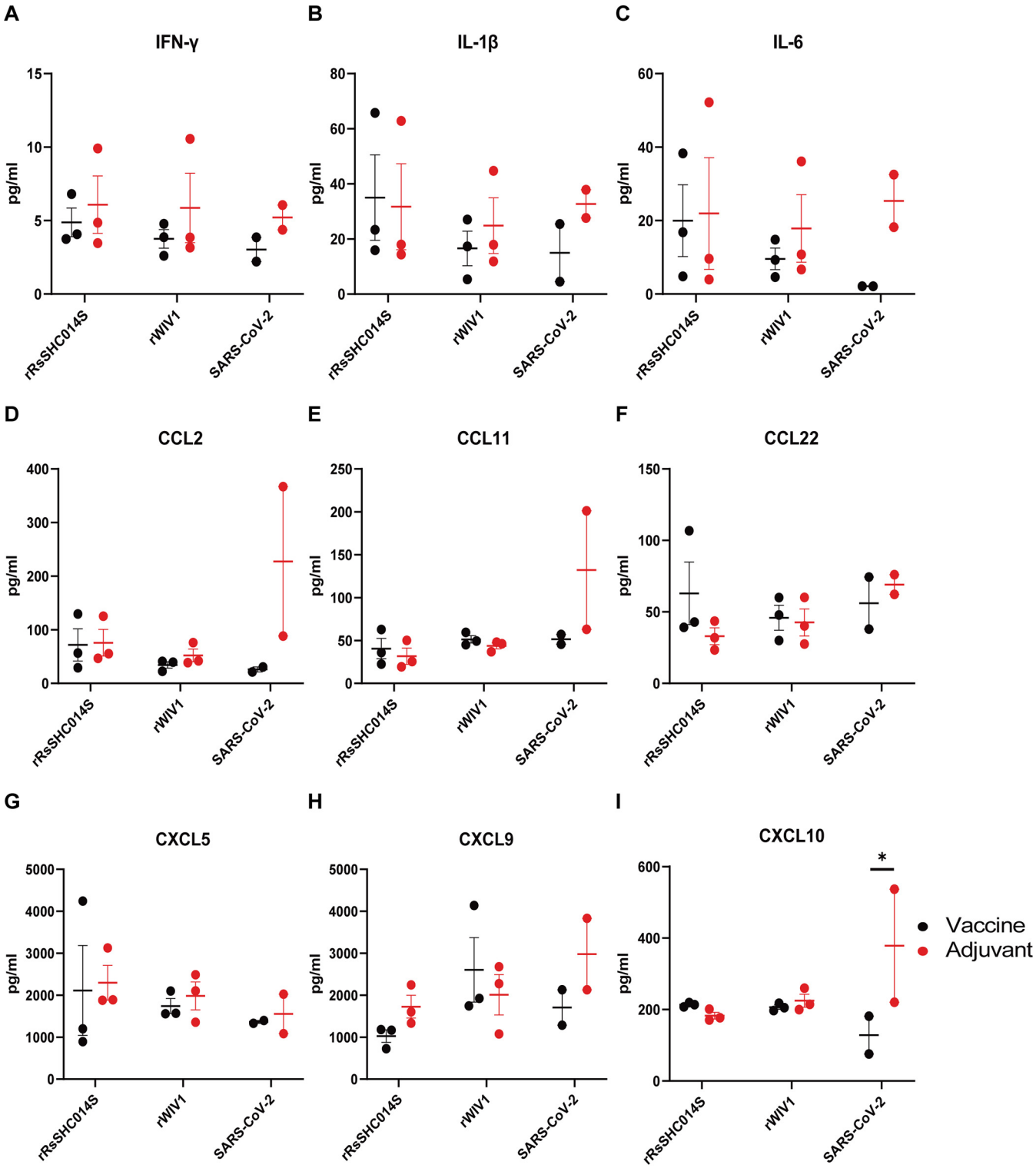

FIG 2 Cytokine/chemokine levels in infected mouse sera. Infected mouse sera at 2 dpi were collected, and the indicated cytokine/chemokine levels were determined using a bead-based flow-cytometric detection assay. Error bars indicate standard errors. Statistical significance was assessed by two-way ANOVA, followed by multicomparison tests (*, $P < 0.05$).

vaccination programs. However, the rapid development and accumulation of mutations in the SARS-CoV-2 genome, particularly in the S protein, challenges the protective efficacy of the current vaccines, which were all developed based on an early epidemic strain. Clinical trial data show that the current vaccines provide effective protection against the following

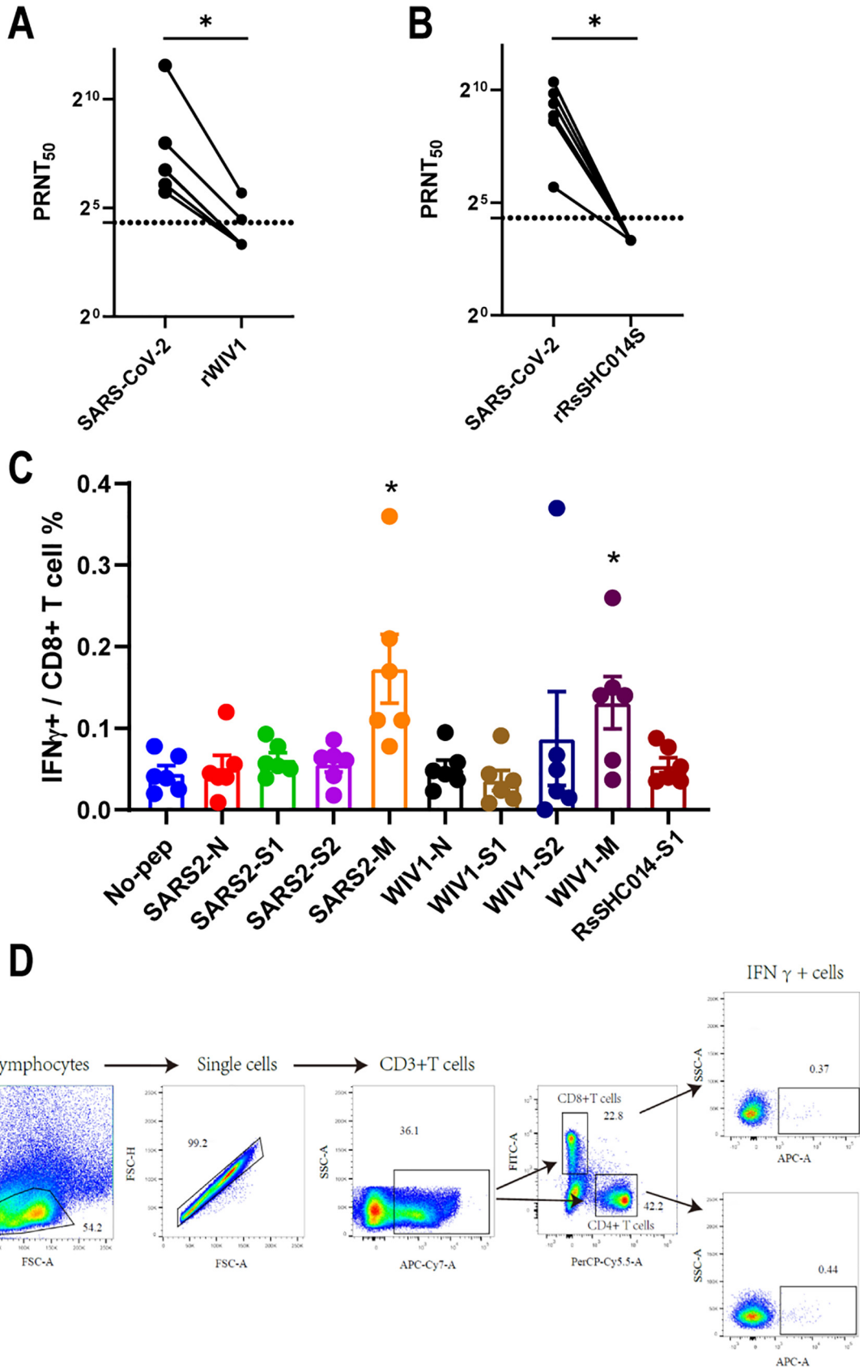

FIG 3 Immune response after vaccination in HFH4-hACE2 mice. (A and B) Sera were collected from infected vaccinated mice, and the $PRNT_{50}$ values against SARS-CoV-2, rWIV1, and rRsSHC014S were determined. (C) An IFN-γ ICS assay was performed using the

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variants of concerns: alpha (B.1.1.7), beta (B.1.351), gamma (P.1), and delta (B.1.617.2), and reduce the risk of infection, hospitalization, and mortality to different levels (32–34). However, the high rate of breakthrough infection caused by variants, particularly the currently dominant delta and omicron variants, raises increasing concern, and there is a need to develop novel therapeutic measures and vaccines with a substantially broader spectrum (35, 36).

Here, we evaluated the cross-protection efficacy of a SARS-CoV-2 IAV against two bat SARSr-CoVs in HFH4-hACE2 mice. We found that the SARS-CoV-2 IAV provides full protection against rWIV1 and partial protection against rRsSHC014S by reducing viral loads in the lungs and brains and evidently suppressing lung damage. A passive serum transfer protection assay using sera of SARS-CoV-2-immunized mice revealed low protection against rWIV1 infection and no protection against rRsSHC014S infection in mice. The $PRNT_{50}$ revealed that neutralizing sera against SARS-CoV-2 exerts marginal cross-neutralization activity only against rWIV1 and not against rRsSHC014S. These data suggest that the neutralizing antibody induced by SARS-CoV-2 IAV has marginal cross-protection activity.

According to the clinical symptoms and the lung viral titers in infected mice, we found that rRsSHC014S was more pathogenic to HFH4-hACE2 mice than rWIV1. This result is in consistent with previous studies in which the mouse-adapted SARS-CoV-based backbone carrying the WIV1 spike (named WIV1-MA15) produced minimal changes in weight loss until late times, whereas the one carrying the RsSHC014 spike (RsSHC014-MA15) infection produced substantial weight loss (10%) in 10-week-old BALB/c mice (24, 25). The spike of RsSHC014 is similar to that of WIV1 in the N-terminal domain but has a distinct RBD, sharing 83 and 76% aa identities to WIV1 and SARS-CoV-2, respectively. The different RBDs of bat SARSr-CoVs may contribute to different pathogenesis *in vivo*. The relatively high pathogenicity of rRsSHC014S may explain the failure of full protection of the SARS-CoV-2 IAV. Our longitudinal surveillance showed that the SARSr-CoVs with the RsSHC014 RBD are circulating in bat populations. Recombination could also occur among RsSHC014-like CoVs and other strains harbored in bats in the same or close colonies (18, 23). Although RsSHC014 is not closely related to SARS-CoV-1 or SARS-CoV-2, this sublineage of SARSr-CoV should never be neglected in both pathogen monitoring and vaccine design in future.

Unlike the mRNA and adenovirus-vectored vaccines that target the S RBD, the SARS-CoV-2 IAV contains full structural proteins, which likely induce a substantially broader immune response. Our results demonstrated that both SARS-CoV-2 and WIV1 M proteins elicit $CD8^+$ T-cell activation, suggesting that the T-cell response plays an important role in the cross-protection against the heterogeneous SARSr-CoVs. This result is consistent with previous study which revealed that the inactivated vaccine (BBIBP-CorV) induced T-cell response targeting not only the S protein but also the N and M proteins (37). Previous studies have shown that the T-cell response has an immunological memory that provides specific defense against reinfection of coronaviruses, including SARS-CoV-1, SARS-CoV-2, and Middle East respiratory syndrome coronavirus, and lasts longer than the antibody response (38, 39). SARSr-CoVs are relatively conserved in the nonstructural proteins encoded by ORF1b and the structural proteins, including the M protein, the envelope protein, and the N protein, as well as the membrane fusion subunit (S2) of the S protein. These conserved proteins can be used as targets for designing vaccines stimulating the T-cell response, which provides cross-protection to diverse SARSr-CoV infections.

SARSr-CoVs are highly divergent in their S proteins, particularly, the RBDs, which are the main targets for developing recombinant protein or vectored vaccines, rendering the design of pan-sarbecovirus vaccines challenging. Although divergent in protein sequences, the S proteins have similar conformational structures that share some common cross-neutralization epitopes. Several teams have identified monoclonal antibodies that exert potent neutralization activity not only to SARS-CoV-2 but also to other SARSr-CoVs, including those

FIG 3 Legend (Continued)
indicated viral structural protein peptide pools. (D) Gating strategy used to analyze T-cell responses. Splenocytes were gated for lymphocytes (FSC-A/SSC-A), single cells (FSC-A/FSC-H), $CD3^+$ (APC-Cy7/SSC-A), and $CD4^+$ or $CD8^+$ (PerCP-Cy5.5/FITC) cells, followed by populations expressing IFN-γ. Error bars indicate standard errors. Statistical significance was assessed using Wilcoxon's matched-pairs signed rank test (A and B) and two-tailed Student *t* tests compared to the no-pep group (C) (*, $P < 0.05$).

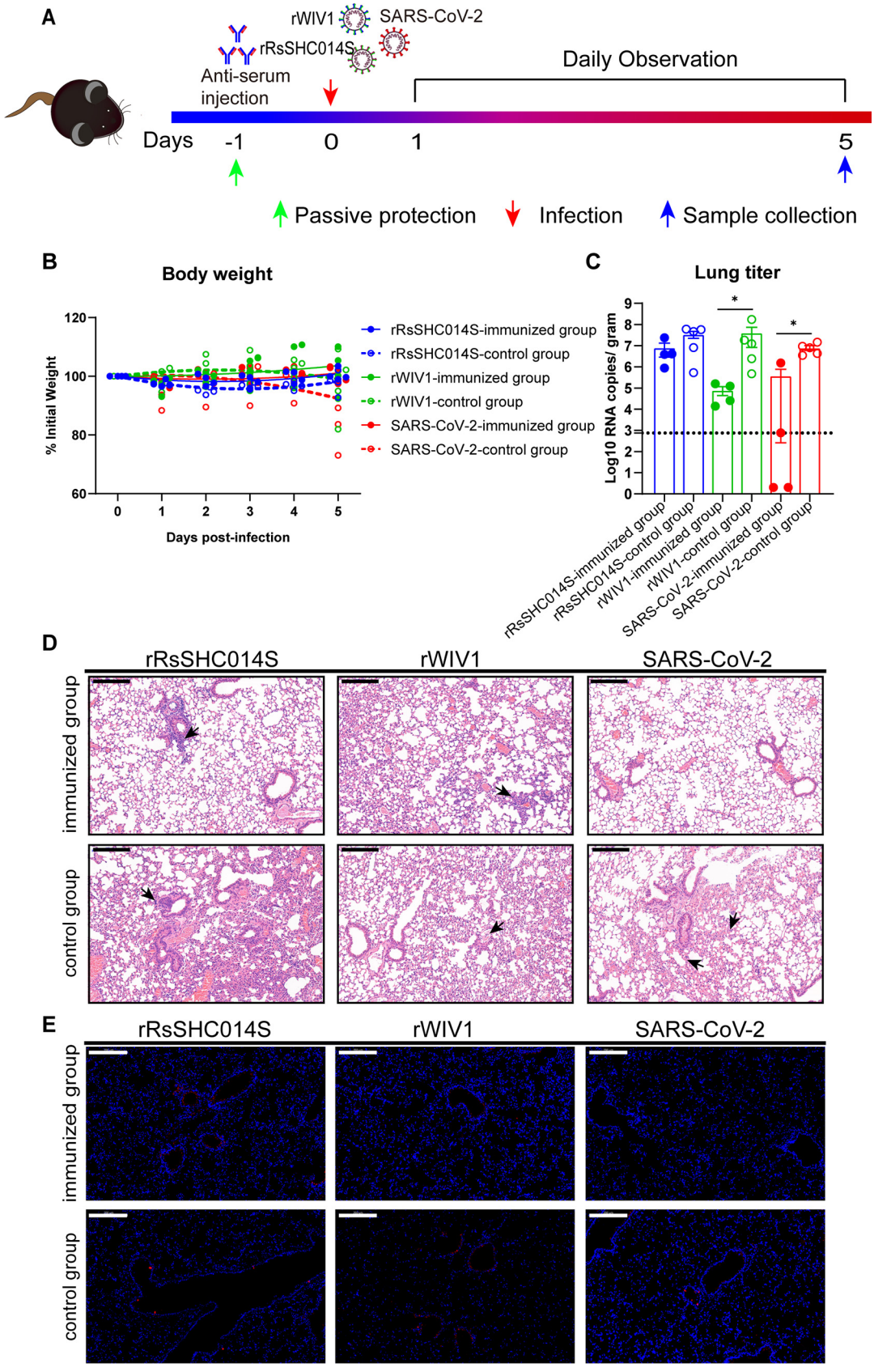

FIG 4 Immunized mouse sera show limited protective efficacy against bat SARSr-CoVs. (A) Passive serum transfer protection experimental scheme. Sera from SARS-CoV-2-vaccinated (immunized group) or nonvaccinated (control group) mice were

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found in bats (40–43). In addition, antibodies from SARS-CoV-1 survivors who had been immunized with the SARS-CoV-2 mRNA vaccine BNT162b2 could cross-neutralize 10 different sarbecoviruses, including seven from the SARS-CoV-2 clade (the original strain of SARS-CoV-2; SARS-CoV-2 variants of concern B.1.1.7, B.1.351, and B.1.617.2; bat coronavirus RaTG13; and pangolin coronaviruses GD-1 and GX-P5L) and three from the SARS-CoV-1 clade (SARS-CoV-1, bat WIV1, and bat RsSHC014) (43). Macaque immunization with a multimeric SARS-CoV-2 RBD nanoparticle elicited cross-neutralizing antibody responses against bat SARSr-CoVs WIV1 and SHC014, SARS-CoV-1, SARS-CoV-2, and SARS-CoV-2 variants B.1.1.7, P.1, and B.1.351 (41). A nucleoside-modified mRNA-lipid nanoparticle vaccine expressing chimeric spikes with admixtures of different SARS-CoV-2 RBD and N-terminal domain modular domains protected vulnerable aged mice against challenges with SARS-CoV-1 and variants of SARS-CoV-2 and WIV1 but did not protect against RsSHC014 infection (44). These findings provide different strategies for designing a pan-sarbecovirus vaccine against SARSr-CoVs or sarbecoviruses.

In conclusion, these study findings suggest that it is feasible to develop a pan-sarbecovirus vaccine by combing epitopes that induce neutralizing antibodies and stimulate the T-cell response.

MATERIALS AND METHODS

Ethics statement. All animal experiments were approved by the Institutional Animal Care and Use Committee of Wuhan Institute of Virology (WIV), Chinese Academy of Sciences (approval WIVA05202015). The animal experiments and protocols were discussed extensively with the biosafety officers and facility managers at the WIV. Viral infections and mouse experiments were performed in an Animal Biosafety Level 3 laboratory.

Virus and cell lines. Bat SARSr-CoV rWIV1 and rRsSHC014S were constructed as described previously (28). SARS-CoV-2 (IVCAS 6.7512) was isolated from a COVID-19 patient (45). All viruses were propagated and titrated in *Cercopithecus aethiops* kidney cells (Vero-E6, ATCC CRL-1586). Vero-E6 cells were cultured in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% Anti-Anti (Invitrogen) at 37°C in the presence of 5% CO_2.

HFH4-hACE2 mice. Transgenic mice expressing the human ACE2 protein (HFH4-hACE2 mice) (kindly provided by Ralph Baric) were propagated and bred at the Laboratory Animal Center of WIV (46). The HFH4-hACE2 mice (6 to 8 weeks old) were randomly assigned to different experiments and treatments in this study.

Animal study and sample collection. For the vaccine protection experiment, HFH4-hACE2 mice were intraperitoneally injected with 5 μg of SARS-CoV-2 IAV and 0.5mg aluminum hydroxide (vaccine group) or 0.5mg aluminum hydroxide with PBS (adjuvant group) following the D0/D14 immunization program (29). At 30 days after the initial injection, the mice were infected with rWIV1, rRsSHC014S, or SARS-CoV-2. At 2 or 6 dpi, the mice were euthanized, and lung and brain tissues were harvested.

For the serum transfer protection experiment, sera were collected from BALB/c mice immunized with SARS-CoV-2 IAV and pooled. HFH4-hACE2 mice were intraperitoneally injected with 200 μL of pooled vaccinated sera. Negative sera collected from healthy BALB/c mice served as a control. One day after injection, the HFH4-hACE2 mice were infected with rWIV1, SARS-CoV-2, or rRsSHC014S via the intranasal route. All mice were euthanized at 5 dpi, and their lung tissues were harvested.

Extraction of viral RNA and qRT-PCR. Mouse organs were homogenized in DMEM, and viral RNA in the samples was quantified by one-step qRT-PCR. Viral RNA was extracted using the QIAamp viral RNA minikit (Qiagen) and then used as the template for qRT-PCR amplification (HiSxript II One-Step qRT-PCR SYBR Green kit; Vazyme). The following primer pairs targeting the SARS-CoV-2 S gene and SARSr-CoV nucleocapsid (N) gene were used (45): RBD-qF1 (5′-CAATGGTTTAACAGGCACAGG-3′)/RBD-qR1 (5′-CTCAAGTGTC TGTGGATCACG-3′) and NP-qF1 (5′-TCGTATGGGTCGCAACTGAG-3′)/NP-qR1 (5′-GCGAGAAGAGGCTTGACTGC-3′). PCRs were run according to the manufacturer's instructions.

Plaque reduction neutralization tests. Neutralizing antibodies titers in mouse sera were measured to determine the $PRNT_{50}$s. In brief, Vero-E6 cells were seeded into 24-well plates 1 day before use. Serum samples were serially diluted and incubated with 100 PFU of SARS-CoV-2 or SARSr-CoVs at 37°C for 0.5 h. The mixtures were then added to Vero-E6 cells and incubated at 37°C for an additional 1 h. The inoculum was removed and the cells were incubated with 0.9% methylcellulose for 5 days. Plaques were counted after crystal violet staining to calculate the $PRNT_{50}$.

FIG 4 Legend (Continued)
intraperitoneally transferred to HFH4-hACE2 mice one the day before infection. (B) Mice were infected with 10^5 PFU of the indicated virus and observed for 5 days. Color-indicated dotted lines represent the fitted curves. (C) Lung viral loads were determined by qRT-PCR. The dotted line indicates the limit of detection. (D) Lung pathological changes at 5 dpi are shown. Inflammatory infiltration was observed in immunized and control mice infected by rWIV1 or rRsSHC014S (black arrow). (E) Viral antigen was detected in both immunized and control mice infected by rWIV1 or rRsSHC014S and in only control mice infected by SARS-CoV-2. Images were acquired using a Pannoramic MIDI system. Scale bars, 200 μm. Error bars indicate standard errors. Statistical significance was assessed using the Mann-Whitney test (*, $P < 0.05$).

Determination of virus titers in lungs. Vero-E6 cells were seeded in 24-well plates 1 day before use. Infected lungs were homogenized in DMEM and 10-fold serially diluted. The cells were inoculated with the tissue dilutions for 1 h. Next, the inoculum was removed, and the cells were incubated with 0.9% methylcellulose for 5 days. PFU were counted after crystal violet staining to calculate the viral titer.

Histopathology and IFA. Tissues were fixed with 4% paraformaldehyde, paraffin embedded, and cut into 3.5-mm sections. For routine histology, the tissue sections were stained using H&E. For IFA, the sections were deparaffinized and rehydrated, followed by EDTA-mediated antigen retrieval. The sections were washed with PBS–0.02% Triton X-100 and then blocked with 5% bovine serum albumin. A primary antibody made in-house (rabbit anti-SARSr-CoV-RP3 N protein at 1:1,000) and secondary antibody (Cy3-conjugated goat-anti-rabbit IgG, 1:150; Abcam) were used. Cell nuclei were stained using 4′,6-diamino-2-phenylindole (Beyotime) at a dilution of 1:100. Images were acquired using a Pannoramic MIDI system (3DHISTECH) and a FV1200 confocal microscope (Olympus).

Flow cytometry. For ICS assay, spleens were collected from vaccinated mice, minced, and passed through 70-μm filters to obtain single-cell suspensions. The splenocytes were lysed using 10$\times$ RBC lysis buffer (eBioscience) and cultured in 96-well plates in the presence of different peptide pools of viral structural proteins and brefeldin A (20 μM; BD Biosciences) at 37°C for 5 to 6 h. Next, the cells were stained for cell surface markers at 4°C in the dark for 30 min, fixed/permeabilized with Cytofix/Cytoperm solution (BD Biosciences), and then stained with intracellular antibodies at 4°C in the dark for 30 min. The following antibodies were used: APC/Cyanine7 anti-mouse CD3ε (BioLegend), PerCP/Cyanine5.5 anti-mouse CD4 (BioLegend), FITC anti-mouse CD8a (BioLegend), and APC anti-mouse IFN-γ (BioLegend). Flow cytometry data were acquired on a BD LSRFortessa flow cytometer and analyzed using the FlowJo software. The cytokine/chemokine levels in mouse sera were determined using a LEGENDplex Multi-Analyte Flow assay kit (BioLegend) according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed using Prism 8.0.2 for Windows (GraphPad). Significant differences between multiple groups were determined using two-way analysis of variance (ANOVA). Significant differences between two experimental groups were determined using two-tailed Student *t* tests or Mann-Whitney test as appropriate. *P* values of <0.05 were considered significant.

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