Development of a SARS-CoV-2 nucleocapsid specific monoclonal antibody

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A B S T R A C T

To help fight COVID-19, new molecular tools specifically targeting critical components of the causative agent of COVID-19, SARS-Coronavirus-2 (SARS-CoV-2), are desperately needed. The SARS-CoV-2 nucleocapsid protein is critical for viral replication, integral to viral particle assembly, and a major diagnostic marker for infection and immune protection. Currently the limited available antibody reagents targeting the nucleocapsid protein are not specific to SARS-CoV-2 nucleocapsid protein, and sequences for these antibodies are not publicly available. In this work we developed and characterized a series of new mouse monoclonal antibodies against the SARS-CoV-2 nucleocapsid protein, with a specific clone, mBG86, targeting only SARS-CoV-2 nucleocapsid protein. The monoclonal antibodies were validated in ELISA, Western blot, and immunofluorescence analyses. The variable regions from six select clones were cloned and sequenced, and preliminary epitope mapping of the sequenced clones was performed. Overall, these new antibody reagents will be of significant value in the fight against COVID-19.

1. Introduction

Over the course of the last year, the novel SARS-CoV-2 coronavirus has spread dramatically across the world, causing the severe respiratory illness termed COVID-19. There have been over 87 million reported cases of COVID-19 globally as of December 2020 (D-19 Dashboard John H, 2994), and over 1.7 million reported deaths attributed to this devastating disease. SARS-CoV-2 is a respiratory droplet-borne pathogen (Nardell and Nathavitharana, 2020) and is easily transmitted between individuals in close proximity, leading to explosive spread and a dire need for rapid diagnostic testing to help control outbreaks.

Testing for COVID-19 infection currently focuses primarily on detection of viral genomic RNA present in patient respiratory samples, including nasopharyngeal swabs and nasal samples. Because COVID-19 is a respiratory disease, detection of viral genomic RNA in patient nasal samples is a positive indicator of both infection and the potential for an infected individual to spread the virus to others. The current diagnostic for detecting viral genomic RNA is quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), which can sensitively detect the presence of viral RNA in samples (Corman et al., 2020; Wang et al., 2020; Zhai et al., 2004) and can be automated to test large numbers of samples in parallel. This workhorse assay can provide exquisitely sensitive and specific detection of SARS-CoV-2 infection, but faces challenges. Those challenges include the need for a centralized laboratory for testing, significant pre-processing of samples, the high cost of

reverse-transcription quantitative PCR reagents, and the need for sophisticated real-time capable thermocyclers for performing the PCR procedure (Li et al., 2020a). Fortunately, RNA is only one of a number of analytes that can provide significant clinical value for diagnosing infection. The coronavirus nucleocapsid protein is one such analyte that can be used in rapid, specific, and inexpensive diagnostic methods for symptomatic patients.

Coronavirus RNA genomes are coated with nucleocapsid protein within both virions and infected cells. The nucleocapsid (N) protein is a ~50 kDa protein that forms dimers that oligomerize on viral RNA, providing protection of the viral genome from cellular RNA decay enzymes and compacting the viral genome into a small enough package to fit within virion particles (Saikatendu et al., 2007; Zúñiga et al., 2007; Chang et al., 2009; Zeng et al., 2020). There have been estimates that between 720 and 2200 nucleocapsid monomers are present for every viral RNA genome copy within virion particles (Zeng et al., 2020; Cavanagh, 1983; Neuman et al., 2011; Escors et al., 2001; Hogue and Brian, 1986; Liu and Inglis, 1991), making the nucleocapsid protein an intriguing analyte for viral infection. Several publications from the original SARS-CoV outbreak in 2003–2004 indicated that detection of nucleocapsid in patient serum samples is diagnostic for early SARS disease, and the amount of detectable SARS-CoV nucleocapsid antigen present in patient samples tracked well with viremia (Che et al., 2004; Li et al., 2005; Shi et al., 2003; Zhu et al., 2006; Peiris et al., 2003). More recent data from the SARS-CoV-2 pandemic indicate that N protein is

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found in very low but detectable amounts in patient serum (Li et al., 2020b), but N protein has been found in greater amounts in patient nasopharyngeal swab and anterior nares swab samples (Diao et al., 2020). Given the high copy number of the N protein compared to viral genomes and the relative stability of N protein in patient samples, detection of this antigen can serve as a valuable orthogonal diagnostic marker compared to genome detection by RT-qPCR in diagnostic assays such as ELISA or point-of-care lateral flow assays.

Detection of protein analytes requires specific antibodies, and since SARS-CoV-2 has emerged very recently, no SARS-CoV-2 specific antibodies have been reported in the literature. Due to the significant homology between SARS-CoV and SARS-CoV-2, new antibodies need to be produced for the research community that may have increased specificity and utility for detecting SARS-CoV-2 nucleocapsid protein or for potential therapeutic use (Lee et al., 2008, 2010; Dutta et al., 2008). Additionally, there are no published sequences for SARS-CoV or SARS-CoV-2 specific antibodies, and the only way to get these antibodies is from commercial sources. Validated and publicly available anti-N antibody sequences can lead to the development of new molecular tools such as scFv, chimeric antibodies, and other applications that are not currently possible due to the lack of available sequences.

Here we report the generation and characterization of a panel of monoclonal antibodies targeting the SARS-CoV-2 N protein. We expressed and purified a truncated recombinant N proteins from all major human coronaviruses, used the recombinant SARS-CoV-2 antigen to immunize mice and generate a panel of hybridomas, and tested the resulting clones for activity in Western blot, ELISAs, and immunofluorescence assays with N protein from SARS-CoV-2 infected cells. Cross-reactivity of the antibodies against N protein from the six other representative human coronaviruses (SARS-CoV, HuCoV-OC43, HuCoV-HKU1, and HuCoV-NL63, and HuCoV-229E) was tested for assessing the ability for these antibodies to function in specific diagnostic methods versus a controlled research capacity. We determined the V_H and V_L sequences of the top 6 antibody clones and performed epitope mapping to identify antigenic regions within the N protein. Overall, our data provides strong evidence for the specificity and utility of the SARS-CoV-2 specific mBG86 monoclonal antibody and an antibody toolkit for the study of SARS-CoV-2 N protein.

2. Materials and methods

2.1. *Expression and purification of coronavirus N proteins*

Amino acid sequences for coronavirus N proteins (SARS-CoV-2 (YP_009724397.2), SARS-CoV (ABI96968.1), MERS (YP_009047211.1), HuCoV-NL63 (ABI20791.1), HuCoV-229E (N protein_073556.1), HuCoV-HKU1 (AYN64565.1), HuCoV-OC43 (QBP84763.1) were obtained from GenBank for sequence alignments. For generation of *E. coli* expression plasmids, amino acid sequences from each virus (SARS-CoV-2 (AA133-419), SARS-CoV (AA133-422), MERS-CoV (AA122-413), HuCoV-OC43 (AA147-448), HuCoV-NL63 (AA100-377), HuCoV-HKU1 (AA146-441), and HuCoV-229E (AA102-389), were used to generate bacterial codon optimized DNA gBlocks using the Integrated DNA Technologies web server tool. A list of gBlocks and PCR primers can be found in Table S1. Each gBlock was cloned into the pET28a T7 expression vector using the New England Biolabs NEBuilder Assembly 2X Master Mix according to the manufacturer's instructions. The resulting clones (SARS-CoV-2 = pBG690 | SARS-CoV = pBG700 | MERS-CoV = pBG706 | HuCoV-OC43 = pBG701 | HuCoV-NL63 = pBG702 | HuCoV-HKU1 = pBG703 | HuCoV-229E – pBG705) were sequence verified by Sanger sequencing (Genewiz).

Nucleocapsid proteins were expressed in BL21 DE3 pLys *E. coli* and purified by nickel affinity and gel filtration chromatography on a HiLoad 16/600 Superdex 200 pg column essentially as previously described (Geiss et al., 2009) with the exception of using 25 mM HEPES (pH 7.5) as the buffer and changing the final NaCl concentration to 500 mM in all of

the buffers to reduce N protein oligomerization. N proteins were concentrated to ~2 mg/ml in gel filtration buffer (25 mM Hepes pH 7.5, 500 mM NaCl, 1 mM DTT, 0.1 mM AEBSF, and 10% glycerol (v/v)) using 10 K Centricon concentrators, flash frozen in liquid nitrogen, and stored at −80 °C until use.

2.2. *N protein antibody generation and initial screening of monoclonal antibodies*

All immunizations were intraperitoneal in a final volume of 200 μl containing 25 μg of recombinant N protein antigen. Two 6-week old female BALB/c mice (Jackson Laboratories) were primed with antigen emulsified in an equal volume of complete Freund's adjuvant (Millipore-Sigma). The mice were boosted 2 and 4 weeks later with antigen emulsified in incomplete Freund's adjuvant. Mice were bled from the tails at 2 and 6 weeks to verify seroconversion by ELISA. A final boost of antigen in PBS at 8 weeks followed by euthanasia and spleen retrieval 4 days later. Final antibody titer was determined to be > 6400 at the time of fusion. On the day of splenocyte collection, immunized mice were euthanized by CO_2 asphyxiation followed by exsanguination. Mouse carcasses were sterilized with 70% ethanol before the spleen was removed and eviscerated. After passing through a 16-gauge needle, the splenocytes were washed and fused with Sp2/0 Ag14 myeloma cells using the ClonaCell™-HY Hybridoma Kit (StemCell Technologies). Following fusion, hybridoma cells were rested for 24 h before being resuspended and plated in ten 10-cm plates with ClonaCell™-HY Semi-Solid Medium D and allowed to propagate for 10 days at 5% CO_2 at 37 °C. Eleven days after fusion, individual colonies were selected and transferred to individual wells in a 96-well plate using a 10 μl micropipetter. 920 colonies were harvested and grown for 5 day at 5% CO_2 and 37 °C.

2.3. *Primary hybridoma screen*

Hybridomas were screened using indirect enzyme-linked immunosorbent assay (ELISA) for activity against SARS-CoV-2 N protein. 96-well plates were coated with 2 μg/ml N protein diluted in 1X PBS and incubated overnight at 4 °C. Plates were blocked with SuperBlock™ T20 (TBS) for 1 h shaking at room temperature. After three washes with 0.1% Tween (v/v) in 1X PBS (Hyclone), supernatant from the 920 hybridoma colonies were incubated on the plates for 1 h shaking at room temperature. After three additional washes the plates were incubated with HRP conjugated goat anti-mouse polyclonal antibodies (Abcam ab97023) diluted at 1:10,000 in 1X PBS for 1 h at room temperature, shaking. The plates were washed three additional times before being developed with 1-Step™ Ultra TMB-ELISA Solution (ThermoFisher). The reaction was stopped after 4 min by adding an equal volume of 2M H_2SO_4. Absorbance at 450 nm (A_{450}) was determined for each well using a PerkinElmer Victor X5 multilabel plate reader. Absorbances were corrected against the PBS negative control and organized by absorbance on Microsoft Excel. Ninety-two colonies with the highest absorbance at 450 nm were selected for further testing.

2.4. *Bacteria cross-reactivity screen*

One 96-well plate was coated with 200ng/well of recombinant N protein and another plate was coated with 200ng/well of *E. coli* lysate (BL21 DE3 pLys) diluted in 1X PBS. The aforementioned ELISA protocol was performed using the selected 92 parent hybridoma culture supernatants as the primary antibody. Following A_{450} determination and correction in relation to the Medium E negative control, any colonies with reactivity towards the bacterial lysate protein excluded from further screening.

2.5. His-tag cross-reactivity screen

96-well plates were coated with 200ng/well of recombinant N protein and control plates were coated with 200ng/well of recombinant 6xHis-tagged SARS-CoV-2 spike protein receptor binding domain (RBD) produced using the FreeStyle 293 Expression System (Thermo Fisher) from BEI Resources (Cat# NR-52366) as described (Stadlbauer et al., 2020). The ELISA protocol described previously was performed using the selected 92 parent hybridoma culture supernatants as the primary antibody. Following A_{450} determination and correction in relation to the Medium E negative control, any colonies with reactivity towards the His tags were removed from consideration.

2.6. SARS-CoV-2 virus

SARS-CoV-2 coronavirus (Isolate USA-WA1/2020) was obtained from BEI Resources. Stocks of virus were grown in Vero-E6 cells in DMEM (Gibco) supplemented with 10% fetal bovine serum (Atlas Biologicals) and 25 mM HEPES (pH 7.5) in BSL-3 containment at the Colorado State University Regional Containment Biocontainment Laboratory. Virus containing media was stored at −80 °C in single use aliquots. Viral titers were performed using plaque assay as described (Case et al., 2020).

2.7. Immunofluorescence assay

Vero cells were plated at a concentration of 3.5×10^4 cells/well in a 96-well plate. The cells were inoculated with SARS-CoV-2 in BSL-3 at a MOI of 0.1 and allowed to absorb for 1 h at room temperature. Unabsorbed virus was washed with 1X PBS, cells were overlaid with 1X DMEM supplemented with 2 mM glutamine, non-essential amino acids and 2% fetal bovine serum, and the cells were incubated for 24 h at 5% CO_2 and 37 °C. Infected cells were briefly washed with 1X PBS then fixed with either 4% paraformaldehyde (w/v) (PFA) or methanol for 10 min before being washed three more times. PFA fixed cells were permeabilized by incubating with 0.5% Tween (v/v) in 1X PBS for 20 min. Plates of infected fixed cells were blocked with 4% nonfat dry milk powder (w/v) dissolved in 1X PBS + 0.1% Tween 20 (v/v) for 1 h at room temperature, shaking. Supernatant from the 18 parent clones along with rabbit anti-N protein polyclonal control antibody (Fagre et al., 2020) were then incubated for 1 h shaking at room temperature and then washed three times with 1X PBS + 0.1% Tween 20 (v/v). FITC-conjugated goat anti-mouse secondary antibodies or Alexa 488-conjugated goat anti-rabbit secondary antibodies at a dilution of 1:2000 in 1X PBS +0.1% Tween 20 (v/v) were added to the appropriate wells, with all wells receiving Hoescht staining at 1:4000. After 1 h of incubating the secondary antibodies and stains in the dark at room temperature, the plates were washed three times with 1X PBS +0.1% Tween 20 (v/v) with the last wash being 50 μl of 1X PBS. Cell labeling and fluorescence were observed with a Celigo high-content imaging cytometer (Nexcelcom) and a Nikon Diaphot 200 fluorescence microscope.

2.8. Western blot

The ability for the initial parental clones to detect linear epitopes was assessed via western blotting. Recombinant N protein and recombinant spike RBD proteins were resolved on a 12% gel in triplicate at 120 V for 1 h before being transferred to PVDF membranes for 1 h at 100 V. Blots were blocked with 4% nonfat dry milk powder (w/v) blocking solution in 1X PBS. Supernatants were diluted 1:5 in blocking solution (4% nonfat dry milk in 1X PBS+ 0.1% Tween 20 (v/v)) before being applied to blocked blots overnight, shaking at 4 °C. Blots were washed three times with blocking solution for 5 min each before being incubated for 1 h at room temperature with goat anti-mouse HRP-conjugated secondary antibodies diluted in blocking solution. After three additional washes

with 1X PBS, the blots were developed with 1-Step™ Ultra TMB-Blotting Solution (ThermoFisher) before being quenched in deionized water and imaged.

Reactivity towards endogenously produced N protein from an active SARS-CoV-2 infection was assessed with infected cell lysates on western blots. Vero cells were infected at 0.1 MOI with SARS-CoV-2 in BSL-3 and allowed to incubate for 48 h before cells were trypsinized, centrifuged, and resuspended in RIPA buffer. These samples were then diluted in 2X Laemmli Buffer and boiled for 15 min before being resolved on an SDS-PAGE gel alongside uninfected Vero cell lysate and processed for Western blot analysis as described above.

2.9. Human coronavirus cross-reactivity screen

96-well plates were coated with 2 μg/ml of recombinant SARS-CoV-2, SARS-CoV, MERS-CoV, HuCoV-OC43, HuCoV-HKU1, HuCoV-NL63, and HuCoV-229E N protein. These plates were used to test the 18 parent clone antibodies for reactivity using the described indirect ELISA protocol. The resulting reactivities were normalized against SARS-CoV-2 reactivity for analysis.

Western blots were performed using the top six mBG clones and the recombinant human coronavirus N proteins to determine the specificity of the antibodies in linearized protein. The previously described Western blot protocol was performed using 10 μg/well of N protein for the mBG67 and mBG86 gels and 1 μg/well of N protein per well for mBG17, mBG21, mBG22, and mBG57 gels due to parent supernatant concentration differences. A control blot from gels loaded with 1 μg/well N protein was labeled using anti-6xHis tag mouse monoclonal antibody (Sigma SAB2702218).

2.10. Isotyping

The isotype of the antibodies produced by parent hybridomas was assessed using the Pierce™ Rapid Antibody Isotyping Kit plus Kappa and Lambda (catalog no. 26179) for mouse antibodies according to the provided procedure. Briefly, antibodies were diluted 1:100 in the provided sample diluent before being applied to the lateral flow assay and the corresponding bands observed after 10 min.

2.11. Sequencing of Anti-N protein monoclonal antibody genes

RNA from each hybridomas 17, 21, 22, 57, 67, and 86 was isolated and stored at −80 °C following Trizol (Invitrogen) and phenol-chloroform extraction. Isolation of kappa, lambda, and heavy chain RNA sequences for hybridoma colonies was accomplished using a previously reported monoclonal antibody sequencing protocol (Meyer et al., 2019). Primers specific for the 3' constant region of either the kappa, lambda, or heavy chain RNA sequences outlined in the protocol were used in conjunction with SMARTScribe Reverse Transcriptase. Amplification of the RT product is accomplished with PCR using primers specific to the universal sequence and a region of the kappa, lambda, or heavy chain sequences that were offset to the primers used during reverse transcription. Successfully produced PCR products were then cloned into the NEB pMiniT 2.0 vector using the NEB PCR Cloning Kit. Five kappa and heavy chain clones were sequenced for each hybridoma by Genewiz. Clone sequences were analyzed by IgBLAST (Ye et al., 2013) for antibody framework regions (FMW) and complementary-determining regions (CDR).

2.12. Epitope mapping

Primers described in Table S1 in were used to create 50 amino acid deletions from the pBG690 plasmid used to produce SARS-CoV-2 N protein amino acids 133–419. The primers produced recombinant N protein variant sequences corresponding to proteins Δ133–179, Δ180–229, Δ230–279, Δ325–379, and Δ381–419. These PCR products
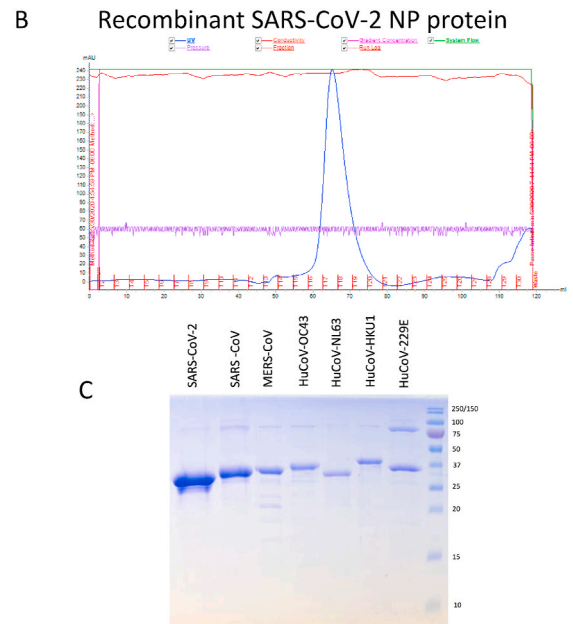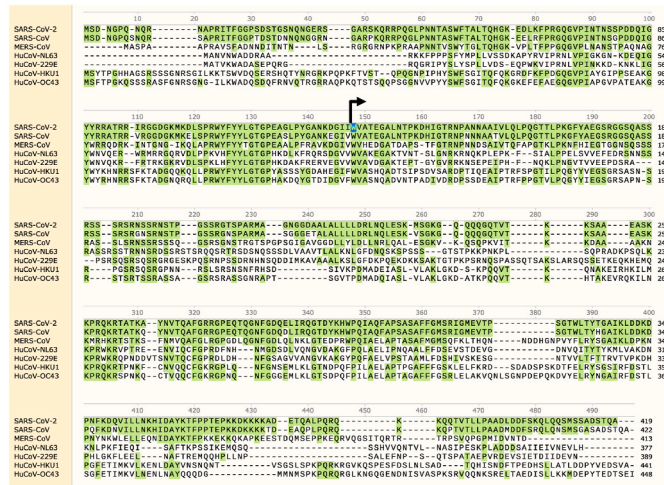
Fig. 1. Production and characterization of truncated SARS-CoV-2 nucleocapsid protein. A) Sequence alignment of human coronavirus N proteins. The arrow denotes the start of the expressed proteins. **B)** Size exclusion chromatograph of nickel-column purified SARS-CoV-2 NP (133–419). **C)** SDS-PAGE gel of purified recombinant Coronavirus nucleocapsid proteins. Each recombinant nucleocapsid protein starts at the same tryptophan residue as SARS-CoV-2 AA-133.
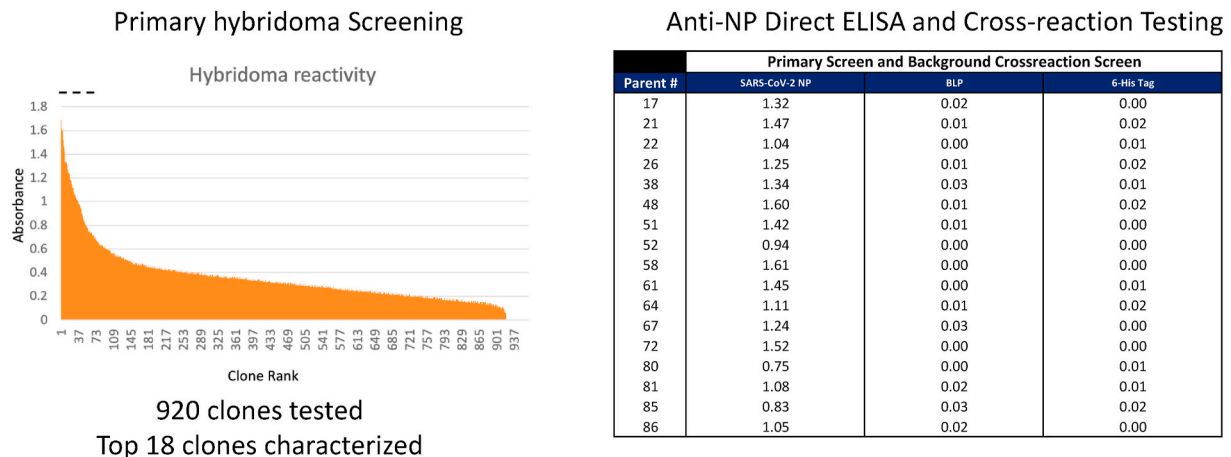

Figure 2. Screening of Anti-Nucleocapsid Clones. A) Direct ELISA analysis of 920 clones picked from hybridoma fusion. **B)** Verification ELISA of top 18 anti-nucleocapsid monoclonal antibody clones and counter screening against bacterial lysate (BLP) and 6-His-tagged SARS-CoV-2 Spike RBD domain. Averages are presented following background subtraction.

were circularized using NEBuilder, cloned into DH5-alpha *E. coli*, and clones sequenced. For protein expression, verified plasmids were transformed into BL21 *E. coli* cells with kanamycin and chloramphenicol selection. Cultures were incubated overnight in LB broth with 10% glucose (w/v), and recombinant N protein production was induced with 0.4 mM IPTG alongside uninduced transformed variants for 5 h. Cells were centrifuged, resuspended in Laemmli sample buffer, boiled for 5 min, and then resolved on a 10% SDS-PAGE gel. Protein lysates were then transferred to a PVDF membrane for Western blot analysis using the indicated monoclonal antibody according to the aforementioned Western blot protocol.

2.13. Virion-derived N protein detection by sandwich ELISA

SARS-CoV-2 virions particles were collected in infected Vero cell supernatant at 5.5×10^5 PFU/ml and inactivated with IGEPAL CA-630 (0.1% (v/v)) for 30 min at 4 °C. Inactivated viral stocks were serially diluted from 1:10 to 1:10,000 in 1X PBS. 96 well plates were coated with 0.1 μg/ml of anti-N protein rabbit polyclonal antibody overnight and then blocked with 4% (w/v) dry milk powder blocking solution dissolved in 1X PBS + 0.1% Tween 20 (v/v) for 1 h at room temperature. Following three washes with 1X PBS +0.1% Tween 20 (v/v), the diluted stocks of inactivated SARS-CoV-2 were applied to the capture antibody-coated plates in 50 μl triplicate aliquots and incubated at 37 °C for 90 min. No N protein was applied to control wells with only diluent being applied to control for false positive reporting of monoclonal antibody reactivity. The plate was washed three additional times, and 50 μl of mBG17 or mBG86 parent supernatant was applied to each of the deactivated virus dilutions in triplicate and then incubated for 1 h at room temperature. Following this the remaining steps followed were derived from the indirect ELISA protocol described earlier. Reported signal was corrected for using the wells in which no inactivated virus was applied.

Table 1
Cross-Reactivity Screening of Anti-Nucleocapsid Monoclonal Antibody Clones. Left: Direct ELISA analysis of top 18 clones against SARS-CoV2, SARS-CoV, MERS-CoV, HuCoV-OC43, HuCoV-NL63, HuCoV-HKU1, and HuCoV-229E nucleocapsid proteins. Average signals are corrected against background signal **Right:** Relative binding of monoclonal antibodies to nucleocapsid proteins compared to SARS-CoV-2.

Human Coronavirus Cross-Reactivity Screen

Parent #	SARS-CoV-2 Corrected Average		Standard Deviation	SARS-CoV Corrected Average		Standard Deviation	MERS-CoV Corrected Average		Standard Deviation	OC43 Corrected Average		Standard Deviation	NL63 Corrected Average		Standard Deviation	HKU1 Corrected Average		Standard Deviation	229E Corrected Average		Standard Deviation
17	0.832	±	0.034	0.872	±	0.065	0.004	±	0.003	0.003	±	0.002	0.365	±	0.041	0.004	±	0.001	0.019	±	0.002
21	0.743	±	0.097	0.302	±	0.033	0.011	±	0.001	0.006	±	0.002	0.339	±	0.027	0.006	±	0.003	0.025	±	0.006
22	0.754	±	0.113	0.779	±	0.057	0.036	±	0.005	0.012	±	0.002	0.339	±	0.023	0.011	±	0.003	0.025	±	0.003
26	0.734	±	0.077	0.893	±	0.056	0.001	±	0.001	0.004	±	0.005	0.394	±	0.036	0	±	0.002	0.018	±	0.002
38	0.849	±	0.071	0.83	±	0.039	0.012	±	0.002	0.006	±	0.003	0.327	±	0.01	0.005	±	0.002	0.024	±	0.007
48	0.838	±	0.045	0.843	±	0.071	0.005	±	0.002	0.009	±	0.011	0.367	±	0.014	0.003	±	0.001	0.019	±	0.001
51	0.727	±	0.103	0.767	±	0.03	0.001	±	0.002	0	±	0.001	0.258	±	0.008	0.001	±	0.001	0.002	±	0.001
52	0.183	±	0.008	0.166	±	0.011	0	±	0.001	0	±	0.001	0.026	±	0.001	0	±	0.001	0	±	0.001
57	0.525	±	0.116	0.554	±	0.024	0	±	0.001	0	±	0.001	0.113	±	0.014	0	±	0.001	0.002	±	0.001
58	0.759	±	0.074	0.915	±	0.037	0.006	±	0.003	0.006	±	0.001	0.425	±	0.027	0.01	±	0	0.03	±	0.001
61	0.858	±	0.033	0.174	±	0.01	0	±	0.001	0.001	±	0.001	0.061	±	0.004	0	±	0.001	0.002	±	0.001
67	0.861	±	0.019	0.094	±	0.043	0.002	±	0.003	0.001	±	0.002	0.038	±	0.037	0	±	0.001	0	±	0.002
72	0.89	±	0.018	0.093	±	0.092	0	±	0.001	0	±	0.002	0.07	±	0.051	0	±	0.002	0	±	0.002
80	0.844	±	0.054	0.83	±	0.016	0	±	0.001	0.002	±	0.001	0.386	±	0.01	0.002	±	0.001	0.012	±	0.002
83	0.687	±	0.046	0.782	±	0.107	0	±	0.001	0	±	0.001	0.217	±	0.007	0	±	0.002	0.004	±	0.001
81	0.874	±	0.049	0.802	±	0.045	0.036	±	0.002	0.002	±	0.001	0.25	±	0.009	0	±	0.001	0.014	±	0.003
85	0.802	±	0.059	0.838	±	0.039	0.046	±	0.002	0.004	±	0.001	0.27	±	0.028	0.004	±	0.005	0.015	±	0.003
86	0.806	±	0.044	0.017	±	0.008	0.003	±	0.002	0.004	±	0.002	0.005	±	0.002	0.003	±	0.002	0.01	±	0.006
Rb Anti-NP	0.745	±	0.077	0.821	±	0.036	0.417	±	0.026	0.322	±	0.039	0.643	±	0.027	0.376	±	0.036	0.367	±	0.035
Ms Anti-His	0.652	±	0.05	0.612	±	0.04	0.663	±	0.041	0.652	±	0.033	0.655	±	0.038	0.805	±	0.039	0.718	±	0.039

Human Coronavirus Cross-Reactivity Screen

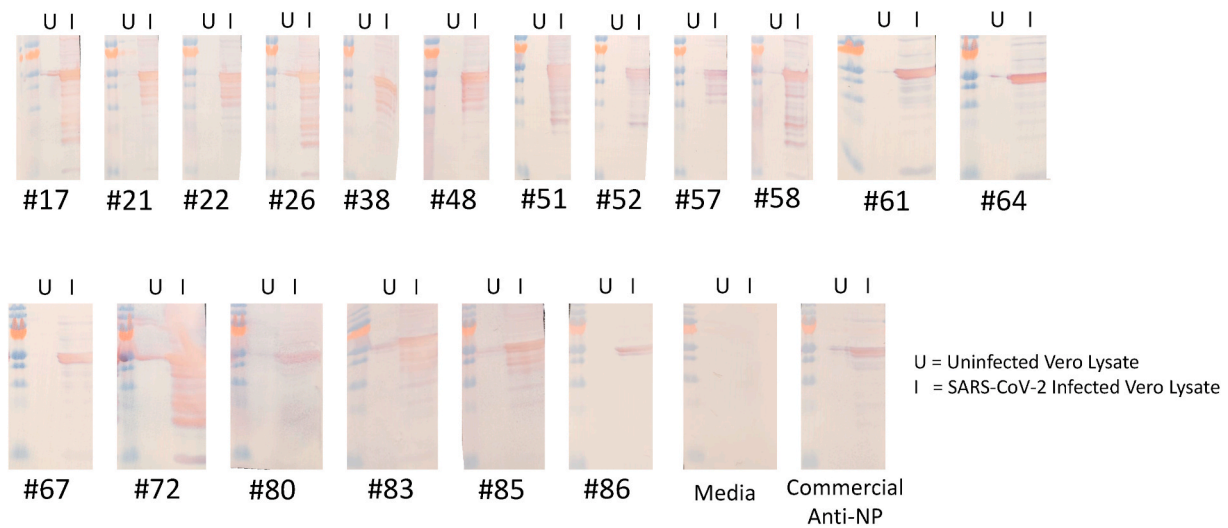| Parent #	SARS-CoV-2	SARS-CoV	MERS-CoV	HuOC43	HuNL63	HuHKU1	Hu229E
17	++++	++++	–	–	++	–	–
21	++++	++	–	–	++	–	–
22	++++	++++	–	–	++	–	–
26	++++	+++++	–	–	++	–	–
38	++++	++++	–	–	++	–	–
48	++++	++++	–	–	++	–	–
51	++++	++++	–	–	+	–	–
52	++++	++++	–	–	+	–	–
58	++++	++++	–	–	+	–	–
61	++++	+++++	–	–	++	–	–
64	++++	+	–	–	+	–	–
67	++++	+	–	–	–	–	–
72	++++	+	–	–	+	–	–
80	++++	++++	–	–	++	–	–
81	++++	+++++	–	–	+	–	–
83	++++	++++	–	–	+	–	–
85	++++	++++	+	–	+	–	–
86	++++	–	–	–	–	–	–
Rb Anti-NP	++++	++++	++	++	+++	++	++
Ms Anti-His	++++	++++	++++	++++	++++	+++++	++++

Fig. 3. Western Blot Analysis of Anti-Nucleocapsid Monoclonal Antibody Clones Against Uninfected (U) or SARS-CoV-2 infected (I) Vero cells.

U = Uninfected Vero Lysate
I = SARS-CoV-2 Infected Vero Lysate

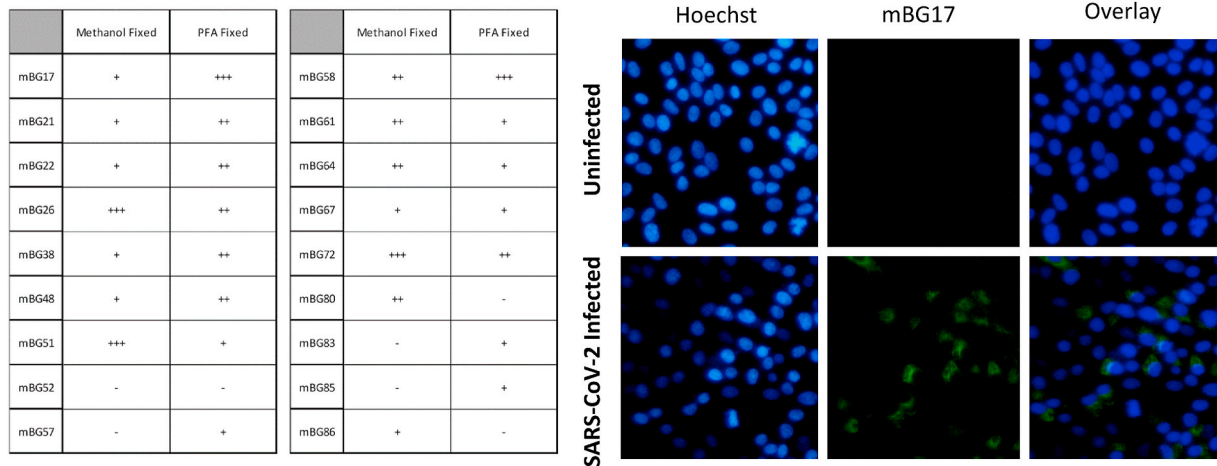|	Methanol Fixed	PFA Fixed		Methanol Fixed	PFA Fixed
mBG17	+	+++	mBG58	++	+++
mBG21	+	++	mBG61	++	+
mBG22	+	++	mBG64	++	+
mBG26	+++	++	mBG67	+	+
mBG38	+	++	mBG72	+++	++
mBG48	+	++	mBG80	++	-
mBG51	+++	+	mBG83	-	+
mBG52	-	-	mBG85	-	+
mBG57	-	+	mBG86	+	-

Fig. 4. Immunofluorescence Analysis of Anti-Nucleocapsid Monoclonal Antibody Clones. A) Relative reactivities of clones in SARS-CoV-2 infected Vero cells fixed with methanol or paraformaldehyde. **B)** representative images of uninfected and SARS-CoV-2 infected Vero cells (paraformaldehyde fixed) processed for immunofluorescence analysis with mBG17.

3. Results

This project was focused on developing novel antibodies that can specifically detect SARS-CoV-2 nucleocapsid protein for use in research and diagnostic testing efforts. Recombinant SARS-CoV-2 N protein was produced as antigen for hybridoma production. The N-terminal domain of human coronavirus N proteins have several conserved regions that may contribute to monoclonal cross-reactivity (Fig. 1A). Previous work developing monoclonal antibodies against MERS coronavirus N protein found that removal of the N-terminal domain improved antibody specificity and increased recombinant protein solubility (Yamaoka et al., 2016). Therefore, we developed a bacterial expression plasmid that produces AA133-419 of the SARS-CoV-2 (isolate USA-WA1/2020) N protein with a N-terminal T7 leader sequence to improve translation efficiency (pBG690). Expression of recombinant N protein in BL21 DE3 pLys *E. coli* was relatively robust, but we found that the recombinant protein formed large molecular weight oligomers at NaCl concentrations below 300 mM (data not shown). Purification of nickel-affinity purified N protein on a Superdex 200 gel filtration column in 500 mM NaCl resolved these aggregation issues and produced protein migrated as the predicted molecular weight of a truncated N dimer (Fig. 1B). Recombinant SARS-CoV, MERS-CoV, HuCoV-OC43, HuCoV-NL63,

HuCoV-HKU1 and HuCoV-229E N protein were produced using the same process for downstream cross-reactivity screening. SDS-PAGE analysis of purified coronavirus N proteins showed that the proteins were >98% pure and migrated at the expected molecular weight (Fig. 1C). This purified SARS-CoV-2 antigen was found to be antigenic and produced rabbit polyclonal antibodies that reacted with N protein in deer mouse infections in parallel work (Fagre et al., 2020). This recombinant SARS-CoV-2 N protein was used to immunize BALB/c mice according to the protocol described above to generate immunized mice, from which spleens were collected and hybridoma clones were produced.

For primary screening of hybridoma colonies, supernatant was collected from the 920 colonies and tested for the presence of N protein specific antibodies by ELISA following 4 min of TMB development (Fig. 2A). After determining reactivity of produced monoclonal activities against the antigen in ELISA via absorbance at 450 nm and correcting based on the negative control, the highest scoring 92 hybridoma colonies were selected for further testing and expansion as "parent" colonies. Due to the bacterial system used for N protein immunogen production, we then screened these 92 parents for reactivity against bacterial proteins. Plates were coated with bacterial lysate protein (BLP) and tested by ELISA in parallel to N protein-coated plates to determine

Table 2
Immunoglobulin Heavy (A) and Light Chain (B) Amino Acid Sequences of Top 5 Hybridoma Clones. Sequences defined by IgBlast following hybridoma RNA extraction, template switch RT-PCR, bacterial cloning, and sequencing. Three key three amino acid mutations between the heavy chains of mBG67 and mBG86 are highlighted in red that appear to alter antibody specificity.

Clone	Isotype	Chain	FRM-1	CDR-1	FRM-2	CDR-2	FRM-3	CDR-3	FRM4-4
17	IgG1	Heavy	EVKLEESGGGLVQPGGSMKFSCVAS	GFTFSDYW	MNWVRQSPDKGLEWVAE	IRLKSNNYAT	HYAASVKGRFTISRDDSKSSVYLQMNNLRAEDSGIYYC	TRSAMDY	WGQGTSVTVSS
21	IgG2b	Heavy	QIQLVQSGPELKKPGETVKISCKAS	GYTFTDYS	MHWVKQAPGKGSKWMGW	INTETGEP	TYADDFKGRFAFSLETSASTAYLQINNLKNEDTATYFC	ALRR	WGQGTLVTVSA
22	IgG2b	Heavy	QIQLVQSGPELKKPGETVKISCKAS	GYTFTDYS	MHWVKQAPGKGSKWMGW	INTETGEP	TYADDFKGRFAFSLETSASTAYLQINNLKNEDTATYFC	ALRR	WGQGTLVTVSA
57	IgG1	Heavy	QIQLVQSGPELKKPGETVKISCKAS	GYTFTDYS	MHWVKQAPGKGSKWMGW	INTETGEP	TYADDFKGRFAFSLETSASTAYLQINNLKNEDTATYFC	ALRR	WGQGTLVTVSA
67	IgG1	Heavy	EVQLVESGGGLVQPGGSLKLSCAAS	GFTFSNYG	MSWVRQTPDKRLELVAT	INRNGGST	YYLDSVKVRFTISRDNAKSTLFLQLSSLKSDDTAMYYC	ARIYDFDEDYFDV	WGAGTTVTVSS
86	IgG1	Heavy	EVQLVESGGGLVQPGGSLKLSCAAS	GFTFSNYG	MSWVRQTPDKRLELVAT	INRNGGST	YYLDSVKVRFTISRDNAKSTLFLQMSSLKSEDTAMYYC	ARIYDYDEDYFDV	WGAGTTVTVSS
17	IgG1	Kappa	DIVMSQSPSSLAVSVGEKITMSCKSS	QSLLYTSDQKNY	LAWFQQKPGQSPKLLIF	WAS	TRDSGVPDRFTGSGSGTDFLTISSVKAEDLAVYYC	QQFYNYPRT	FGGGTKLEIK
21	IgG2b	Kappa	IVMTQTPKFLLVSAGDRVTITCKAS	QSVSND	VAWFQQKPGQSPKLLIY	FAS	NRYTGVPDRFTGSGYGTDFTFTTTVQAEDLAVYFC	QQDYSSPWT	FGGGTKLEIK
22	IgG2b	Kappa	IVMTQTPKFLLVSAGDRVTITCKAS	QSVSND	VAWFQQKPGQSPKLLIY	FAS	NRYTGVPDRFTGSGYGTDFTFTTTVQAEDLAVYFC	QQDYSSPWT	FGGGTKLEIK
57	IgG1	Kappa	DIVMTQAAPSEPVTPGESVSISCGSS	KSLLHSNDNTY	LYWFLQRPGQSPQLLIY	RMS	NLASGVPDRFTGSGSGTAFTLRISRVEAEDVGVYYC	MQHLENPLG	VRWRHQAGNQT
67	IgG1	Kappa	QIVLTQSPAIMSASLGERVTMTCTAS	SSVSSSY	LHWYQQKPGSSPKLWIY	STS	NLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYC	LQYHRSPWT	FGGGTKLEIK
86	IgG1	Kappa	QIVLTQSPAIMSASLGERVTMTCTAS	SSVSSSY	LHWYQQKPGSSPKLWIY	STS	NLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYC	LQYHRSPWT	FGGGTKLEIK

which parents had residual bacterial reactivity. Of these, parents 5,7, 23, 43,53, 54, 65, 77, and 79 demonstrated specificity for bacterial proteins and were thus eliminated from further testing. The remaining parents were then tested for reactivity against the 6xHis tag using 6x-His tagged soluble spike receptor binding domain as the counter screening antigen. Parent 55 showed reactivity to the 6xHis tag and was thus eliminated from further testing. 18 top-performing parents were selected for further testing and given the prefix mBG (Fig. 2B).

The top 18 parents were tested for cross-reactivity against the six other human coronaviruses known to be represented in the human population. This assessment was performed to determine which antibodies were only reactive towards SARS-CoV-2 N protein. The recombinant SARS-CoV, MERS-CoV, HuCoV-OC43, HuCoV-NL63, HuCoV-HKU1 and HuCoV-229E N protein were expressed and purified for determining the top 18 parents' specificity compared to SARS-CoV-2 N protein by indirect ELISA (Table 1). With the exception of mBG86 there was strong cross reactivity with SARS-CoV N protein. Cross reactivity with NL63 N protein was similar in that mBG86 lacked reactivity to this N protein variant while other parents maintained some reactivity, albeit less than they demonstrated for SARS-CoV. mBG 21, 22, 57, and 67 all had reduced reactivity towards NL63 N protein. The anti-His control showed equal reactivity towards all N proteins tested. From the indirect ELISA, we determined that mBG86 was specific for SARS-CoV-2 N proteins.

Western blots were performed to determine reactivity of the selected 18 parents towards linear or semi-linear epitopes. All parents demonstrated specific reactivity for recombinant SARS-CoV-2 N protein (Supplemental Fig. 1). The multiple bands on each blot corresponds to oligomers of N protein and degraded protein. mBG 67, 80, and 86 demonstrated two band labeling each, suggesting a specificity for linear epitopes on the N protein while the other parents labeling suggests conformational epitopes. Western blots comparing reactivity towards SARS-CoV-2 infected Vero cell lysates and uninfected Vero cell lysates (Fig. 3) demonstrated a high specificity towards antigen in infected cells and the endogenously produced N protein for the top 18 clones.

To test the capability of these antibodies for cellular localization of N in SARS-CoV-2 infected Vero cells, the ability for the top 18 parents to bind to endogenous N protein following PFA or methanol fixation was determined by immunofluorescence assay (IFA). Signals intensity and subcellular localization were comparable to a previously described rabbit anti-N protein polyclonal antibody (Fagre et al., 2020) (Fig. S2). Results indicated that the parent antibodies that demonstrated reactivity to fixed infected cells were non-reactive towards uninfected fixed cells. N protein was found predominantly in the cytoplasm in SARS-CoV-2 infected cells as expected (Fig. 4B). IFA positive clones varied in effectiveness between methanol and paraformaldehyde fixation methods (Fig. 4A).

Based on the activity of clones in ELISA, Western blot, and IFA analysis, we chose six clones (mBG17, mBG21, mBG22, mBG57, mBG67, and mBG86) for sequence analysis using the method outlined in Meyer et al. (2019). Five separate clones for each V_H or V_L were sequenced, and consensus sequences for each clone were assembled, translated, and subjected to IgBLAST analysis to determine framework and complementary determining regions (Table 2). mBG21 and 22 presented identical sequences, suggesting a common B-cell origin. Isotype tests revealed each of the five parents, with the identical isotypes of mBG 21 and 22 supporting the prediction of a common origin. Clones mBG21/22, and mBG57 shared identical heavy chains but different kappa chains. Clone mBG17 possessed unique heavy and light chains. mBG67 and mBG86 possessed identical kappa light chains and heavy chains separated by three mutations in framework 3 of the heavy chain. This mutation may be the source of the improved specificity of mBG86 compared mBG67 observed in Table 1 and merits further study.

We sought to determine preliminary epitope ranges for the representative clones mBG17, mBG22, mBG57, and mBG67 using SARS-CoV-2 N protein deletion mutants in Western blot analysis. These clones were
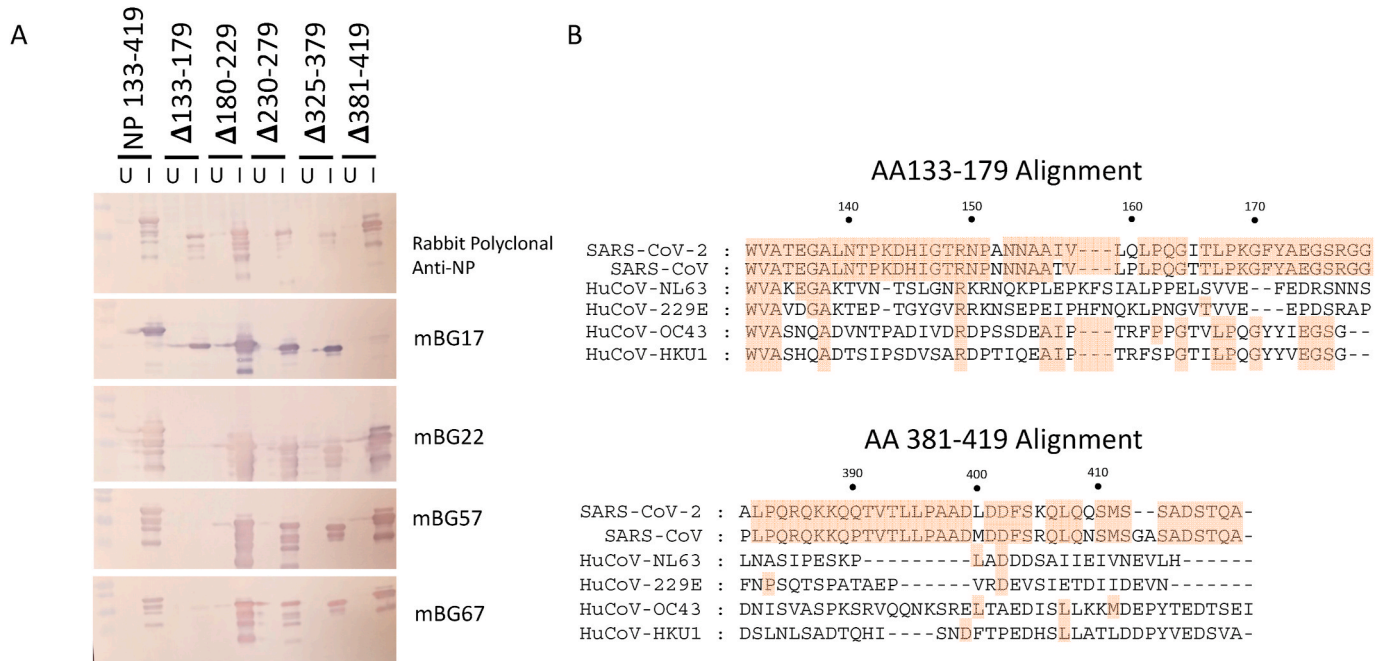
Fig. 5. Epitope Mapping Using N Protein Deletions. A) Western blot analysis of selected antibody reactivity against SARS-CoV-2 nucleocapsid protein deletions. Bacterial lysates of non-induced protein expression cultures (U) and induced protein expression cultures (I) were run side by side. **B)** Sequence alignments of NP AA133-179 and AA381-419 regions with heterologous human coronavirus nucleocapsid proteins.
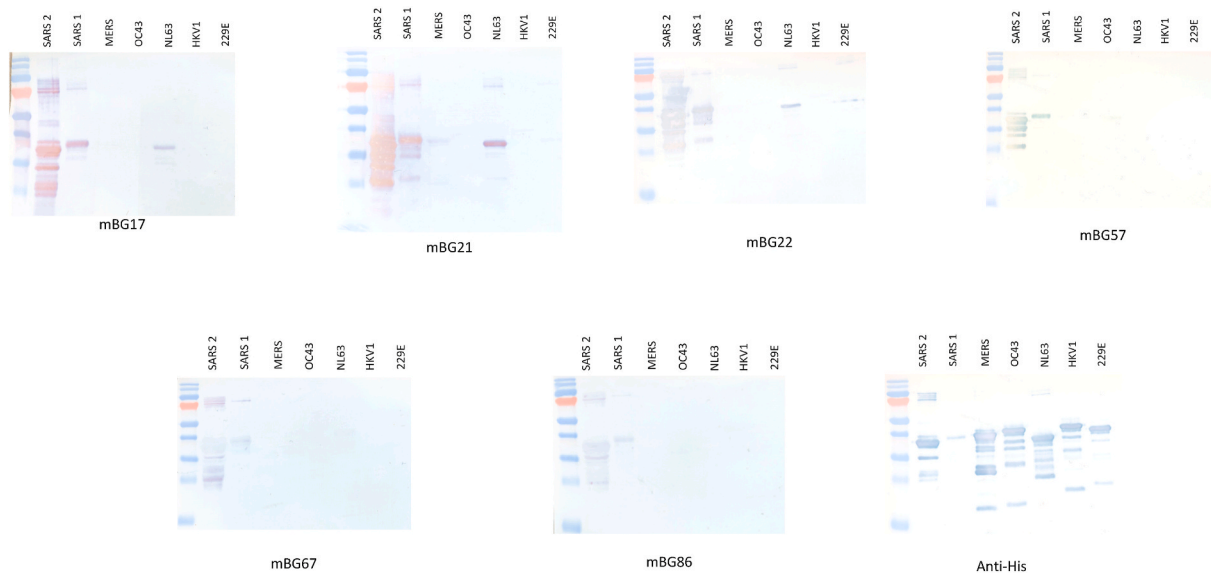

Fig. 6. mBG Western Blot Reactivity to Linearized Recombinant Human Coronavirus N Proteins. Recombinant human coronavirus western blots show the reactivity of the selected top mBG antibodies. SDS-PAGE gels for mBG67 and mBG86 were loaded with 10 μg/well recombinant N protein while the other gels were loaded with 1 μg/well as mBG67 and mBG86 appear to bind weakly to linearized epitopes compared to mBG17, mBG21, and mBG22.

selected from the six selected top clones due to mBG22 and mBG67's sequence similarity to mBG21 and mBG86, respectively. Recombinant N protein expression plasmids, each with a different 50 amino acid deletion, were constructed and used to narrow down the range of epitope locations for mBG17, mBG22, mBG57, and mBG67 (Fig. 5). Clones mBG22, mBG57, and mBG67 showed strong reactivity in Western blot analysis against all clones except the Δ133–179 deletion, suggesting that mBG21/22, mBG57, and mBG67/86 epitope resides within AA133-179 range. mBG17 showed strong reactivity towards all deletions except Δ381–419, indicating that clone mBG17 is likely within AA381-419 at the C-terminal end of the N protein. Additional peptide mapping is

needed to determine the exact amino acid sequences for each epitope.

With the selected top six hybridoma clones we screened for Western blot cross-reactivity against the heterologous recombinant coronavirus proteins (Fig. 6). The results largely mirrored the indirect ELISA data, with mBG17, 21, 22, and 57 possessing cross reactivity with SARS-CoV and NL63 N protein. mBG67 and mBG86 produced weak additional bands against SARS-CoV N protein that indicate slight reactivity towards the linearized SARS-CoV protein in western blots. Of note, western blots for mBG67 and mBG86 needed significantly more protein for bands to be detected, which were generally lower than signals from mBG17, 21, 22, and 57 that were loaded with less protein. Overall, these results
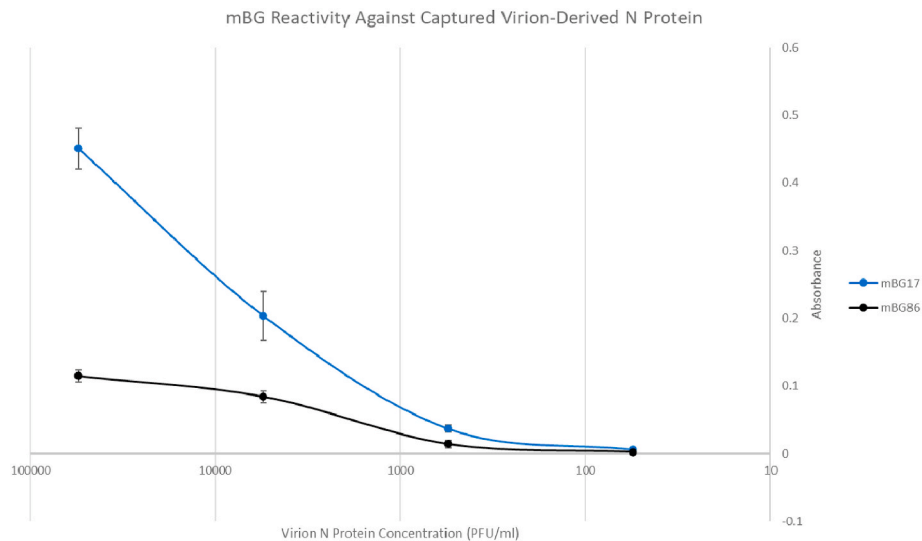
Fig. 7. Reactivity of mBG17 and mBG86 Against Captured Virion-Derived N Proteins. Virion-derived N protein serially diluted from 5.5×10^5 PFU/ml stock and captured by anti-NP rabbit polyclonal antibody. mBG17 and mBG86 used in tandem with goat anti-mouse HRP-conjugated antibody to report on mBG effectiveness in capture ELISA.

support that mBG67 and mBG86 appear to have a significant preference for conformational epitopes whereas clones mBG17, 21, 22, and 57 appearing to have a strong preference towards linear epitopes.

Antibodies from clones mBG17 and mBG86 selected for use in sandwich ELISA to determine the monoclonal antibodies effectiveness at detecting captured virion-derived N protein from SARS-CoV-2 virions. The rabbit polyclonal antibody was effective in capturing N protein and the monoclonal mBG antibodies selectively labeled captured antigen as opposed to binding to the capture antibody. Both mBG17 and mBG86 showed proficiency at reporting captured virion N protein with the former being able to detect 5500 PFU/ml and the latter detecting 55,000 PFU/ml at the parent supernatant concentration (Fig. 7). mBG17's higher signal compared to mBG86 is similar to the IFA data in Fig. 4, and may indicate that the epitope for mBG17 (AA381-419) may be more accessible in N protein derived from infection than the mBG67/86 epitope (AA133-179). Given the propensity for N proteins to aggregate in vitro and the oligomerization of N protein on viral genomes during replication, additional steps (such as increasing ionic concentration or degrading RNA) may be needed to improve mBG86 accessibility in viral samples. Future studies will assess how to increase the sensitivity of these antibodies in various applications.

4. Conclusions

With SARS-CoV-2 spreading globally amidst a dearth of effective diagnostics, treatments, and reagents to tackle pandemic, it is more important than ever that tools for detection and research are developed and validated. The mBG86 antibody characterized in this paper has been selected and validated for high specificity towards SARS-CoV-2 nucleocapsid protein across several diagnostic and research assays. The other five selected top hybridoma clones (mBG 17, 21, 22, 57, and 67) all show activity against SARS-CoV-2 N protein with minor cross reactivity towards SARS-CoV and HuCoV-NL63. These antibodies will be useful for laboratory research where the infection of cell culture and model organisms can be controlled for. The specific mBG86 antibody can be used in the future for rapid, accurate, and inexpensive diagnostic assays for bed-side diagnosis of COVID patients. Future studies can utilize these antibodies for studies determining N protein structure, intracellular interactions, diagnostic development, and potential therapeutics. Preliminary epitope mapping experiments revealed ranges of amino acids that contain partial or full epitopes utilized by antibodies from the selected six top mBG antibodies. The sequences for the FMW and CDR

regions of these antibodies shown in Table 2 be used for the production of recombinant single-chain variable fragment (scFv) antibodies that may be used for observing real-time N protein production (Zhao et al., 2019), heavy and light genes cloned into antibody expression vectors for non-hybridoma eukaryotic expression systems, or used in neutralizing treatments (Nakanaga et al., 1986). Overall, we hope that these data will be useful to the wider research community for fighting the ongoing COVID-19 pandemic.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2021.01.003.

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