Virologica Sinica 39 (2024) 414–421

Contents lists available at ScienceDirect

Virologica Sinica

journal homepage: www.keaipublishing.com/en/journals/virologica-sinica
www.virosin.org

Research Article

Characterization of humoral immune responses against SARS-CoV-2 accessory proteins in infected patients and mouse model

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ARTICLE INFO

Keywords:
SARS-CoV-2
Accessory protein
Humoral immune responses
COVID-19
Mouse model

ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, encodes several accessory proteins that have been shown to play crucial roles in regulating the innate immune response. However, their expressions in infected cells and immunogenicity in infected humans and mice are still not fully understood. This study utilized various techniques such as luciferase immunoprecipitation system (LIPS), immunofluorescence assay (IFA), and western blot (WB) to detect accessory protein-specific antibodies in sera of COVID-19 patients. Specific antibodies to proteins 3a, 3b, 7b, 8 and 9c can be detected by LIPS, but only protein 3a antibody was detected by IFA or WB. Antibodies against proteins 3a and 7b were only detected in ICU patients, which may serve as a marker for predicting disease progression. Further, we investigated the expression of accessory proteins in SARS-CoV-2-infected cells and identified the expressions of proteins 3a, 6, 7a, 8, and 9b. We also analyzed their ability to induce antibodies in immunized mice and found that only proteins 3a, 6, 7a, 8, 9b and 9c were able to induce measurable antibody productions, but these antibodies lacked neutralizing activities and did not protect mice from SARS-CoV-2 infection. Our findings validate the expression of SARS-CoV-2 accessory proteins and elucidate their humoral immune response, providing a basis for protein detection assays and their role in pathogenesis.

1. Introduction

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has become one of the most threatening global health problems (Wu F. et al., 2020). Since the COVID-19 outbreak in December 2019, it has led to over 772 million confirmed cases with about 7 million deaths (https://covid19.who.int/). Growing evidence supports the notion that SARS-CoV-2 dysfunction patients' innate immune response and experience "cytokine storm", which is characterized by the poor induction of type I interferon (IFN-I) response and the excessive levels of pro-inflammatory cytokines (such as TNF-a, IL-6) in the plasma (Blanco-Melo et al., 2020; Huang et al., 2020; Qin et al., 2020; Tan et al., 2020).

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https://doi.org/10.1016/j.virs.2024.04.005
Received 5 November 2023; Accepted 19 April 2024
Available online 26 April 2024

SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus belonging to betacoronavirus. The SARS-CoV-2 genome is approximately 30 kb in length, and generally encodes sixteen non-structure proteins (NSP1–16), four structure proteins [spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins], the remaining sequence encodes several putative SARS-CoV-2-specific proteins (Gordon et al., 2020; Kim et al., 2020; Wu A. et al., 2020). These putative proteins (named accessory proteins) based on bioinformatics prediction, have no sequence homology with other coronaviruses except SARS-CoV-1. Due to the genetic relation between SARS-CoV-2 and SARS-CoV-1, at least six accessory proteins (3a, 6, 7a, 7b, 8, and 10) were annotated (GenBank: NC_045512.2). Based on the data of transcriptome (Kim et al., 2020) and proteomics (Bojkova et al., 2020), proteins 3a, 6, 7a, 7b, 8 RNA sequence, and proteins 3a, 6, 7a, 8, 9b were detected in cells infected with SARS-CoV-2, but just one read of protein 10 RNA sequence was detected in the transcriptome. However, several researchers suggested that protein 10 is indeed expressed during infection because protein 10 can be found in immune cells of COVID-19 patients (Liu et al., 2020), and their T cells can react to protein 10 *in vitro* (Bacher et al., 2020). Li et al. (2021) revealed that proteins 3b and 9b exhibit a higher antibody positivity rate in COVID-19 patients by proteome microarray assay, whereas proteins 3a, 6, and 7a show a low antibody positivity rate. In addition, Asmaa Hachim et al. detected antibodies recognizing proteins 3a, 3b, 7a, 7b, and 8 in COVID-19 patients using luciferase immunoprecipitation system (LIPS) assay (Hachim et al., 2020, 2021). However, the accessory proteins have not yet been experimentally verified for expression and their immunogenicity still has not been fully understood.

Growing studies have revealed that SARS-CoV-2 accessory proteins play critical roles in agitating host innate immune response. Proteins 3a (Ren et al., 2020) and 7b (Yang et al., 2021) can induce cell apoptosis. Proteins 3a (Minakshi et al., 2009), 3b (Konno et al., 2020), 6 (Li et al., 2020; Miorin et al., 2020), and 9b (Jiang et al., 2020; Han et al., 2021; Wu et al., 2021) antagonize interferon response. Protein 7 can increase monocyte chemotaxis and reduce neutrophils chemotaxis (Wang et al., 2021). Protein 8 can mediate MHC-I degradation (Zhang et al., 2021). Insights of accessory protein characteristics could provide implications for SARS-CoV-2 pathogenesis and better drug and vaccine design.

In this study, nine accessory proteins (3a, 3b, 6, 7a, 7b, 8, 9b, 9c, and 10) were predicted according to the SARS-CoV-2 genome employing a bioinformatics algorithm (Wu F. et al., 2020). Antibodies against these proteins were examined by multiple detection methods, and the immunogenicity of these proteins in mice was further evaluated. This study provides valuable information on the detections of SARS-CoV-2 accessory proteins and their roles in pathogenesis.

2. Materials and methods

2.1. Virus, cells, and mice

The SARS-CoV-2 virus used in this research was an original strain isolated from COVID-19 patients in Guangzhou (Accession numbers: MT123290) and passaged on Vero E6 cells. All work with SARS-CoV-2 was conducted in the Guangzhou Customs District Technology Center Biosafety Level 3 (BSL-3) Laboratory. Huh7 cells, BHK-21 cells, 293T cells, A549 cells, HeLa cells, and Vero81 cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% fetal bovine sera (FBS). Caco-2 cells were grown in MEM (GIBCO, Grand Island, NY) supplemented with 20% FBS. Specific pathogen-free (SPF) 6–8 week-old female BALB/c mice were purchased from Hunan SJA Laboratory Animal Co. (Hunan, China). All protocols were approved by the Institutional Animal Care and Use Committees of Guangzhou Medical University.

2.2. Construction of expression vectors and venezuelan equine encephalitis replicon particles (VRPs)

Nine putative accessory proteins nucleotide sequences (with Flag tag in C-end) were synthesized according to SARS-CoV-2 sequence (GenBank: MT123290), which was named ORF3a (25396-26223), ORF3b (25527-25700) ORF6 (27205-27390), ORF7a (27397-27762), ORF7b (27759-27890), ORF8 (27897-28262), ORF9b (28287-28580), ORF9c (28737-28958), and ORF10 (29561-29678), respectively, followed by being cloned into pCAGGS vector. VRPs expressing the SARS-CoV-2 accessory protein or GFP were constructed as previously described (Deming et al., 2006).

2.3. Detection of antibody responses using LIPS

pcDNA3.1-Nanoluc-SARS2 RBD-10 × His were gifts from Prof. Linfa Wang (Duke-NUS Medical School, Singapore). Based on SARS-CoV-2 genome (GenBank: MT123290), four structure proteins (S, E, M, N) and nine accessory proteins (3a, 3b, 6, 7a, 7b, 8, 9b, 9c, 10) were amplified, then replace the SARS2 RBD with interest proteins. The constructed pcDNA3.1-Nanoluc luciferase plasmid containing the SARS-CoV-2 antigen of interest were transfected into HEK-293T cells using FuGENE 6 (Promega) as per the manufacturer's instructions. Cells were collected 48 h later, lysed and sonicated and Nanoluc-antigen yields were measured using a luminometer plate reader (PerkinElmer) according to the protocol of Burbelo et al. (2009). Each Nanoluc-fusion antigen is tested for its luminometer units (LU) yield and then standardized to 10^7 LU per antigen in each well before LIPS assay, thus controlling for varying yields of Nanoluc-tagged proteins during transfection in the assay.

The LIPS assays were performed following the protocol of Burbelo et al. (2009) with the following modifications. Briefly, Nanoluc-antigen (10^7 per well) and sera (heat-inactivated and diluted 1:100) were incubated for 2 h with shaking at 800 rpm. Ultralink protein A/G beads were added to the mixture in a 96-well microtiter plate and incubated for 2 h with shaking at 800 rpm. The entire mixture was then transferred into HTS plates and washed as previously described. The plate was read using QUANTI-Luc Gold substrate (Invivogen). Experimental controls include PBS or sera from age-matched SARS-CoV-2-negative healthy donor collected before the COVID-19 pandemic. The background corresponds to the LU signal from each Nanoluc-fusion antigen with protein A/G and substrate with PBS.

2.4. Detection of SARS-CoV-2 accessory protein antibody in sera of COVID-19 patient

BHK-21 cells were seeded on sterile glass coverslips in a 24-well plate and infected with VRP-ORFs at a multiplicity of infection (MOI) of 1 for 24 h. After infection, the cells were fixed with 4% paraformaldehyde, the sera of COVID-19 patient diluted using primary antibody dilution buffer (beyotime), was used as the primary antibody. An immunofluorescence assay (IFA) was then performed as described below.

2.5. SARS-CoV-2 infection and immunofluorescent staining

Huh7 cells and Caco-2 cells were seeded on sterile glass coverslips in a 24-well plate. Cells were infected with SARS-CoV-2 at 0.1 MOI for 48 h, then fixed with 4% paraformaldehyde for 2 h at room temperature (RT, 25 °C ± 2 °C). Cells were permeabilized by 0.2% Triton X-100 for 10 min, then stained with rabbit anti-accessory protein antibody and mice anti-SARS-CoV-2 N protein antibody for 2 h at RT. Following staining with Alexa Fluor 488 AffiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch) and Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch), and incubating with nuclear staining dye (DAPI, Sigma-Aldrich) in dark, coverslips were mounted on a slide for imaging (LSM 880 confocal microscope, Zeiss). All primary antibody information used in this study was shown in Supplementary Table S1.

2.6. SARS-CoV-2 Focus Reduction Neutralization Test (FRNT)

Vero E6 cells were seeded in 96-well plates one day before infection. Sera samples were serially diluted from 10- to 1,280-fold, and mixed with an equal volume of SARS-CoV-2 containing 200 FFU. After incubation at

37 °C for 1 h, aliquots were added to Vero E6 cells and incubated at 37 °C in 5% CO_2 for 1 h. Inocula were then removed before adding 150 μL 0.8% carboxymethylcellulose per well. After 24 h, cells were fixed with 4% paraformaldehyde and permeabilized and then incubated with SARS-CoV-2 N protein antibody, followed by an HRP-labeled goat anti-rabbit secondary antibody. The foci were visualized by TrueBlue™ Peroxidase Substrate and recorded using CTL ImmunoSpot S6 Universal Analyzer. $FRNT_{50}$ were half-maximum neutralizing titers.

2.7. Mouse immunization and infection

Mice were vaccinated twice at 4-week intervals with 1×10^5 IU of VRP expressing SARS-CoV-2 accessory proteins (3a, 3b, 6, 7a, 7b, 8, 9b, 9c, and 10) in 50 μL of PBS subcutaneously through footpad. Sera were harvested at 14 days after boosting. A total of 150 μL of sera was transferred intravenously (i.v.) into the SARS-CoV-2 infection murine model (Sun et al., 2020) 1 day before SARS-CoV-2 infection. Lungs were harvested and homogenized at day 2 post infection, and the virus was titered by focus forming assay (FFA). Titers are expressed as FFU/g (4 mice per group). Data are representative of two independent experiments. The FFA for SARS-CoV-2 quantification was performed as previously described (Sun et al., 2020).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software, version 8.00. Nonparametric two-sided Mann–Whitney U-tests were used to analyze antibody levels between COVID-19 patients and health donors (Fig. 1), and a Student's *t*-test was used to analyze the differences in mean

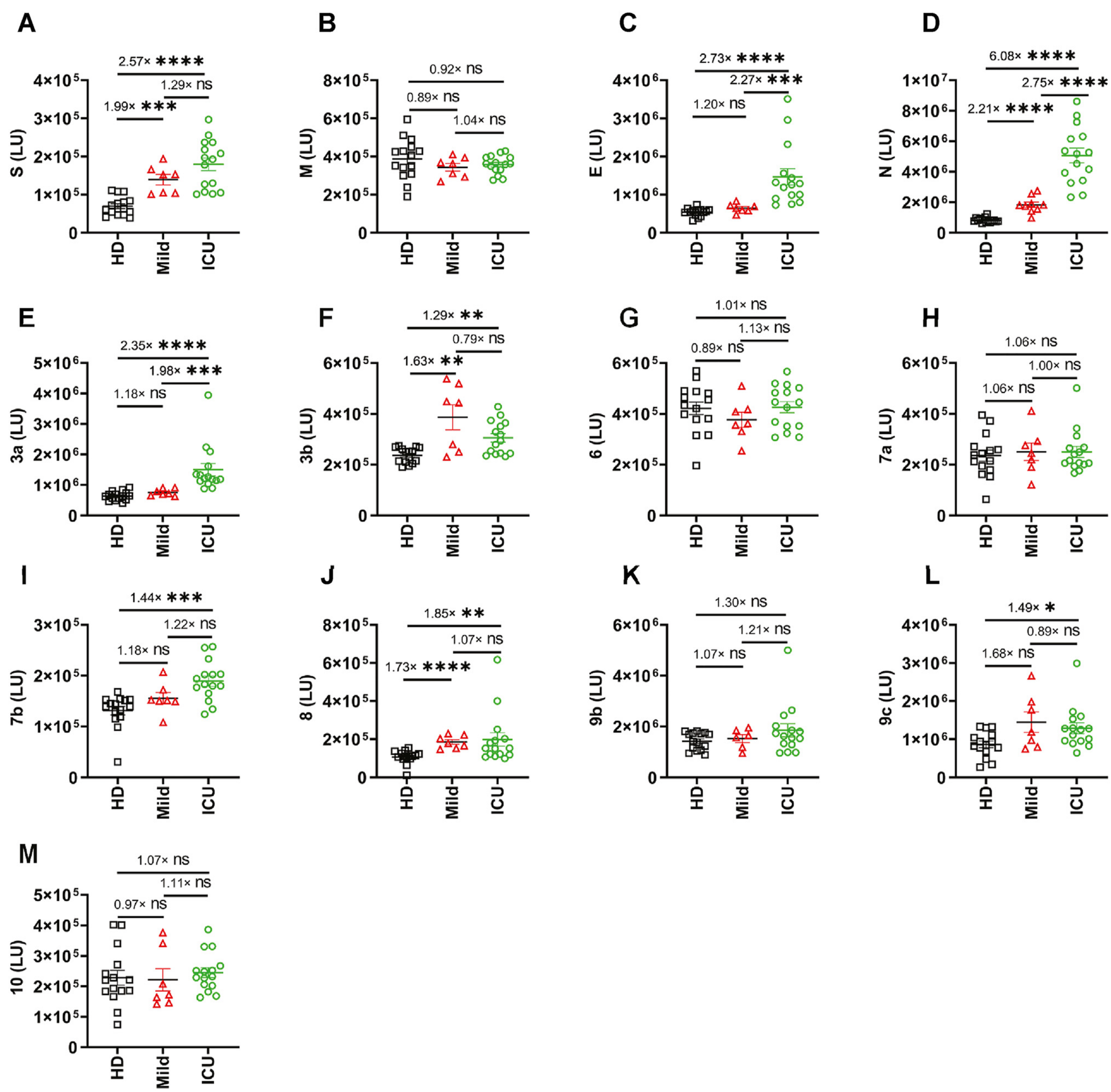

Fig. 1. Profiling accessory protein antibody responses in COVID-19 patients. Antibodies against structural proteins [S (**A**), M (**B**), E (**C**), N (**D**)] and accessory proteins [3a (**E**), 3b (**F**), 6 (**G**), 7a (**H**), 7b (**I**), 8 (**J**), 9b (**K**), 9c (**L**), and 10 (**M**)] were measured by LIPS from sera from patients with different severity levels of the COVID-19 disease [mild (red), ICU (green)], and age-matched healthy donor (HD) controls (black), LU: luminometer units. The geometric mean of the fold change is shown above each plot. *P* values were calculated using nonparametric two-sided Mann–Whitney U-tests. *shows statistical significance between COVID-19 patients versus healthy donors. ns: no significance, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

values in Fig. 4. All results are expressed as means ± standard errors of the means (SEM). P values of $\leq$0.05 were considered statistically significant (*, $P \leq 0.05$).

3. Results

3.1. Profiling accessory protein antibody responses in COVID-19 patients

Asmaa Hachim et al. showed that there are significantly higher accessory protein antibody levels in COVID-19 patients, such as proteins 3a, 3b, 7a, 7b, and 8, using luciferase immunoprecipitation system (LIPS) assay (Hachim et al., 2022), and found antibodies against proteins 8 and 3b can be used as serological markers of early and late SARS-CoV-2 infection. There is a knowledge gap on whether the disease severity is correlated with accessory protein antibody levels. We first tested the structure protein antibodies in Mild and ICU patients' sera ('Mild' denotes cases characterized by mild clinical features and the absence of pneumonia symptoms. 'ICU (critical illness)' refers to individuals whose respiratory failure requiring mechanical ventilation and need for ICU care, as per the Chinese Government Diagnosis and Treatment Guideline). The results showed that there were higher levels of S, N, and E protein antibodies in COVID-19 patients, and the S and N

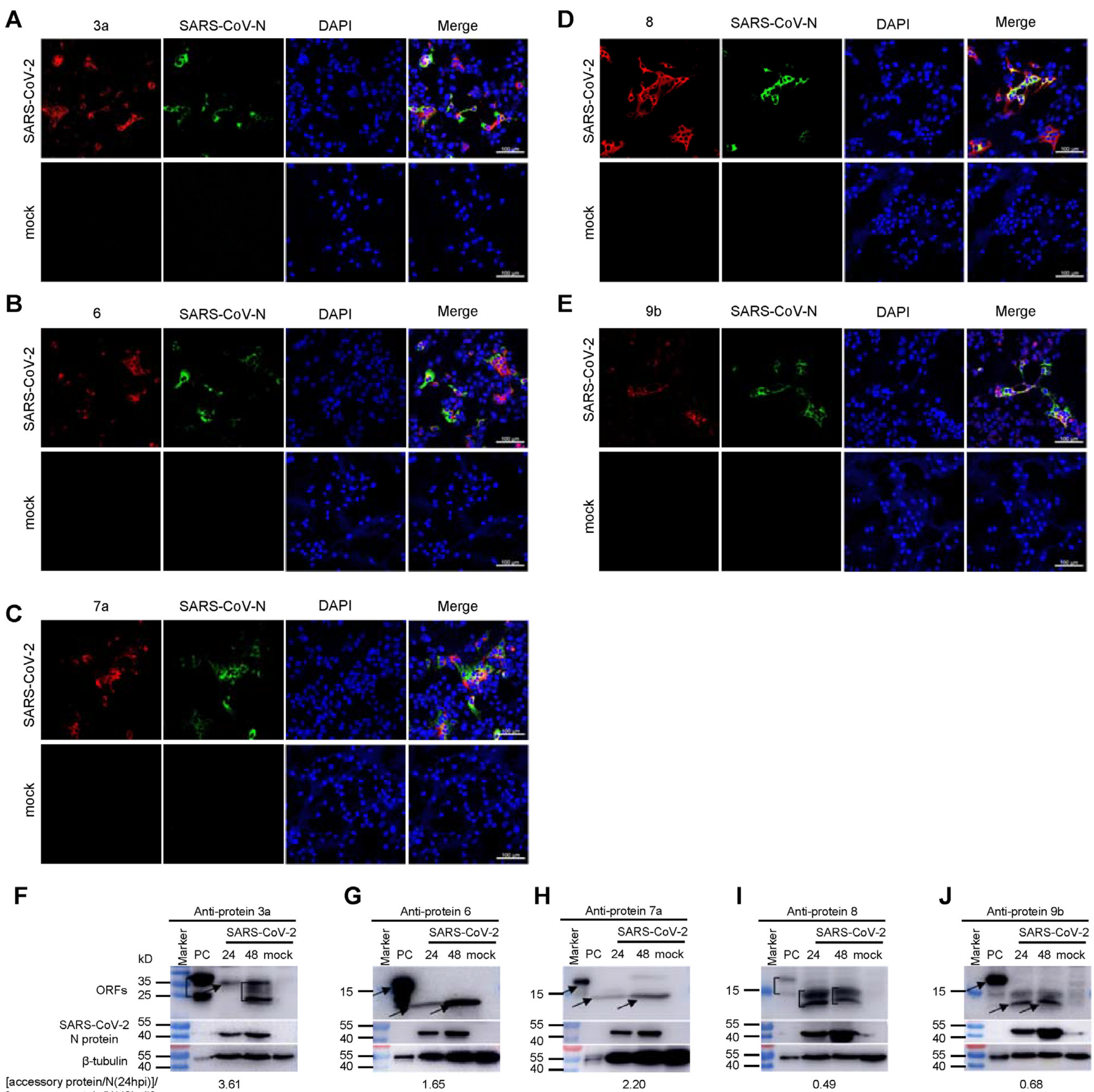

Fig. 2. Detection of accessory protein expression in Huh7 cells infected with SARS-CoV-2. Huh7 cells were infected with SARS-CoV-2 at MOI 0.1. **A–E** cells were fixed at 48 h post infection (hpi) and subjected to an immunofluorescence assay (IFA) using an indicted rabbit anti-accessory antibody (red) and with a mouse anti-SARS-CoV-2-nucleoprotein (N) (green). Cell nuclei were stained with DAPI (blue) and examined by confocal microscopy. **F–J** Cell extracts were collected at 24 and 48 hpi for Western blot (WB) analysis using an indicted rabbit anti-accessory antibody and mouse anti-SARS-CoV-2-N antibody. Expression of β-actin in cells used as internal control. Cell extracts from uninfected cells were used as a negative control, and VRP-ORFs infected BHK21 cells as a positive control (PC). The proteins from VRP-ORFs-transfected cells all contain a 3 × HA tag, which showed larger size compared with that from the virus infected cells.

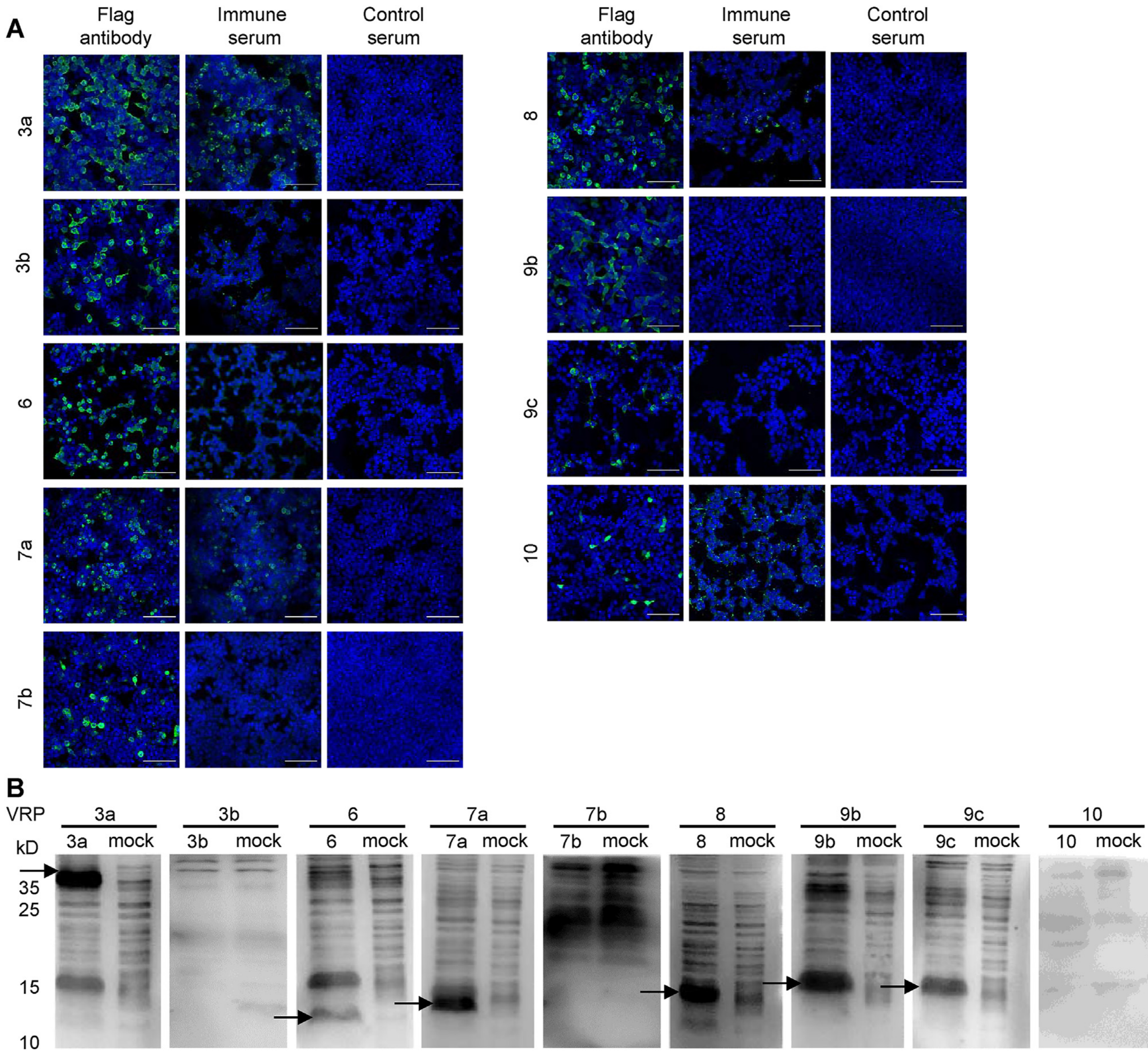

Fig. 3. Evaluation of antibodies against accessory proteins in sera of VRP-ORFs immunized mice. Six-week-old female BALB/c mice were vaccinated twice at 4-week intervals with 10^5 infectious units of VRP expressing indicted accessory proteins in 50 μL of PBS. Antibodies in the sera of vaccinated mice were evaluated two weeks after boosting by IFA (**A**) and WB (**B**). **A** 293T cells were transfected with pCAGGS-accessory proteins-Flag tag using Lipofectamine 2000 and fixed at 36 hpi, corresponding immune sera were used as a prime antibody, and accessory protein expression was monitored with Flag antibody. Cell nuclei were stained with DAPI (blue) and examined by confocal microscopy. **B** 293T cells were transfected with pCAGGS-accessory proteins-Flag tag using Lipofectamine 2000 and cell extracts were collected at 36 hpi for Western blot (WB) analysis using the indicted immune sera, and cell extracts from mock 293T were used as control.

protein antibodies were positive correlation with disease severity (Fig. 1A–D). ICU patients had higher S and N antibody levels than the mild patients. There was no significant difference between COVID-19 patients and healthy donors for proteins 6, 7a, 9b, and 10 specific antibodies (Fig. 1G, H, K, and M). While COVID-19 patients had higher levels of antibodies against proteins 3a, 3b, 7b, 8 and 9c (Fig. 1E, F, I, J, and L). Of note, antibody levels for proteins 3a, 7b, and E were only found in ICU patients. Therefore, we hypothesize that predicting the severity of the disease may also be possible through the detection of antibodies against these accessory proteins. Then we measured accessory protein-specific antibodies in five severe COVID-19 patient sera by IFA and WB, respectively. All five sera contained high levels of SARS-CoV-2 S protein antibodies detected by LIPS. However, only protein 3a antibody can be detected, antibodies against other proteins were undetectable in all these five patients using IFA (Supplementary Fig. S1). The antibodies were also monitored by WB assay, and the results were consistent with IFA (Supplementary Fig. S2), suggesting antibody levels against these proteins are relatively low *in vivo* and LIPS is a more sensitive serological method than IFA and WB.

3.2. *Expression of putative accessory proteins in cells infected with SARS-CoV-2*

To investigate the expression properties of accessory proteins within virus-infected cells, we infected Huh7 (Fig. 2A–J) and Caco-2 (Supplementary Fig. S3) cells with the same viral strain observed in patients. The expressions of proteins 3a, 6, 7a, 8, and 9b were identified using IFA and WB with commercially available antibodies (Supplementary Table S1). Proteins 3b, 7b, and 9c remain unidentified due to the absence of corresponding antibodies. These findings suggested the successful expression and detection of certain SARS-CoV-2 accessory proteins within infected cells.

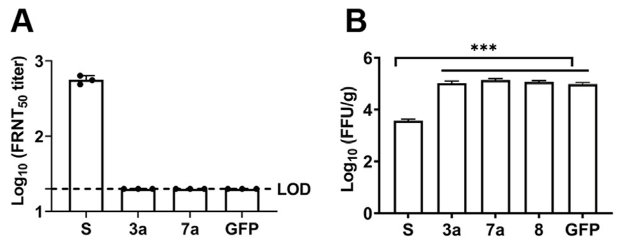

Fig. 4. VRP-ORFs immunized mice sera absence neutralizing activity *in vitro* and cannot protected against SARS-CoV-2 challenge in mice. **A** 2-fold serially diluted VRP-ORFs, -spike protein (positive control), or -GFP (negative control) immunized mice sera were mixed with an equal volume of SARS-CoV-2 (100 FFU), respectively. After incubation at 37 °C for 1 h, aliquots were added to Vero cells and the neutralizing ability of the sera was evaluated by $FRNT_{50}$. **B** 150 μL of VRP-ORFs, -spike protein (positive control), or -GFP (negative control) immunized mice sera was transferred into the SARS-CoV-2 infection murine model (Tang et al., 2023) intravenously (i.v.) 1 day before SARS-CoV-2 infection. Lungs were harvested and homogenized at day 2 post infection, and the virus was titered by focus forming assay. All data are representative of two independent experiments. Statistical significance was analyzed using the Student's *t*-test. $^{***}P < 0.001$.

3.3. Immunogenic analysis of SARS-CoV-2 accessory proteins in mice

To determine whether these SARS-CoV-2 accessory proteins can elicit detectable antibodies in an immunization setting, venezuelan equine encephalitis replicon particles (VRPs) vaccination of mice was performed. VPRs could induce high level of foreign gene expression and efficiently induce humoral responses in mice, as we described previously (Zhao et al., 2016; Sun et al., 2020). VRP vectors expressing accessory proteins fused with 3 × HA tag were generated as described previously (Pushko et al., 1997; Zhao et al., 2016) (Supplementary Fig. S4A, B). VRP-GFP was used as a control. Accessory proteins with a Flag tag were also generated and validated using eukaryotic expression plasmids, and proteins 3b, 7b, and 10 were fused with a 3 × Flag tag to facilitate expression (Supplementary Fig. S4A, C). Immune sera from mice vaccinated with different VPRs were evaluated using 293T cells transfected accessory protein. The results showed that antibodies of proteins 3a, 3b, 7a, and 8 could be detected by IFA (Fig. 3A), while antibodies against proteins 3a, 6, 7a, 8, 9b, and 9c can be detected by WB (Fig. 3B). Nevertheless, the presence of antibodies against proteins 7b and 10 were not observed through both WB and IFA assays, suggesting a lower level of immunogenicity for these proteins.

3.4. Accessory protein vaccinations did not induce neutralizing antibodies

Our previous study showed that passive transfer of sera from VRP-SARS-CoV-2-S-immunized mice could accelerate virus clearance in the lungs of SARS-CoV-2 infected mice (Sun et al., 2020). In SARS-CoV-1, some accessory proteins, such as 3a and 7a, were involved in the composition of the viral structure (Ito et al., 2005; Huang et al., 2006), and induced neutralizing antibodies (Akerström et al., 2006). Here, we evaluated the neutralizing activity of mouse immune sera against the corresponding proteins, 3a and 7a in the SARS-CoV-2 (Fig. 4A). We found that neither of these sera from VRP-ORFs immunized mice could neutralize SARS-CoV-2 *in vitro*, suggesting SARS-CoV-2 accessory proteins 3a and 7a could not induce neutralizing antibodies. Additionally, we also conducted *in vivo* experiments to assess the efficacy of these sera in virus clearance. The findings indicated that VRP-ORFs immunized mouse sera did not affect virus clearance in BALB/c mice at day 2 post infection as well (Fig. 4B).

4. Discussion

Hachim et al. (2020) observed higher antibody levels for proteins 3a, 3b, 7a, 7b, and 8 using LIPS assay in COVID-19 patients. In contrast, Li et al. (2021) found minimal IgG positivity rate to proteins 3a, 6, and 7a through a microarray-based assay. In our study, we identified antibody responses to proteins 3a, 3b, 7b, 8, and 9c using the LIPS assay, but we did not detect antibodies against proteins 7a and 6. Due to potential sequence variations or expression limitations in certain strains (Lam et al., 2020; Tang et al., 2023), we examined the expression of accessory proteins from the same SARS-CoV-2 strain that had infected the patients under investigation. We successfully detected the expression of the indicated proteins including proteins 7a and 6 in Huh7 and Caco-2 cells that were infected with SARS-CoV-2, suggesting that the absence of antibodies in patients' sera may not be related to the expression of these proteins. It's noteworthy that our research reveals the cytoplasmic localization of the protein 9b (Fig. 2E, Fig. 3, Supplementary Fig. S3). In contrast, other studies suggest a possible localization of this protein in both the cell nucleus and the cytoplasm (Sharma et al., 2011; Zhang et al., 2020). This inconsistency might stem from variations in detection timing or the employed expression systems. Additionally, we also employed alternative methods for the detection of protein antibodies. Although we observed a notable difference in antibody levels against proteins 3a, 3b, 7b, 8, and 9c in the sera of severe patients using the LIPS method, only protein 3a antibodies can be detected by IFA or WB. This may be related to the lower levels of antibodies against other proteins, which further highlights that the LIPS assay is a more sensitive antibody detection technique compared to WB and IFA.

Next, we vaccinated mice with VRP expressing individual accessory proteins. Mice vaccinated with VRP-ORF3a, 6, 7a, 8, 9b, and 9c induced measurable antibody responses, indicating that these proteins exhibit robust immunogenicity. Based on the analysis above, we hypothesize that the absence of antibodies against certain accessory proteins in the sera of COVID-19 patients may be attributed to the disease stage and individual variations, necessitating further investigation.

In SARS-CoV-1, several accessory proteins induce neutralizing antibodies (Tang et al., 2023), consequently, we evaluated the neutralizing activity of antibodies against SARS-CoV-2 accessory proteins 3a, 7a, and 8, as these proteins can be detected using IFA. Unfortunately, antibodies induced by these proteins show no virus-neutralizing activity *in vitro*. Given the pivotal roles of proteins 3a, 7a, and 8 in innate immunity (Arshad et al., 2022; Matsuoka et al., 2022), does the neutralization of these proteins in SARS-CoV-2-infected mice affect the clearance of the virus? However, the passive transfer of these sera does not substantially influence the clearance of the virus in mice. Therefore, we conclude that in SARS-CoV-2, the antibody against accessory proteins 3a, 7a, and 8 does not exert a neutralizing effect and cannot effectively reduce the viral load in the mice. Another possibility is that the concentration of neutralizing antibodies is not high enough to achieve an effective neutralizing effect, and the reason behind that could be investigated in future research.

Research conducted by Asmaa Hachim et al (Hachim et al. 2020) has shown that antibodies against proteins 8 and 3b serve as accurate serological markers for early and late stages of SARS-CoV-2. Is it possible to predict severe disease through antibody level? From Fig. 4, we found that antibodies against the proteins are either detectable in both mild and ICU patients or undetectable in both, except for proteins 3a and 7b which are only detectable in sera of ICU patients. Therefore, antibodies against proteins 3a and 7b may serve as potential markers for predicting the severity and disease progression.

5. Conclusions

In summary, antibodies against proteins 3a, 3b, 7b, 8, and 9c were identified in the sera of COVID-19 patients using LIPS. Interestingly, only antibodies against protein 3a were detected using WB and IFA. Notably, antibodies against proteins 3a and 7b hold promise as potential markers for predicting disease severity and progression. A thorough analysis was conducted to evaluate the expression of SARS-CoV-2 accessory proteins within viral-infected cells and their immunogenicity in mice. The expressions of proteins 3a, 6, 7a, 8, and 9b were confirmed, and proteins 3a, 6, 7a, 8, 9b, and 9c could induce humoral immune responses in mice. However, it is noteworthy that sera from mice immunized with accessory proteins 3a, 7a, and 8 lacked neutralizing activities. Based on the antibody response to these proteins in COVID-19 patients using different detection methods, we found that LIPS is a more sensitive serological method than IFA and WB. The study provides a basis for further investigation into SARS-CoV-2 accessory proteins, enhancing our understanding of the pathogenesis of the virus, and aiding the development of diagnosis assays and effective treatments.

Data availability

All the data generated during the current study are included in the manuscript.

Ethics statement

The research plan was reviewed and approved by the Ethics Committees of The First Affiliated Hospital of Guangzhou Medical University (Ethics ID ES-2020-65), on the use of human samples for experimental studies. Written informed consent was obtained from all participants enrolled in the studies. All experiments were performed in accordance with relevant guidelines and regulations. The research conformed to the principles of the Helsinki Declaration.

Author contributions

Jincun Zhao: conceptualization, formal analysis, funding acquisition, methodology, validation, visualization, writing-review & editing. Yuming Li: conceptualization, formal analysis, funding acquisition, methodology, validation, visualization, writing-original draft. Jingxian Zhao and Jing Sun: conceptualization, funding acquisition, supervision, writing-review & editing. Yanhong Tang: data curation, formal analysis, methodology, writing-original draft. Xiaoqian Wang, Fengyu Chi, Hu Guo, Yiyun He, Dongdong Liu, XinZhuo Liu, Yin Xi, Zhaoyong Zhang, Airu Zhu, Jie Zheng, Zhen Zhuang: data curation, methodology. Zhao Chen, Fang Li, Wei Ran: data curation. Dong Wang, Liyan Wen, Kuai Yu, Yanjun Zhang, Jianfen Zhuo: methodology. Dongdong Liu: data curation, resources. Yanqun Wang: data curation, funding acquisition, methodology, resources.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

We thank Guangzhou Customs District Technology Center BSL-3 Laboratory to provide the BSL-3 Laboratory to complete the authentic SARS-CoV-2 experiments. We thank the Biobank for Respiratory Disease at the National Clinical Research Center for Respiratory Disease (BRDNCRCRD, Guangzhou, Southern China). We thank Prof. Linfa Wang (Duke-NUS Medical School, Singapore) for the pcDNA3.1-Nanoluc-SARS2 RBD-10xHis plasmid. This work is supported by grants from the National Natural Science Foundation of China (82002127, 81971500, 82025001, 82172240), National Key R&D Program of China (2021YFC2301700, 2022YFC2604100), Guangdong Basic and Applied Basic Research Foundation (2022B1515020059, 2021B1515130005), and R&D Program of Guangzhou Laboratory (EKPG21-30-2).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2024.04.005.

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