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Letter

A pair of SARS-CoV-2 nucleocapsid protein monoclonal antibodies shows high specificity and sensitivity for diagnosis

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Dear Editor,

Severe Acute Respiratory Syndrome Coronavirus Type 2 (SARS-CoV-2) is the pathogen of the coronavirus disease 2019 (COVID-19), which has spread worldwide.

SARS-CoV-2 is an enveloped virus belongs to the family of β-coronavirus (Xu et al., 2020). Its genome is a linear single-stranded positive-sense RNA about 30,000 nucleotides packed inside a virion with a diameter of 100 nm and a volume of about 10^6 nm^3 (Bar-On et al., 2020). The genome of SARS-CoV-2 encodes 29 proteins, including 16 nonstructural, four structural, and nine accessory proteins (Bai et al., 2021). The four structural proteins are spike (S) glycoprotein, envelope (E) protein, membrane (M) protein, and nucleocapsid (N) protein (Naqvi et al., 2020), which are shared with other coronaviruses (Brian and Baric, 2005; Fehr and Perlman, 2015). S glycoprotein is essential for viral infection while N protein facilitates viral RNA packing, replication, virion assembly, and release (Almazán et al., 2004; Peng et al., 2020). SARS-CoV-2 has 96% identity to bats coronaviruses and 91% identity to pangolins coronaviruses in nucleic acid sequence. In contrast, its nucleotide homology to SARS-CoV-1 is 80%, to Middle East Respiratory Syndrome (MERS) Coronavirus (MERS-CoV) is 55% (Bar-On et al., 2020; Zhou et al., 2020). Due to its error-prone RNA dependent RNA polymerase (RdRp), mutation rate of SARS-CoV-2 is relatively high (Ludwig and Zarbock, 2020). Accordingly, multiple strains of SARS-CoV-2 have emerged, such as Alpha, Beta, Gamma, Delta, and Omicron, etc.

The N protein consists of three domains: an amino-terminal RNA-binding domain (NTD), a carboxyl-terminal dimerization domain (CTD), and an intrinsically disordered central Ser/Arg (SR)-rich linker (Kang et al., 2020). The nucleic acid sequences of the N proteins from different coronaviruses and different SARS-CoV-2 strains are relatively conserved (Peng et al., 2020). Besides, the N protein of SARS-CoV-2 is highly immunogenic and abundant in serum of infected patient (Burbelo et al., 2020). It is thus a good serological antigen for diagnosis of SARS-CoV-2 infection (Ni et al., 2020; Xiang et al., 2020; Zeng et al., 2020). SARS-CoV-2 is highly contagious and spreads very quickly, thus it is urgent to develop an accurate diagnosis. However, most of the effects nowadays are devoted to produce blocking antibody through screening B cells from the patients (Cohen-Dvashi et al., 2022; Ju et al., 2020; Sun et al., 2022) while fewer reports on producing antibodies for diagnosis (Wang et al., 2022). In this study, we aimed to produce monoclonal antibody (mAb) against the N protein of SARS-CoV-2, which we hope will be useful for COVID-19 diagnosis.

First, the N protein was cloned into the restriction enzyme sites *Nco* I and *Sal* I of pET-28a (+) to generate the pET-28a (+)-SARS-CoV-2 (N) recombinant plasmid (Supplementary Fig. S1A), which was then transformed into *E. coli* Rosetta (DE3). Expression of the N protein was induced with IPTG (Isopropyl-β-D-thiogalactopyranoside) at the concentration of 1 μmol/L, and the induced N protein was further purified with a Ni-NTA affinity chromatography column (QIAGEN, Cat#30210, Valencia, CA, USA) (Fig. 1A). The purified protein was injected into three BALB/c mice to produce mAb according to a standard procedure (Supplementary Table S1). After immunization, seven hybridoma cell lines stably secreting mAbs against N protein were obtained and named as 1C7, 1D5, 2E11, 2G11, 3C6, 4F10, and 5E11, respectively. Karyotyping confirmed that these mAbs were hybridoma cells (Fig. 1B). The isotypes of these mAbs were determined using a kit from Proteintech (PK 20002, Wuhan, China) (Supplementary Table S2).

We then determined whether these mAbs could be applied for Western blotting (WB). The N protein was cloned into pcDNA 3.1 (+) to generate the pcDNA 3.1 (+)-SARS-CoV-2 (N) expression plasmid (Supplementary Fig. S1B). Cell lysates (50 μg/lane) from 293T cells transfected with pcDNA 3.1 (+)-SARS-CoV-2 (N) plasmid were

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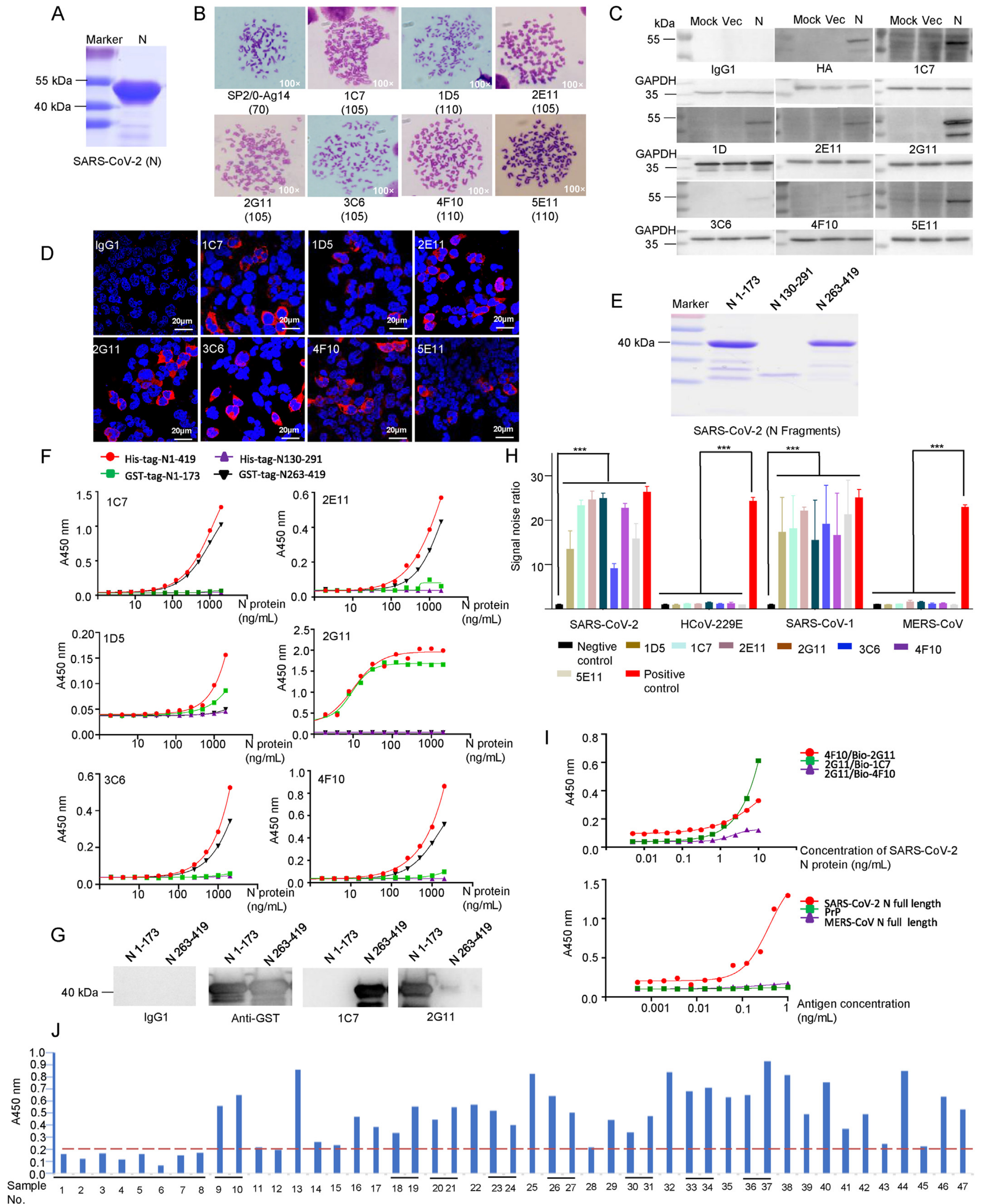

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Fig. 1. Production and characterization of mAbs against N protein of SARS-CoV-2. **A** Coomassie brilliant blue staining of purified N protein. The expressed N protein was purified with a Ni-NTA column and tested with SDS-PAGE assay. **B** Karyotyping of hybridoma cells. The number in parentheses represented the number of chromosomes that each hybridoma cell line came from. **C** WB assay for N protein detection with the seven mAbs. SARS-CoV-2 N protein expressed in 293T cells was separated by SDS-PAGE and transferred to a nitrocellulose membrane. N protein was blotted with different mAbs. Mock: no plasmid transfected; Vec: Empty vector transfected; N: N protein. **D** IF staining with the seven selected mAbs to detect N protein expressed in 293T cells. 293T cells expressing SARS-CoV-2 N protein were fixed with 4% paraformaldehyde and blocked with 3% BSA/PBST. The seven mAbs were then incubated with the fixed cells and bound primary antibodies were detected with alex fluor 555 nm conjugated goat anti-mouse IgG (1:2000). **E** Three truncated N protein fragments were expressed and detected with SDS-PAGE. The recombinant protein was purified with a glutathione-agarose affinity chromatography (Cytiva, Cat#17075601), and stained with Coomassie brilliant blue. **F** ELISA to determine the reaction between different mAbs and different domains of N protein. N protein was coated at different concentrations in the ELISA plate and detected with different mAbs (50 ng/well). Bound mAbs were further detected with HRP conjugated goat anti-mouse IgG (1:8000). **G** WB to determine the domains of N protein recognized by 2G11 and 1C7 mAbs. Purified truncated N proteins (1 μg/lane) were run on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. 2G11 and 1C7 (1 ng/μL) were used to detect the target protein. Mouse IgG1 and an antibody specific against GST used at the same concentration were applied as negative and positive controls, respectively. **H** ELISA to determine the reaction between different mAbs and N proteins of SARS-CoV-2, HCoV-229E, SARS-CoV-1, and MERS-CoV (50 ng/well). Coated N proteins were detected with mAbs (50 ng/well), and bound mAbs were further detected with HRP conjugated goat anti-mouse IgG. ***: $P < 0.001$ (two tailed, Student *t*-test). **I** Determination of mAbs combination pair and its sensitivity for detecting with different concentrations of SARS-CoV-2 N protein. ELISA plate was coated with 4F10, 2G11 (100 ng/well) and then different concentrations of SARS-CoV-2 N protein were added. Bound SARS-CoV-2 N protein was detected with bio-2G11, bio-1C7, or bio-4F10 (50 ng/well) as indicted. Biotinylated antibodies were further detected with HRP conjugated streptavidin. To determine the sensitivity of the 2G11/bio-1C7 mAbs pair, the ELISA was coated with 2G11 (100 ng/well) and different concentrations of SARS-CoV-2 N protein or negative control proteins of PrP and MERS-CoV N protein. The bound proteins were further detected with bio-1C7 (50 ng/well). HRP conjugated streptavidin was used to detect the bound biotinylated mAbs. **J** Determination of specificity and sensitivity of the mAb 2G11 with patient samples. ELISA plate was coated with 2G11 (50 ng/well) and blocked with 3% BSA/PBST. Then 50 ng/well SARS-CoV-2 N protein was loaded. Human plasma (1 μL/well) from eight healthy donors (Sample No. 1–8) and 31 SARS-CoV-2 infected patients (Sample No. 9–47) were then loaded. Sample numbers underlined mean the samples from the same patient but at different dates. Bound human IgG was detected with preabsorbed HRP conjugated goat anti-human IgG. Sample information was listed in Supplementary Table S4. Full experiment details are given in the Supplementary file.

separated by a 12 % SDS-PAGE and transferred onto a nitrocellulose membrane, and cell lysates transfected with the vector pcDNA 3.1 (+) plasmid were used as the negative control. The purified mAbs were used to detect the expression of N protein with a concentration of 1 ng/μL, and mouse IgG1 at the same concentration was used as a negative control. Polyclonal antibody against hemagglutinin (HA) was used as a positive control to identify the molecular weight of the expressed protein (Tianjin Sungene Biotech, Cat#KM8004, Tianjin, China) (Fig. 1C). WB assay showed that all produced mAbs reacted specifically against the N protein of SARS-CoV-2. However, 3C6 showed the weakest reaction while 2G11 is the strongest (Fig. 1C). In addition, 2G11 also identified a protein with smaller molecular weight (Fig. 1C), which is most likely a proteolytic product of the N protein since it was not detected in the vector transfected cells. The seven purified mAbs were further assessed in immunofluorescence (IF) staining assay with a concentration of 10 ng/μL and mouse IgG1 at the same concentration was used as the negative control. We found that all mAbs showed cytoplasmic staining although 2E11, 4F10, 2G11, and 5E11 had occasional nuclei staining (Fig. 1D).

To investigate which domain of N protein was recognized by the mAbs, we cloned the amino-terminal domain (1–173), the central domain (130–291), and the carboxyl-terminal domain (263–419) of SARS-CoV-2 N protein into pGEX-6p-1, respectively (Supplementary Fig. S1C). Primers used for expressing recombinant SARS-CoV-2 N protein were listed inSupplementary Table S3. The SDS-PAGE assay confirmed that all truncated N proteins were successfully expressed (Fig. 1E). Then in the enzyme linked immunosorbent assay (ELISA), the three truncated and the full-length N proteins were used to determine which domain of SARS-CoV-2 N protein was recognized by the mAbs. It turned out that the mAbs (2G11 and 1D5) recognized the amino-terminus of SARS-CoV-2 N protein and the full-length N protein (Fig. 1F). Another four mAbs (1C7, 2E11, 3C6, and 4F10) could only recognize the carboxyl-terminal domain of SARS-CoV-2 N protein and the full-length N protein (Fig. 1F). In contrast, we could not identify which domain of N protein was recognized by mAb 5E11 (result not shown), and the exact reason remains unknown. Since 1C7 and 2G11 seem to recognize distinct domains of N protein with relatively high affinity, we further investigated if these two mAbs could react to the two domains of N protein via WB. The results showed that 2G11 recognized the amino-terminus while 1C7 only recognized the carboxyl-terminal domain of SARS-CoV-2 N protein. As expected, mouse IgG1 used at the same concentration did not show any positive reaction while a mAb specific against GST Tag identified a positive signal with expected molecular weight in WB assay (Fig. 1G).

We then determined whether these mAbs could differentiate N proteins of different coronaviruses with ELISA assay. Thus, the seven mAbs were added into ELISA plate coated with different N proteins (20 ng/well) from MERS-CoV, Human Coronavirus (HCoV)-229E, SARS-CoV-1 and SARS-CoV-2 in an ELISA assay. The result showed that these mAbs did not cross react with the N protein of MERS-CoV and HCoV-229E but cross reacted with that of SARS-CoV-1 (Fig. 1H).

We further assessed the utility of different mAbs combinations in detecting SARS-CoV-2 N protein with double sandwich ELISA (dsELISA). For this purpose, the ELISA plate was coated with 1C7, 4F10, or 2G11, three mAbs with relatively higher reaction in ELISA. Full-length N protein was then diluted and loaded into each well as indicated. Biotin (bio) conjugated 2G11, 1C7, or 4F10 were used individually as detecting antibody in dsELISA, and horseradish peroxidase (HRP) conjugated streptavidin (1:10,000) was loaded to detect the bound biotinylated mAbs. It turned out that 2G11/bio-1C7 was the best combination to detect SARS-CoV-2 N protein (Fig. 1I, upper). We then determined the sensitivity and specificity of the 2G11/bio-1C7 in detecting SARS-CoV-2 N protein. In this case, we applied recombinant prion protein (PrP) and MERS-CoV N protein at the same concentration as SARS-CoV-2 N protein as negative controls. We found that the 2G11/bio-1C7 mAbs combination showed high specificity and sensitivity in detecting SARS-CoV-2 N protein that can be as low as 15 pg/well with an OD450 nm value over 0.2 (Fig. 1I, below), which was comparable to a similar ELISA kit with a sensitivity of 70 pg (RayBiotech). These results imply that the mAbs generated can be used potentially for SARS-CoV-2 detection.

Finally, we used human blood samples to assess the specificity and sensitivity of the mAbs. The ELISA plate was coated with 2G11 and then SARS-CoV-2 N protein was loaded. Forty-seven human plasma samples, among which eight were plasma from healthy donors and 39 others were from SARS-CoV-2 infected patients, were then loaded into the plate. Among the 39 positive samples, 16 were paired samples derived from eight patients at different dates. The bound human IgG was detected with preabsorbed HRP conjugated goat anti-human IgG (1:8000). It turned out that this ELISA had a sensitivity of 97.4% (38/39) and a specificity of 100% (Fig. 1J). Six samples from SARS-CoV-2 infected patients derived at the later dates had a higher IgG reactivity against SARS-CoV-2 N protein (Fig. 1J). However, two samples (24 & 27) derived at the later dates showed weaker IgG reactions. The exact mechanism warrants further investigation.

Here, we report the production of seven mAbs against SARS-CoV-2 N protein. These mAbs can be used successfully for WB and IF assay, and most of them can also be applied in ELISA. Although these mAbs cannot differentiate N protein of SARS-CoV-1 from that of SARS-CoV-2, they cross reacted to N protein of neither MERS-CoV nor HCoV-229E. It is worth noting that the 2G11/bio-1C7 mAbs pair can detect SARS-CoV-2 N protein as low as 15 pg/well. This sensitivity is comparable to that of an ELISA kit available. In addition, we developed an ELISA method to detect human plasma IgG against SARS-CoV-2 N protein, which showed a sensitivity of 97.4% and a specificity of 100%. Although we have identified approximate domains recognized by these mAbs, the exact epitopes reacted with these mAbs remain investigated. In a word, we produced several mAbs against the N protein of SARS-CoV-2, among which a pair shows high specificity and sensitivity for SARS-CoV-2 diagnosis.

Footnotes

This study was conducted according to ARRIVE guidelines and all animal experimental procedures approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Guangzhou Medical University and State Key Laboratory of Respiratory Diseases, China (Protocol Permit Number: 2021243 and 2021338; approved on July 2021 and August 2021). Mice were maintained in Specific Pathogen Free (SPF) animal facility of Laboratory Animal Center, Guangzhou Medical University. The authors declare that they have no conflict of interest.

The SP2/0-Ag14 mouse myeloma cells used in the experiment is a gift from Professor Jin Su of the State key Laboratory of Respiratory Diseases. The plasma of SARS-CoV-2 infected patients or healthy donors and purified N proteins of SARS-CoV-1, MERS-CoV, and HCoV-229E used in ELISA for differentiating the mAbs were provided by Professor Jincun Zhao of the State Key Laboratory of Respiratory Diseases. The Olympus BX53 system microscope for karyotype analysis was provided by Professor Weijie Guan of the State Key Laboratory of Respiratory Diseases. We thank Ms. Yongfeng Zheng for her kind help in the process of using ZEISS confocal laser scanning microscope.

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