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Original article

Expression of water-soluble nucleocapsid protein of SARS-CoV-2 and analysis of its immunogenicity

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Abstract

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to be a major public health concern. Nucleocapsid (N) protein is the most abundant structural protein on SARS-CoV-2 virions and induces the production of antibodies at the early stage of infection. Large-scale preparation of N protein is essential for the development of immunoassays to detect antibodies to SARS-CoV-2 and the control of virus transmission. In this study, expression of water-soluble N protein was achieved through inducing protein expression at 25°C with 0.5 mM IPTG for 12 h. Western blot and ELISA showed that recombinant N protein could be recognized by sera collected from subjects immunized with Sinovac inactivated SARS-CoV-2 vaccine. Four monoclonal antibodies namely 2B1B1, 4D3A3, 5G1F8, and 7C6F5 were produced using hybridoma technology. Titers of all four monoclonal antibodies in ELISA reached more than $1.28\times10^{6.0}$. Moreover, all monoclonal antibodies could react specifically with N protein expressed by transfection of pcDNA3.1-N into BHK-21 cells in IPMA and IFA. These results indicated that water-soluble N protein retained high immunogenicity and possessed the same epitopes as that of native N protein on virions. In addition, the preparation of water-soluble N protein and its monoclonal antibodies laid the basis for the development of immunoassays for COVID-19 detection.

Keywords: SARS-CoV-2, optimization of expression conditions, water-soluble nucleocapsid protein, immunogenicity analysis

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Introduction

Coronavirus disease 2019 (COVID-19) has been prevalent worldwide for more than three years and has had a major impact on global health and economy. Clinical manifestations of COVID-19 include pneumonia, fever, dry cough, fatigue and muscle pain, often accompanied by shortness of breath and dyspnea (Han et al. 2020). In severe cases, acute respiratory distress syndrome, septic shock, multiple organ failure and death may occur (Meyer et al. 2021).

The causative agent of COVID-19 was identified to be severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which could be transmitted from person to person through droplets, direct contact, and aerosols in closed environments (Malik 2020, Leung 2021). The virus causes infections of bronchial epithelial cells and upper respiratory tract cells, which can develop into severe, life-threatening respiratory pathologies and lung injuries (Clementi et al. 2021). Currently, there is no specific prophylactic or therapeutic treatment and the incubation period after infection can reach up to 14 days (Jin et al. 2020, Lauer et al. 2020). Therefore, it is of great importance to carry out rapid screening, early diagnosis, early isolation, and early treatment for subjects suspected of COVID-19.

SARS-CoV-2 is a single strand, positive-sense RNA virus encoding spike protein (S), membrane protein (M), envelope protein (E), and nucleocapsid protein (N) (Zhou et al. 2020). N protein has been found to bind with viral RNA to form a spiral nucleocapsid and be involved in viral genome replication and regulation of cell signaling (Chen et al. 2020, Chen et al. 2020). In addition, it is relatively conservative and accounts for the largest proportion of the structural proteins of the virus (Zeng et al. 2020, Bai et al. 2021). N protein has been reported to be highly immunogenic and antibodies to N protein could be detected at the early stage of infection (Guo et al. 2020, Okba et al. 2020). Hence, it is considered as an excellent diagnostic antigen for the detection of antibodies to SARS-CoV-2.

Until now, the expression of N protein has been realized in mammalian cells including human embryonic kidney cells (HEK-293) and Chinese hamster ovary (CHO) cells, insect cells such as Spodoptera frugiperda (sf21) cells, and *Escherichia coli* (*E. coli*) cells (Bates et al. 2021, Liu et al. 2021, Rump et al. 2021, Tian et al. 2022). Considering the cost and the yield of the final protein product, *E. coli* cells are generally preferred for protein expression. However, high-level expression of foreign protein in *E. coli* cells often leads to aggregation of the protein molecules into inclusion bodies which need refolding to be bioactive (Singh et al. 2015). In this study, we aimed to express recombinant N protein in *E. coli* cells and optimize the culture conditions to harvest water-soluble protein. Additionally, monoclonal antibodies to N protein were produced using hybridoma technology and the immunogenicity of N protein was evaluated by Western blot, enzyme-linked immunosorbent assay (ELISA), immunoperoxidase monolayer assay (IPMA), and immunofluorescence assay (IFA).

Materials and Methods

Ethics statement

Collection of blood samples was approved and supervised by the Ethics Committee of Xinxiang Medical University (XYLL-2021098) in accordance with China's legislation on human welfare.

Serum samples and reagents

Serum samples from six subjects immunized with Sinovac inactivated SARS-CoV-2 vaccine were collected with informed consent and pooled. A pool of six negative sera to SARS-CoV-2 collected in 2018, the hemagglutinin (HA) protein of H3N2 influenza virus which contained a hexahistidine (His_6)-tag, and its cognate monoclonal antibody were previously prepared in our laboratory.

Plasmid construction and transfection

The gene encoding the N protein of SARS-CoV-2 was derived from isolate Wuhan-Hu-1 (GenBank No. NC_045512.2), synthesized chemically by GenScript Biotech Corporation after codon optimization, and inserted into the multiple cloning sites of pET28a and pcDNA3.1(+) using standard molecular cloning technique. Plasmids pET28a-N which contained a His_6-tag and pcDNA3.1-N were verified by gene sequencing.

Baby hamster kidney (BHK-21) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). For expression of N protein in eukaryotic cells, pcDNA3.1-N was transfected into BHK-21 cells using lipofectamine 2000 reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. 0.2 μg of pcDNA3.1-N was mixed with DMEM media in 25 μL for 5 min. Meanwhile, 0.5 μL lipofectamine 2000 was added into 25 μL DMEM media and kept at room temperature for 5 min. The plasmid and the transfection reagent were then mixed and incubated for 20 min. The cells were washed twice with DMEM media before the addition of the transfection complex into each well, slowly, while shaking the plate. 6 h later, the culture media were changed to DMEM containing 10% FBS. The expres-

sion of N protein in transfected cells was examined 36 h post-transfection.

Expression and purification of N protein

E. coli BL21(DE3) cells harboring pET28a-N were cultured in Luria-Bertani (LB) liquid media by shaking at 220 rpm at 37°C for 2-3 h to an optical density of 0.4-0.5 at 600 nm wavelength (OD_{600}). The cells were then placed on an ice bath for 20 min and expression of N protein was induced with a final concentration of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) by shaking at 220 rpm at 16°C or 25°C for 12 h. After centrifugation at 6000 g for 20 min at 4°C, the pelleted cells were resuspended in 1/10 culture volume of Tris buffered saline (pH 8.0) containing 20 mM Tris and 500 mM NaCl (binding buffer) and treated with ultra-sonication on ice for 20 min. The supernatant was harvested by centrifugation at 12000 g for 20 min at 4°C and filtered through a 0.45 μm nitrocellulose filter before purification. The precipitate was resuspended with an equal volume of binding buffer before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Recombinant N protein was purified using nickel--nitrilotriacetic acid (Ni-NTA) affinity chromatography. The column was pre-equilibrated with binding buffer before sample loading. The supernatant was added into the column and washed with binding buffer containing 40 mM imidazole (wash buffer). N protein was then eluted from the column with binding buffer containing 500 mM imidazole (elution buffer) and 0.5 mL collected per tube. Purified protein samples were pooled, dialyzed against 20 mM Tris (pH 8.0) and then stored at -20°C after determination of protein concentration using a Bradford Protein Assay Kit (Sangon Biotech, Shanghai, China). The expression and purification of N protein was examined using 12% SDS-PAGE.

Production of monoclonal antibodies

BALB/c mice were subcutaneously immunized with 50 μg N protein emulsified in Freund's adjuvants four times at two-week intervals. The mouse with the highest antibody titer in ELISA was intravenously injected with 50 μg N protein three days before cell fusion. Splenocytes were then harvested and fused with SP2/0 myeloma cells under treatment with polyethylene glycol 2000 (PEG 2000). The reactivity of the hybridomas with N protein was examined using Western blot, ELISA, immunoperoxidase monolayer assay (IPMA) and immunofluorescence assay (IFA). Ascites in mice were produced for the best hybridomas and IgG was purified from the ascitic fluids using a Pierce™ protein A column (Thermo Fisher Scientific, USA). The subtypes of monoclonal antibodies were determined using a commercial isotyping kit (Proteintech, Wuhan, China).

Western blot

In SDS-PAGE, 15 μL of each sample prepared as above was loaded and then transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% skimmed milk at 37°C for 1 h, the transferred membrane was incubated with 1:5000 diluted monoclonal antibody 2B1B1 or 1:100 diluted Sinovac inactivated SARS-CoV-2 vaccine-immunized human sera at 37°C for 1 h. Horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG or HRP-conjugated goat anti-human IgG (Thermo Fisher Scientific, USA) were used as secondary antibodies and incubated with the membrane at 1:10000 dilution for 1 h. The HRP label was finally exposed to a colorimetric substrate, aminoethyl carbazole (AEC). After each step, the membrane was washed with phosphate-buffered saline containing 0.05% Tween-20 (PBST).

ELISA

Briefly, 96-well microtiter plates were coated with 50 μL purified N protein (2.5 μg/ml) or His_6-tagged HA protein of H3N2 influenza virus (2.5 μg/ml) in carbonate buffered saline (pH 9.6) at 4°C overnight. After washing with PBST, the plates were blocked with 5% skimmed milk at 37°C for 1 h. Serum antibodies, supernatants from hybridomas, or ascitic fluids were used as primary antibodies to incubate with the plates at 37°C for 30 min. Each sample was tested in a two-fold serial dilution manner. Sinovac inactivated SARS-CoV-2 vaccine-immunized human sera (1:100) was used as a positive control, while negative sera to SARS-CoV-2 (1:100) and a monoclonal antibody to the HA protein of H3N2 influenza virus (1:10000) were used as negative controls. After washing six times with PBST, the plates were incubated with 50 μL HRP-conjugated goat anti-mouse IgG (1:10000) or HRP-conjugated goat anti-human IgG (1:10000) at 37°C for 30 min. The plates were then washed again and finally incubated with 50 μL 3,3',5,5'-tetramethylbenzidine (TMB) for color development. The enzymatic reaction was stopped with 50 μL of 2 M H_2SO_4 after 10 min and the plates were read at A450 (OD_{450}) with an automatic plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Each sample was tested in triplicate independently for three times and the mean OD_{450} values were calculated. The cut-off value was set to be the mean OD_{450} value for negative controls (X) plus twice the standard deviation (X+2SD). Antibody titer was expressed as the reciprocal of the highest dilution that gave a positive reaction.

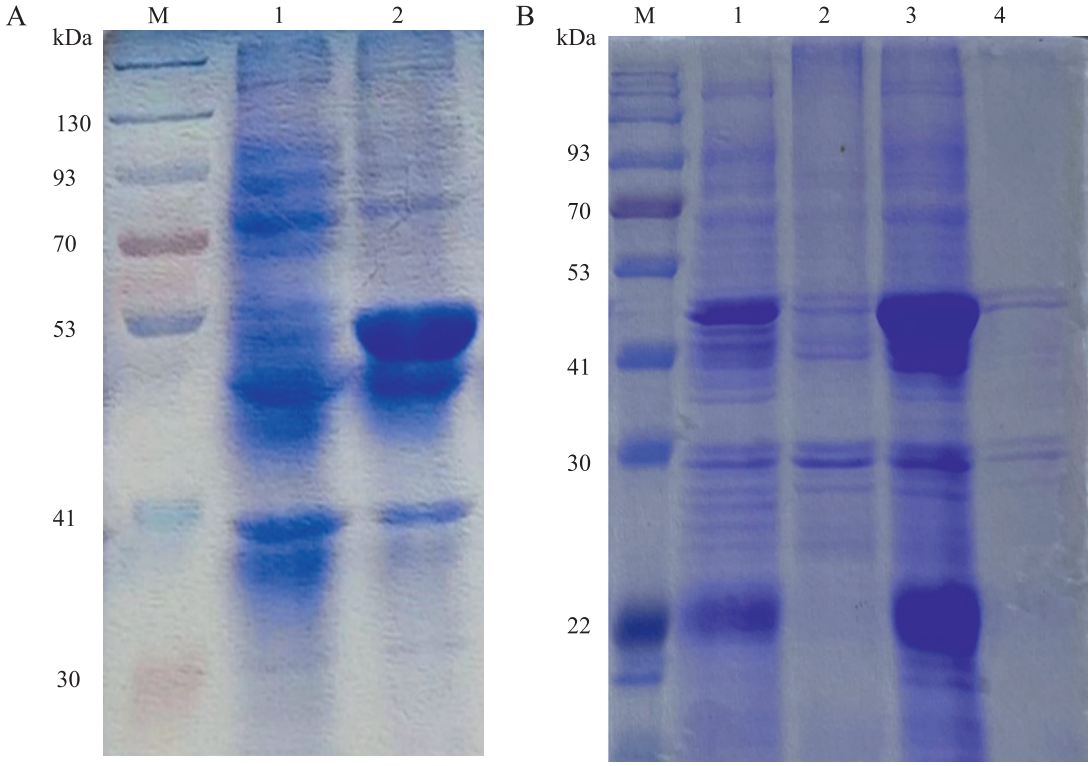

Fig. 1. Optimization of induction temperature for recombinant N protein expression. (A). Lane M: pre-stained protein molecular weight marker; Lane 1: non-induced *E. coli* BL21(DE3) cells bearing pET28a-N; Lane 2: induced *E. coli* BL21(DE3) cells bearing pET28a-N. (B). Lane 1: supernatant of cell lysate from pET28a-N transformed cells induced at 16°C; Lane 2: precipitate of cell lysate from pET28a-N transformed cells induced at 16°C; Lane 3: supernatant of cell lysate from pET28a-N transformed cells induced at 25°C; Lane 4: precipitate of cell lysate from pET28a-N transformed cells induced at 25°C.

IPMA

IPMA was performed as previously described (Wang et al. 2019). Briefly, BHK-21 cells were grown in 96-well cell culture plates to a confluent monolayer within 24 h. After washing twice with DMEM, the cells were transfected with pcDNA3.1-N and grown in DMEM containing 10% FBS for 36 h. The cells were then washed with sterile PBS and fixed with cold methanol containing 2% H_2O_2 for 15 min at room temperature. 5% skimmed milk was added to block available binding sites on the plate at 37°C for 1 h. 50 µL of monoclonal antibodies (1:5000) were added and allowed to incubate with the cells at 37°C for 30 min. Sinovac inactivated SARS-CoV-2 vaccine-immunized human sera (1:100), negative sera to SARS-CoV-2 (1:100), and the monoclonal antibody to HA protein of H3N2 influenza virus (1:5000) were used as controls. After washing with PBST, 50 µL of HRP-conjugated goat anti--mouse IgG (1:5000) or HRP-conjugated goat anti-human IgG (1:5000) was added and incubated with the plate at 37°C for 30 min. Finally, AEC substrate buffer was added to allow color development for 5 min before the addition of double distilled water (DDW) to stop the reaction. The wells were then observed under light microscopy and the presence of red brown precipitates indicated a positive reaction. Reactions between the antibody samples and pcDNA3.1-transfected BHK-21 cells were also tested as described above.

IFA

In brief, IFA was performed similar to that of IPMA. Instead, HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-human IgG were replaced with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:5000) and FITC-conjugated goat anti-human IgG (1:5000). The result was observed under a fluorescent microscope and the appearance of green spots in pcDNA3.1-N transfected cells indicated positive reactions.

Results

Expression of water-soluble N protein

To test the expression of recombinant N protein, *E. coli* BL21(DE3) cells bearing pET28a-N were induced with 0.5 mM IPTG at 37°C for 12 h. Fig. 1A indicates that there was a clear band difference between the induced and uninduced samples. The relative molecular weight of the expressed N protein was esti-

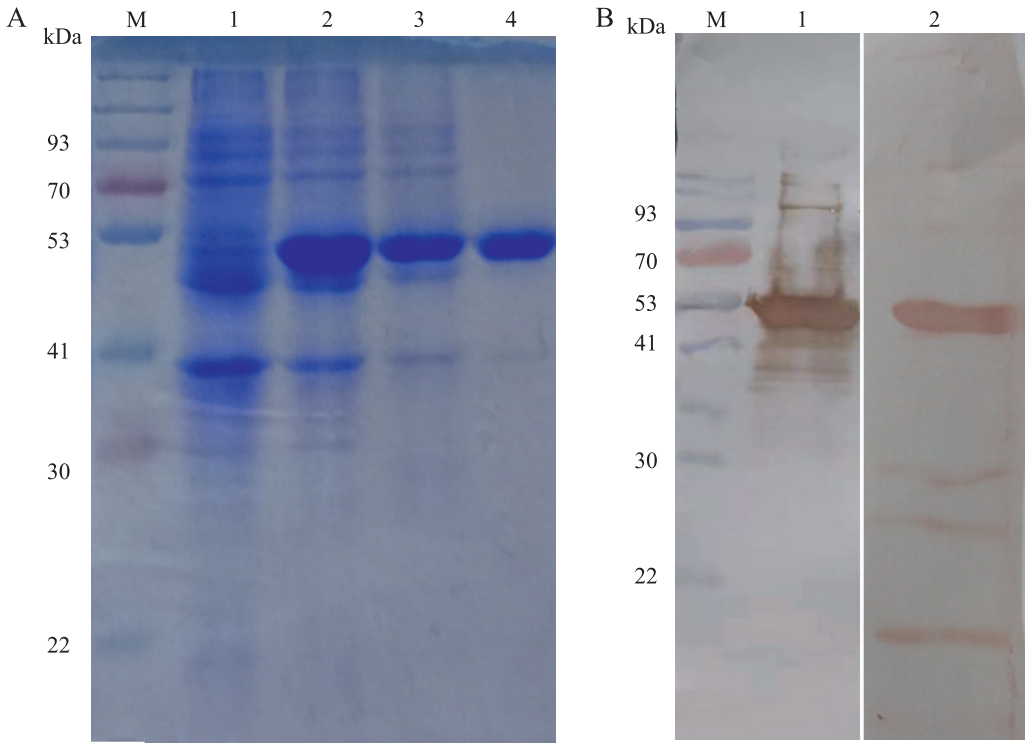

Fig. 2. Purification of recombinant N protein (A) and analysis of its immunogenicity by Western blot (B). (A). Lane M: pre-stained protein molecular weight marker; Lane 1: non-induced *E. coli* BL21(DE3) cells bearing pET28a-N; Lane 2: induced *E. coli* BL21(DE3) cells bearing pET28a-N; Lane 3: supernatant of cell lysate from pET28a-N transformed cells induced at 25°C; Lane 4: purified N protein (8.4 μg). (B). Lane 1: reaction between N protein and monoclonal antibody 2B1B1; Lane 2: reaction between N protein and Sinovac inactivated SARS-CoV-2 vaccine-immunized human sera.

mated to be 50 kDa, which was consistent with that predicted by DNAStar software.

The expression of water-soluble proteins by *E. coli* cells is affected by several factors such as the hydrophilicity of the protein and the expression conditions including temperature, final concentration of IPTG, and time (Gutiérrez-González et al. 2019). To test if expression of water-soluble N protein could be achieved, the induction temperature was optimized. The cells were induced with 0.5 mM IPTG at 16°C or 25°C for 12 h, respectively. Fig. 1B indicates that water-soluble protein appeared in the supernatants of cell lysates at both induction temperatures, whereas a large amount of soluble protein was harvested from cells induced at 25°C. Hence, such an expression condition was determined to obtain water-soluble N protein.

Purification and bioactivity of recombinant N protein

As there existed specific binding between the His_6-tag of recombinant N protein and Ni-NTA resin, affinity chromatography was used for the purification of N protein. It was found that recombinant N protein in the supernatant of cell lysate could bind to the Ni-NTA resin. After washing with 40 mM imidazole, unwanted proteins were removed. Recombinant N protein was then eluted from the column with 500 mM imidazole and its purity was examined using SDS-PAGE (Fig. 2A). After determination of protein concentration using a Bradford Protein Assay Kit, the overall yield of purified N protein was calculated to be over 28.4 mg/L cell culture. Western blot analysis showed that the expressed protein could be recognized by monoclonal antibody 2B1B1 and a pool of Sinovac inactivated SARS-CoV-2 vaccine-immunized human sera (Fig. 2B).

Characterization of monoclonal antibodies to N protein

To produce monoclonal antibodies specific to the N protein of SARS-CoV-2, BALB/c mice were immunized with purified N protein. Splenocytes from mouse with the highest antibody titer in ELISA were fused with SP2/0 myeloma cells under treatment with PEG 2000. After cell fusion, fourteen monoclonal antibodies specific to N protein were obtained and four of these were subjected to the preparation of ascitic fluids, namely 2B1B1, 4D3A3, 5G1F8, and 7C6F5. Indirect ELISA showed that the four monoclonal antibodies were able to bind to the N protein with high affinity, but not to the HA protein of the H3N2 influenza virus

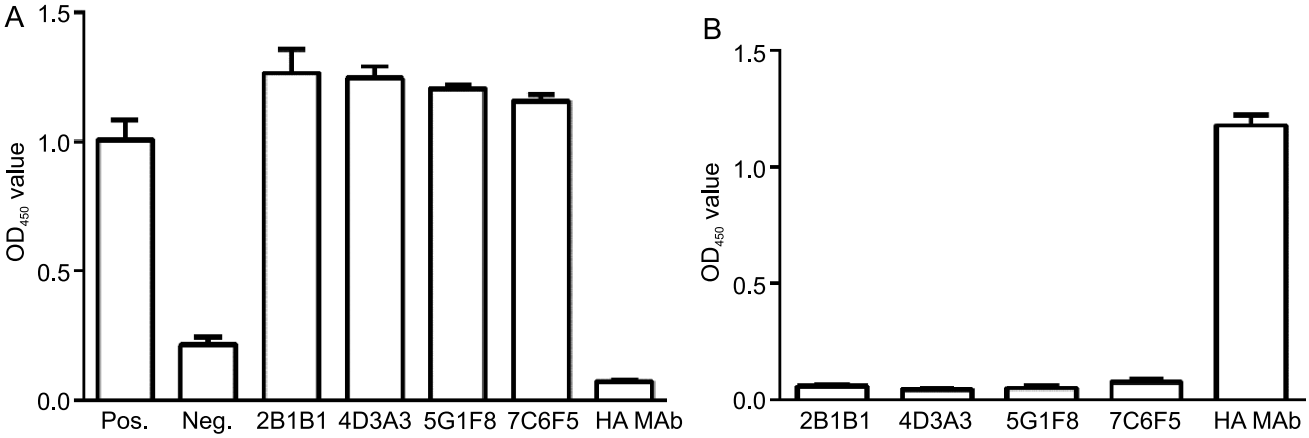

Fig. 3. Reactivity of monoclonal antibodies with N protein of SARS-CoV-2 (A) and HA protein of H3N2 influenza virus (B). Pos. represents Sinovac inactivated SARS-CoV-2 vaccine-immunized human sera; Neg. represents negative human sera to SARS-CoV-2; HA MAb represents a monoclonal antibody to the His_6-tagged HA protein of H3N2 influenza virus.

Table 1. Titers of antibodies determined using ELISA.

MAb	Supernatant	Ascitic fluid
2B1B1 (IgG1/κ)	1×640	$2.56\times10^{6.0}$
4D3A3 (IgG2a/λ)	1×320	$1.28\times10^{6.0}$
5G1F8 (IgG1/κ)	1×640	$1.28\times10^{6.0}$
7C6F5 (IgG1/κ)	1×640	$2.56\times10^{6.0}$

(Fig. 3). In addition, ELISA revealed that there existed a specific reaction between Sinovac inactivated SARS-CoV-2 vaccine-immunized human sera and purified N protein, while no reaction between HA MAb and N protein was found (Fig. 3). Antibody subtypes and titers in the culture supernatant and ascitic fluids were determined using ELISA and were listed in Table 1. It should be noted that antibody titers for the four monoclonal antibodies all reached over $1.28\times10^{6.0}$ in ELISA.

To test the specificity of the monoclonal antibodies, IPMA was performed which examined the reactions between monoclonal antibodies and pcDNA3.1-N transfected cells (Fig. 4). The results showed that 2B1B1, 4D3A3, 5G1F8, and 7C6F5 were able to react with eukaryotically-expressed N protein in transfected cells. The antibody against the HA protein of the H3N2 influenza virus did not bind to the transfected cells, indicating that all four monoclonal antibodies were specific to the N protein of SARS-CoV-2. IFA also showed that the four monoclonal antibodies could specifically bind to transfected cells while the control antibody showed no reaction (Fig. 5). All monoclonal antibodies to the N protein had no reactions with pcDNA3.1-transfected cells in both IPMA and IFA (data not shown).

Discussion

The prevention and control of COVID-19 transmission relies on the development of effective diagnostic methods. At present, quantitative reverse-transcription polymerase chain reaction (qRT-PCR), colloidal gold-based paper test, and chemiluminescence immunoassay have been used for the detection of nucleic acids, serum IgM and IgG, and viral antigens of SARS-CoV-2, respectively (Liao et al. 2020, Mak et al. 2020, Liu et al. 2021, Lv et al. 2021). Among them, qRT-PCR is recognized as the gold standard. However, nucleic acid detection is generally time consuming and requires a high level of biosafety in the laboratory, which is not convenient for grass-roots hospitals to carry out. In contrast, the detection of serum antibody has been shown to be more biologically safe and produces fewer false negative results than nucleic acid detection (Ji et al. 2020). Therefore, antibody detection is an important supplement to the nucleic acid test and can be applicable for wide use in clinics.

For the specific and sensitive detection of antibodies to SARS-CoV-2, the preparation of pure viral protein antigen is a prerequisite. In addition, the viral proteins to be used in immunoassays should be highly immunogenic and be able to elicit the production of antibodies at an early stage of infection. The N protein of SARS-CoV-2 could meet these requirements. It is the most abundantly expressed protein during infection and has been reported to induce antibody production as early as day one post-symptom onset (Guo et al. 2020). Moreover, several immunodominant epitopes have been identified on the N protein and has proposed for use for the development of peptide-based

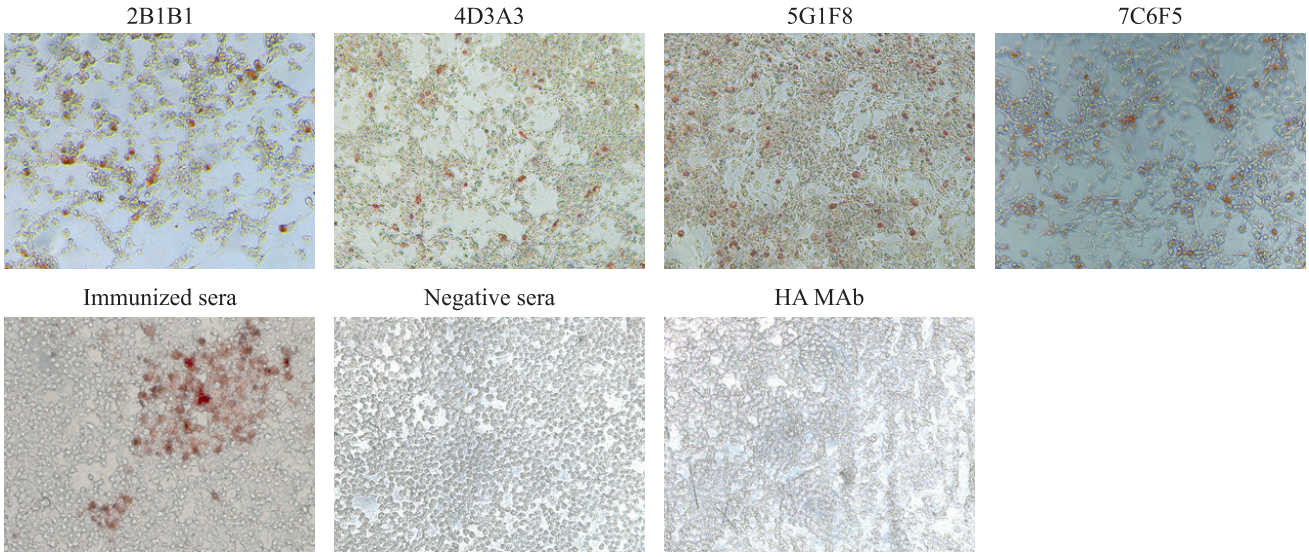

Fig. 4. Reactivity of monoclonal antibodies with pcDNA3.1-N transfected cells in IPMA. BHK-21 cells were transfected with pcDNA3.1-N and used to test the reactivity of antibody samples with eukaryotically-expressed N protein of SARS-CoV-2. Positive reactions were revealed with immunoenzymological staining methods and images were taken at ×100 magnification.

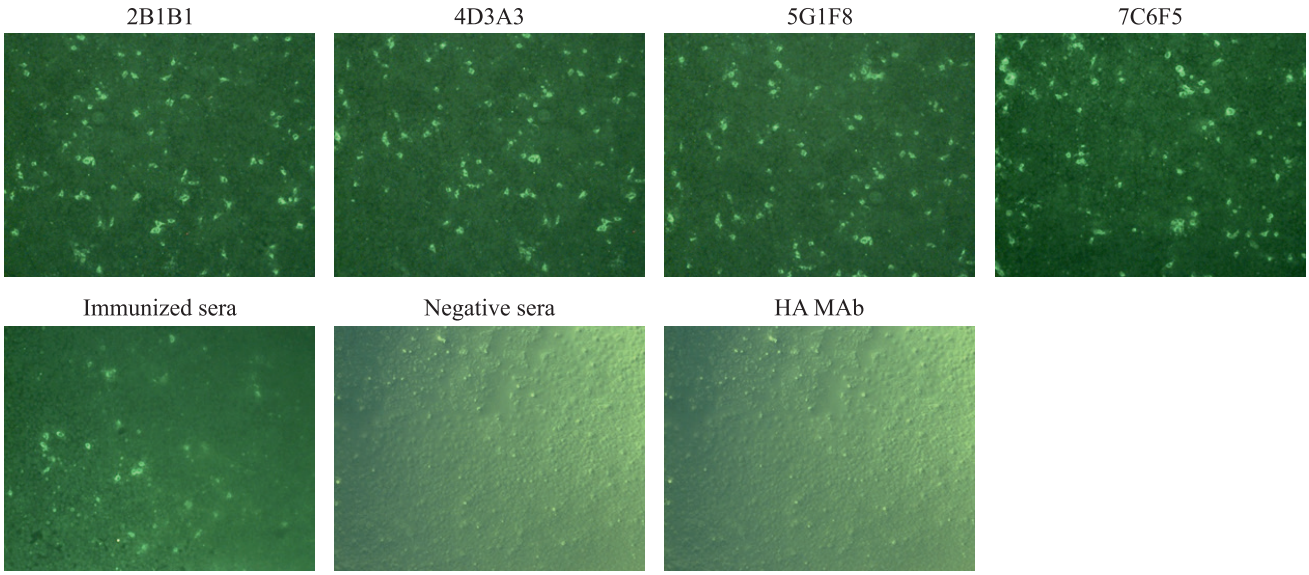

Fig. 5. Reactivity of monoclonal antibodies with pcDNA3.1-N transfected cells in IFA. BHK-21 cells were transfected with pcDNA3.1-N and used to test the reactivity of antibody samples with eukaryotically-expressed N protein of SARS-CoV-2. Images were taken at ×100 magnification.

immunoassays (Tian et al. 2022, Vashisht et al. 2023). To express foreign protein, *E. coli* cells are usually used, which allows the harvest of protein at high yield and low cost. However, expression of proteins in *E. coli* cells under certain induction conditions often leads to aggregation due to the misfolding of protein molecules. Among the expression conditions, temperature is demonstrated to be the most significant factor as expression of insoluble proteins has been shown to be correlated with higher temperatures (Gutiérrez-González et al. 2019). Our previous studies also found that expression of water-soluble proteins could be obtained through lowering the induction temperature as 37°C is the optimal temperature for *E. coli* growth, and expression at 37°C often causes accumulation of insoluble proteins (Jin et al. 2012, Wang et al. 2017). Therefore, the induction temperature was optimized to obtain water-soluble N protein in this study. The results showed that expression of N protein in water-soluble forms was realized at both 16°C and 25°C. However, induction at 16°C was found to severely affect the amount of N protein expressed, whereas expression at 25°C guaranteed both the expression of water-soluble N protein and the yield. In addition, the induction time was prolonged to 12 h to ensure the yield.

Correct display of epitopes on viral proteins is essential for the recognition by B cell receptors and the induction of antibodies *in vivo*. Therefore, to assure the

expressed N protein is bioactive, it is essential to test its reactivity with SARS-CoV-2 vaccine-immunized human sera. Western blot and ELISA showed that there were specific reactions between N protein and Sinovac inactivated SARS-CoV-2 vaccine-immunized human sera, indicating that the recombinant N protein prepared in this study possessed the same epitopes as that expressed on the native N protein of the virus.

The immunogenicity of N protein was examined by immunization of mice and the production of monoclonal antibodies. Four monoclonal antibodies were generated which showed specific reactions with N protein in Western blot and ELISA. In addition, ELISA showed that all four monoclonal antibodies had no reaction with the His_6-tagged HA protein of H3N2 influenza virus, which indicated that the monoclonal antibodies to N protein did not react with the His_6-tag, further proving their specificity. Since the N protein used for immunization of mice was expressed in *E. coli*, a prokaryotic expression system, the specificity of monoclonal antibodies to SARS-CoV-2 was confirmed by testing their reactivity with eukaryotically-expressed N protein. IPMA showed that 2B1B1, 4D3A3, 5G1F8, and 7C6F5 were able to bind to BHK-21 cells transfected pcDNA3.1-N, while a control antibody against the HA protein of the H3N2 influenza virus did not react with pcDNA3.1-N transfected cells, indicating that all four monoclonal antibodies were specific to the N protein of SARS-CoV-2. This result was confirmed further by IFA which also showed that the four monoclonal antibodies possessed specific reactions with pcDNA3.1-N transfected cells. These data demonstrated that recombinant N protein was highly immunogenic in mice and induced the production of high-affinity antibodies.

In summary, expression of water-soluble N protein was achieved in the *E. coli* expression system by optimizing the induction temperature. Western blot and ELISA showed that recombinant N protein could be specifically recognized through Sinovac inactivated SARS-CoV-2 vaccine-immunized human sera and had good biological activity. Meanwhile, we prepared monoclonal antibodies against N protein using hybridoma technology and characterized their specificity with ELISA, IPMA, and IFA. It was found that monoclonal antibodies could specifically react with recombinant N protein and pcDNA3.1-N transfected cells. These results indicate that recombinant N protein possesses high immunogenicity and shares the same epitopes with the native N protein on the virus. The preparation of water-soluble N protein and its cognate monoclonal antibodies could greatly facilitate the development of immunoassays for detecting antibodies to SARS-CoV-2 and the viral antigen.

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