

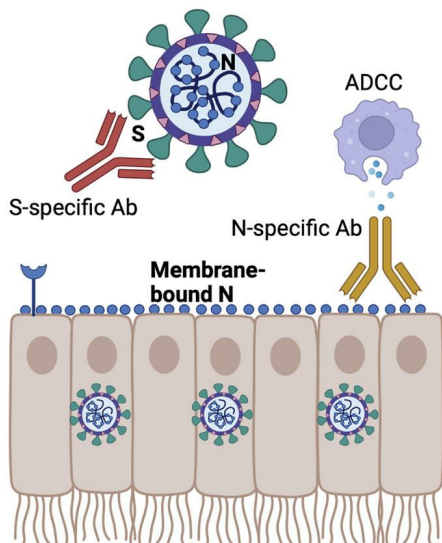
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Improved control of SARS-CoV-2 by treatment with nucleocapsid-specific monoclonal antibody

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Summary:

The SARS-CoV-2 spike protein is the main antigen in all approved COVID-19 vaccines and is also the only target for monoclonal antibody therapies. Immune responses to other viral antigens are generated after SARS-CoV-2 infection, but their contribution to the antiviral response remains unclear. Here, we interrogate whether nucleocapsid-specific antibodies can improve protection against SARS-CoV-2. We first immunized mice with a nucleocapsid-based vaccine, and then transferred sera from these mice into naïve mice, followed by challenge with SARS-CoV-2. We show that mice that received nucleocapsid-specific sera or a nucleocapsid-specific monoclonal antibody (mAb) exhibited enhanced control of SARS-CoV-2. Nucleocapsid-specific antibodies elicited NK-mediated antibody-dependent cellular cytotoxicity (ADCC) against infected cells. These findings provide the first demonstration in the coronavirus literature that antibody responses specific to the nucleocapsid protein can improve viral clearance, providing a rationale for the clinical evaluation of nucleocapsid-based monoclonal antibody therapies to treat COVID-19.

Keywords:

SARS-CoV-2 vaccines; COVID-19; spike antigen; nucleocapsid antigen; antibodies; mAb, passive immunization; convalescent plasma therapy.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has infected more than 600 million people and continues to spread around the globe. Although vaccines and monoclonal antibody therapies can prevent severe disease and death, breakthrough infections can occur, highlighting the need for improving current vaccines and available treatments (1-9). The SARS-CoV-2 spike protein is critical for viral entry, making this protein an important antigen present in all SARS-CoV-2 vaccines and the only target for all monoclonal antibody therapies. Besides spike-specific immune responses, other antigen-specific immune responses are elicited during natural SARS-CoV-2 infection (10-13), but their role at protecting against infection remains unclear.

In particular, it is unknown if antibodies specific to internal viral proteins such as the nucleocapsid protein, which does not play a role in viral entry, can confer protection against SARS-CoV-2. Knowing if other antigen-specific antibodies are protective could facilitate the development of more potent vaccines and monoclonal antibody therapies for coronavirus infections. In this study, we studied nucleocapsid-specific immune responses in a cohort of COVID-19 patients, and we interrogated whether nucleocapsid-specific antibody responses elicited by a novel nucleocapsid-based vaccine could confer protection against a SARS-CoV-2 challenge in K18-hACE2 mice. Interestingly, we show that nucleocapsid-specific humoral responses and a nucleocapsid-specific monoclonal antibody can mediate antibody-dependent cellular cytotoxicity (ADCC) and help control SARS-CoV-2 infection when given as pre-exposure prophylaxis. Together, these data warrant the clinical evaluation of

nucleocapsid-specific monoclonal antibody therapies for the treatment of SARS-CoV-2 and suggest that inclusion of nucleocapsid in next generation vaccines could confer an additional immunological benefit.

Results

Adaptive immune responses elicited by a nucleocapsid vaccine help control a SARS-CoV-2 infection

All approved COVID-19 vaccines express the spike protein of SARS-CoV-2. Immune responses against other antigens, for example against the nucleocapsid antigen, are not elicited after SARS-CoV-2 vaccination, but can be induced after natural SARS-CoV-2 infection. As shown in **Figure 1A-1B**, we detected nucleocapsid-specific antibody responses in the plasma of COVID-19 patients, but not in the plasma of individuals before the 2019 pandemic. Similar antibody responses were detected to an irrelevant viral antigen (Influenza) in SARS-CoV-2 exposed and unexposed individuals (**Figure 1C**). Although COVID-19 patients show nucleocapsid-specific immune responses, it is still unclear whether nucleocapsid-specific immune responses can play an antiviral role *in vivo*. In particular, it is unknown whether antibodies against nucleocapsid (an internal viral protein that is not target of neutralization) could have an effect during a SARS-CoV-2 infection.

We previously showed that a nucleocapsid-based vaccine does not confer significant protection against an intranasal SARS-CoV-2 challenge when given as a “single vaccine,” without a spike-based vaccine (14). In that prior paper, we evaluated viral loads at a very early time post-infection (day 3 post-infection) to measure breakthrough infection. In our follow up studies, we evaluated viral control at later times post-infection. We vaccinated K18-hACE2 mice intramuscularly with an adenovirus serotype 5 vector

expressing SARS-CoV-2 nucleocapsid (Ad5-N) at a dose of 10^{11} PFU per mouse. We utilized K18-hACE2 mice because they are susceptible to SARS-CoV-2 and are widely used to evaluate vaccines (14-19). Two weeks post-vaccination, we detected nucleocapsid-specific CD8 T cell responses (**Figure 2A**) and antibody responses (**Figure 2B**) in mice vaccinated with the nucleocapsid vaccine. Then we challenged these K18-hACE2 mice intranasally with a high dose (4×10^5 PFU) of SARS-CoV-2 (isolate USA-WA1/2020), and at day 5 post-challenge we quantified viral loads in lungs by RT-qPCR. Interestingly, the mice that received the nucleocapsid vaccine showed significantly lower weight loss (**Figure 2C**) and lower viral loads (**Figure 2D**) compared to control vaccinated mice. Altogether, our prior paper showed that a nucleocapsid vaccine does not prevent breakthrough infection and does not significantly reduce viral titers during the hyperacute phase (day 3 post-infection) (14), but our new data in **Figure 2D** show that a nucleocapsid vaccine may facilitate viral control at later times (day 5 post-infection).

Nucleocapsid-specific T cell responses have been suggested to contribute to viral control following SARS-CoV-2 infection (20, 21), but the role of nucleocapsid-specific antibody responses remains elusive (since nucleocapsid-specific antibodies do not target a neutralizing epitope on SARS-CoV-2).

Immunogenicity of a nucleocapsid prime-boost vaccine regimen

To understand the role of nucleocapsid-specific humoral responses during SARS-CoV-2 infection, we developed a prime-boost vaccine regimen that elicited high levels of nucleocapsid-specific antibodies, which were later used in passive immunization experiments. We primed C57BL/6 mice intramuscularly with an adenovirus serotype 5 vector expressing SARS-CoV-2 nucleocapsid (Ad5-N) (14, 21, 22) at a dose of 10^{11} PFU per mouse, followed by booster with 100 μ g of nucleocapsid protein three weeks later to generate high titers of nucleocapsid-specific antibody responses. As controls, we immunized mice with an “empty” Ad5 vector (Ad5-Empty) followed by a PBS boost. 2 weeks post-boost, we measured nucleocapsid-specific immune responses (**Figure 3A**). Mice immunized with the nucleocapsid vaccine regimen exhibited robust nucleocapsid-specific CD8 T cell responses (**Figure 3B**), memory B cell responses (**Figure 3C**), and antibody responses (**Figure 3D**).

Prior studies have suggested that nucleocapsid-specific T cells can help clear SARS-CoV-2 infection via killing of infected cells (14, 20-22). However, it is still unclear if nucleocapsid-specific antibodies play any role in antiviral control, since the nucleocapsid is an internal viral protein that is not involved in viral entry. We performed focus reduction neutralization titer (FRNT) assays using live SARS-CoV-2 to examine if nucleocapsid-specific antibodies prevent SARS-CoV-2 infection (**Figure 4A**). We used live SARS-CoV-2 instead of a pseudovirus, because live virus contains all viral proteins, including nucleocapsid. As positive control, we used sera from mice that received a spike-based adenovirus vaccine (Ad5-S, similar to the CanSino vaccine and the Sputnik vaccine). As expected based on prior studies (22, 23), sera from mice that received the

spike-based vaccine completely prevented SARS-CoV-2 infection, even when the sera were diluted 450-fold (**Figure 4B**). However, sera from mice that received the nucleocapsid-based vaccine did not exert any antiviral effect in this in vitro infection assay (**Figure 4B-4C**). Taken together, only spike-specific (and not nucleocapsid-specific) antibodies can block SARS-CoV-2 infection, consistent with the widely established notion that spike-specific antibodies are protective, as they can block the first step in the SARS-CoV-2 life cycle: entry into host cells.

Nucleocapsid-specific antibodies help clear a SARS-CoV-2 infection in vivo

Antibody responses exert antiviral functions by various mechanisms, including viral neutralization and Fc-dependent effector mechanisms. Although nucleocapsid-specific sera did not prevent SARS-CoV-2 infection in vitro, we reasoned that it could confer protection in vivo via effector mechanisms. To test this hypothesis, we performed a passive immunization study to evaluate whether the specific transfer of nucleocapsid-specific antibodies can confer a clinical benefit (**Figure 5A**). We adoptively transferred 500 μ L of nucleocapsid-specific sera (week 2 post-boost; same mice from **Figure 3A**) into naïve K18-hACE2 mice. One day after sera transfer, we challenged these K18-hACE2 mice intranasally with a low dose (10^3 PFU) of SARS-CoV-2, followed by evaluation of weight loss and viral loads at day 4 post-challenge. Following this low dose viral challenge, we did not observe weight loss (**Figure 5B**). However, the mice that received nucleocapsid-immune sera showed a significant reduction in viral titers relative to control, by RT-qPCR and focus forming assays (**Figure 5C-5D**). These

findings demonstrate that even though nucleocapsid-specific humoral responses do not neutralize SARS-CoV-2 in vitro, they can exert antiviral effects in vivo.

The studies above suggest that nucleocapsid-specific antibody can help clear a SARS-CoV-2 infection. However, sera contain other molecules that could mediate antiviral control besides antibodies, such as complement and cytokines. To ascertain the specific contribution of nucleocapsid-specific antibody, we treated K18-hACE2 mice with a nucleocapsid-specific monoclonal antibody (mAb) or an isotype control antibody, and on the next day, mice received a low dose intranasal SARS-CoV-2 challenge (10^3) followed by evaluation of viral loads in lungs at day 7 post-infection (**Figure 6A**). With this low dose viral challenge, there was no weight loss by day 7 (**Figure 6B**). Consistent with our prior results, treatment with nucleocapsid-specific mAb resulted in a significant improvement in viral control by RT-qPCR (163-fold lower viral titers, relative to control) (**Figure 6C**). Improved antiviral control in the mice treated with nucleocapsid-specific mAb was also observed by another virological quantification method, focus forming assays (**Figure S1**). In separate experiment, we quantified lung viral loads at a more acute time point (day 4) and we observed a similar improvement in viral control in mice that received the nucleocapsid-specific mAb (**Figure S2**). We did not observe statistically significant differences in CD8 T cell responses (Figure S3).

We also performed viral challenges using a high dose of SARS-CoV-2 (5×10^4 PFU) that normally results in severe weight loss and immunopathology (**Figure 6D**). We sacrificed mice at day 5 post-infection, because this high viral challenge dose causes severe

pneumonia and lethality within approximately a week of challenge. Interestingly, treatment with nucleocapsid-specific mAb mitigated weight loss (**Figure 6E**) and resulted in a significant improvement in viral control (**Figure 6F**). In addition, mice treated with nucleocapsid-specific mAb exhibited lower levels of inflammatory IL-6 cytokine in circulation (**Figure 6G**), and showed reduced lung immunopathology (**Figure 6H-6I**) relative to control mice. Altogether, these data demonstrate that nucleocapsid-specific antibodies contribute to the control of a SARS-CoV-2 infection and can mitigate COVID-19 disease progression.

Although nucleocapsid specific antibodies failed to neutralize cell-free SARS-CoV-2 in vitro, we and others have recently shown that nucleocapsid is expressed on the surface of SARS-CoV-2 infected cells, and therefore has the potential to mediate ADCC (24, 25). To understand whether this could be the mechanism of action in the current study, we performed ADCC assays using sera from mice immunized with the nucleocapsid vaccine. We also examined ADCC activity with the nucleocapsid-specific mAb. Interestingly, we observed that N-specific antibodies from immune sera or mAb bind to SARS-CoV-2 infected cells (**Figure 7A**). Moreover, nucleocapsid-specific sera and nucleocapsid-specific mAb triggered ADCC activity by NK cells, evidenced by in vitro CD107a degranulation (**Figure 7B**). These findings suggest that nucleocapsid-specific antibodies confer antiviral protection via recognition of infected cells displaying the nucleocapsid antigen on their surface, which triggers Fc-mediated antibody-mediated effector functions.

Discussion:

The SARS-CoV-2 spike protein mediates viral entry by binding to the ACE2 receptor, and therefore, this protein is considered the most important antigenic target for vaccines and monoclonal antibody therapies. All approved SARS-CoV-2 vaccines target the spike protein with the goal of generating spike-specific antibodies that block viral entry. However, it is unclear if antibodies of other specificities (e.g. internal viral proteins that do not mediate viral entry) can play a role in antiviral protection. In particular, nucleocapsid-specific antibodies are generated after SARS-CoV-2 infection and also after immunization with experimental nucleocapsid-based vaccines (14, 22), but it is unclear if nucleocapsid-specific antibody responses can confer any protection in vivo.

To the best of our knowledge, the data presented here provide the first demonstration that nucleocapsid-specific humoral responses can help clear a SARS-CoV-2 infection. These findings may be important for the development of next-generation SARS-CoV-2 vaccines encoding other antigens besides the spike protein, and for developing new mAb therapies. Current monoclonal antibody therapies for COVID-19 target only the spike protein, and many of these therapies have lost efficacy against variants, since the spike protein is highly variable. Although the widely distributed Omicron variant exhibits mutations in the nucleocapsid, the vast majority of mutations are focused in the spike. Similarly, most of the genetic diversity in human immunodeficiency virus (HIV) (and other rapidly mutating viruses) occurs in the envelope proteins that mediate viral entry, motivating the development of mAb therapies targeting exclusively these surface viral

proteins for prevention and treatment of HIV infection (26, 27). Our data provide a rationale for the clinical evaluation of mAb therapies targeting internal viral proteins, which may provide another layer of immune protection by engaging antibody effector mechanisms. Although nucleocapsid-specific antibodies do not prevent breakthrough infection with SARS-CoV-2, they help control infection. Following initial infection, virus can transmit via cell-to-cell interactions and this type of infection is resistant to neutralizing antibodies, but not antibody effector mechanisms that target cell-surface antigens (28). It is therefore possible that nucleocapsid-specific monoclonal antibodies could also synergize with spike-specific monoclonal antibodies, which are currently a standard of care given either as pre-exposure prophylaxis or post-exposure prophylaxis in high-risk individuals.

In a prior paper we demonstrated that a nucleocapsid-based vaccine confers limited protection against a SARS-CoV-2 challenge when given as a “single vaccine” without a spike-based vaccine. At first glance, this prior study appears to contradict our results, but in that prior study we used a high challenge dose of SARS-CoV-2 (5×10^4 PFU) and evaluated viral loads during the hyperacute phase of infection (72 hr), which may have been too early to observe virologic differences, given that the efficacy of nucleocapsid-specific antibodies may rely on the recruitment and effector mechanisms of immune cells. The antiviral effects of nucleocapsid-specific antibodies may also vary depending on the virus inoculum. Following low dose viral challenges (10^3 PFU), which better represent what humans encounter during a natural infection, nucleocapsid-specific antibodies elicited a 163-fold reduction in viral titers (**Figure 6C**). However, following

high dose viral challenges (5×10^4 PFU), nucleocapsid-specific antibodies elicited only a 3-fold reduction in viral titers (**Figures 2D and 6F**). Although the antiviral effect was more limited during the high dose viral challenge, there was an overall reduction in emaciation and inflammation, following treatment with nucleocapsid-specific antibodies. For more than a century, convalescent plasma therapy has been used to treat many infectious diseases. Convalescent plasma therapy has also shown efficacy against COVID-19, but its benefits are more apparent in patients who receive plasma with high antibody titers, which can only be obtained from convalescent donors early after infection (29, 30). A limitation of our study is that we only performed adoptive sera transfers early after nucleocapsid vaccination using a prime-boost regimen that elicits high antibody responses. It is unknown if a similar level of protection would be observed if the passive immunizations were performed using nucleocapsid-immune sera from later time points post-vaccination when antibody titers decline. We detected memory B cells, suggesting that the nucleocapsid-specific antibody response was long-lived, but future studies are needed to evaluate the durability of nucleocapsid-specific antibody responses. Recent work from our laboratory and others have shown that the nucleocapsid protein can be expressed on the surface of infected cells, and can trigger antibody dependent cellular cytotoxicity (ADCC) (24, 25). Although these studies did not evaluate *in vivo* viral control, their observation is consistent with our findings that nucleocapsid-specific antibodies could exert a virological effect by engaging effector mechanisms, independent of neutralization. This notion was corroborated by our ADCC studies, which demonstrated that both vaccine-induced and monoclonal nucleocapsid-specific antibodies trigger degranulating activity in NK cells. Taken together, these data

provide insights for next-generation vaccines and for expanding the armamentarium of mAb-based therapies for COVID-19, as well as other viral diseases.

Lead contacts

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Pablo Penaloza-MacMaster (ppm@northwestern.edu) and/or Justin Richner (richner@uic.edu)

Materials availability

For Ad5-SARS CoV-2 nucleocapsid viral vector access, contact David Masopust at masopust@umn.edu.

Animals and ethics statement: Mice were purchased from Jackson laboratories and were housed at Northwestern University or University of Illinois at Chicago (UIC) animal facility. All procedures were performed with the approval of the center for comparative medicine at Northwestern University and the UIC IACUC. Adult mice, approximately half females and half males were used for the immunogenicity experiments included in this study. For the challenge studies, female mice were used.

Method Details

Cell lines

Adenoviral vectors were propagated using HEK293 cells purchased from ATCC (cat # CRL-1573). Vero E6 cells were used to propagate SARS-CoV-2 isolate USA-WA1/2020 (BEI resources, NR-52281). Cells were not authenticated as they were purchased from a reputable vendor and a certificate of analysis was obtained.

Mice and vaccinations:

6-8-week-old mice were used in these studies. Wild type C57BL/6 mice were used for immunogenicity studies, and K18-hACE2 mice (on C57BL/6 background) were used for challenge studies. These mice express the human ACE2 protein behind the keratin 18

promoter, directing expression in epithelial cells. K18-hACE2 mice were purchased from Jackson laboratories (Stock No: 034860). Mice were immunized intramuscularly (50 μ L per quadriceps) with an Ad5 vector expressing SARS-CoV-2 nucleocapsid protein (Ad5-N) at 10^{11} PFU per mouse, and N protein; diluted in sterile PBS. Ad5-N was a kind gift of the Masopust/Vezys laboratory (21). This is a non-replicating Ad5 vector (E1/E3 deleted). The vector contains a CMV (Cytomegalovirus) promoter driving the expression of the respective proteins. The Ad5 vector was propagated on trans-complementing HEK293 cells (ATCC), purified by cesium chloride density gradient centrifugation, titrated, and then frozen at -80 °C.

SARS-CoV-2 virus and infections:

SARS-Related Coronavirus 2, Isolate USA-WA1/2020, NR-52281 was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH. Virus was propagated and tittered on Vero-E6 cells (ATCC). In brief, Vero cells were passaged in DMEM with 10% Fetal bovine serum (FBS) and Glutamax. Cells less than 20 passages were used for all studies. Virus stocks were expanded in Vero-E6 cells following a low MOI (0.01) inoculation and harvested after 4 days. Viral titers were determined by plaque assay on Vero-E6 cells. Viral stocks were used after a single expansion (passage = 1) to prevent genetic drift (a small proportion of the virus stock contained mutations in the furin cleavage site). K18-hACE2 mice were anesthetized with isoflurane and challenged with 10^3 PFU of SARS-CoV-2 intranasally. Mouse infections were performed at the University of Illinois at Chicago (UIC) following BL3 guidelines with approval by the UIC Institutional Animal Care and Use Committee (IACUC).

SARS-CoV-2 RNA quantification:

Lungs were harvested from infected mice and homogenized in PBS. RNA was isolated with the Zymo 96-well RNA isolation kit (Catalog #: R1052) following the manufacturer's protocol. SARS-CoV-2 viral burden was measured by RT-qPCR using Taqman primer and probe sets from IDT with the following sequences: Forward 5' GAC CCC AAA ATC AGC GAA AT 3', Reverse 5' TCT GGT TAC TGC CAG TTG AAT CTG 3', Probe 5' ACC

CCG CAT TAC GTT TGG TGG ACC 3'. A SARS-CoV-2 copy number control was obtained from BEI (NR-52358) and used to quantify SARS-CoV-2 genomes.

Focus Forming Assay (FFA) and Focus Reduction Neutralization Titer (FRNT) Assay using live SARS-CoV-2:

Quantification of SARS-CoV-2 by FFA was performed by serial dilution of viral stocks or lung homogenate. Dilutions were added onto a monolayer of Vero cells in a 96 well plate. One hour after infection, cells were overlaid with 1% (w/v) methylcellulose in 2% FBS, 1X MEM. Plates were fixed for 30 minutes with 4% PFA 24 hr after infection. Staining involved 1° anti-SARS guinea pig (1:15,000, NR-10361 from BEI Resources) and 2° goat anti-guinea pig HRP (200 ng/ml) in Perm Wash Buffer (0.1% Saponin, 0.1% BSA, in PBS). Treatment with TrueBlue peroxidase substrate (KPL) produced FFU that were quantified with an ImmunoSpot® ELISpot plate scanner (Cellular Technology Limited). For FRNT assays, serial dilutions of heat-inactivated serum from vaccinated mice were incubated with 100 FFU of live SARS-CoV-2 (isolate USA-WA1/2020) for one hour at 37°C before infecting a monolayer of Vero cells in a 96-well plate. Viral foci were determined as above for FFA.

Reagents, flow cytometry and equipment:

Single cell suspensions were obtained from PBMCs or tissues. Dead cells were gated out using Live/Dead fixable dead cell stain (Invitrogen). SARS-CoV-2 nucleocapsid protein was biotinylated and conjugated to streptavidin-PE for detection of nucleocapsid-specific memory B cells on MACS-purified B cells. MHC class I monomers (D^b219, LALLLLDRL; and K^bVL8, VNFNFNGL) were used for detecting virus-specific CD8 T cells, and were obtained from the NIH tetramer facility located at Emory University. MHC monomers were tetramerized in-house. Cells were stained with fluorescently-labeled antibodies against CD8α (53-6.7 on PerCP-Cy5.5), CD44 (IM7 on Pacific Blue), and K^bN219 (PE). Fluorescently-labeled antibodies were purchased from BD Pharmingen, except for anti-CD44 (which was from Biolegend). Flow cytometry samples were acquired with a Becton Dickinson Canto II or an LSRII and analyzed using FlowJo v10 (Treestar).

SARS-CoV-2 nucleocapsid specific ELISA:

Binding antibody titers were quantified using ELISA as described previously (31, 32), using nucleocapsid protein as coating antigens. In brief, 96-well flat bottom plates MaxiSorp (Thermo Scientific) were coated with SARS-CoV-2 nucleocapsid protein, washed and blocked. Serial sera dilutions were performed. Absorbance was measured using a Spectramax Plus 384 (Molecular Devices). Antibody levels were reported as endpoint titer using serial 3-fold dilutions.

Sera transfers and monoclonal antibody therapies:

For the passive immunization studies, 500 μ L of N-specific immune sera or irrelevant sera were transferred one day before SARS-CoV-2 challenge. Control mAb (IgG2a, clone C1.18.4) and Anti-N mAb (clone 1C7C7) were purchased from Leinco. Mice received 800 μ g of the respective antibody clone diluted in PBS one day before SARS-CoV-2 challenge. Sera transfers and mAb therapy were administered intraperitoneally.

ADCC assays:

293-ACE2 cells were made by transducing 293 cells with a lentivirus expressing human ACE2, and selected in Blasticidin for 7 days, as previously described (33). Effector NK cells expressing mouse CD16 (NKL-mCD16) were a kind gift from O.Aguilar and were maintained as previously described (34). In brief, 293-ACE2 cells were mock-infected or infected with SARS-CoV-2 (England2 strain) at MOI=5. After 24 hr, 293-ACE2 cells (25,000/well) were cultured with effectors (NKL-mCD16, 50,000/well), together with sera or mAb (1C7) in the presence of CD107a-FITC (Biolegend) and Golgi-stop (BD Biosciences). 5h later cultures were washed and stained with live/dead fixable aqua, and CD56-BV605 (Biolegend). CD107a levels were analyzed on an Attune flow cytometer (Thermo Fisher). Cells were gated on the live/CD56+ population, and the percentage of CD107a+ cells was calculated. All sera/mAb samples were incubated with both infected and mock-infected cells to control for non-specific activation. Samples were tested over a 3-fold dilution series beginning at 1:30, to account for “hooking” effects. Every sample was tested in technical duplicate and averaged.

Detecting binding of N-specific antibodies to infected cells:

293-ACE2 cells were infected with SARS-CoV-2 (England2 strain) at MOI=5. After 24 hr, cells were detached with TrypLE, and stained with primary N-specific mAb (or N-specific immune sera) as indicated, for 30 min at 4°C. After washing, cells were incubated with a secondary antibody (anti-mouse Alexa-Fluor 647) for 30 min at 4°C. Cells were washed, fixed in 4% PFA, and analyzed on an Attune flow cytometer.

Statistical analysis:

Statistical analyses are indicated on the figure legend. Dashed lines in data figures represent limit of detection. Statistical significance was established at $p \leq 0.05$ and was generally assessed by Mann Whitney tests, unless indicated otherwise in figure legends. Data were analyzed using Prism (Graphpad).

Study approval

Human specimens. All protocols used for participant recruitment, enrollment, blood collection, sample processing, and immunological assays with human samples were approved by the IRB of Northwestern University (STU00212583). All participants voluntarily enrolled in the study by signing an informed consent form after receiving detailed information about the clinical study.

Author Contributions:

T.D. and S.S. performed the vaccination and immunogenicity experiments. J.C. performed the challenge experiments. Y.R.C helped with the H&E studies. K.B. and R.J.S. designed and performed the ADCC assays. P.P.M. and J.R. designed the immunogenicity and challenge experiments and secured funding. P.P.M. wrote the paper, with feedback from all authors.

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Declaration of Interests: The authors declare that no conflicts of interests exist.

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Figure 1

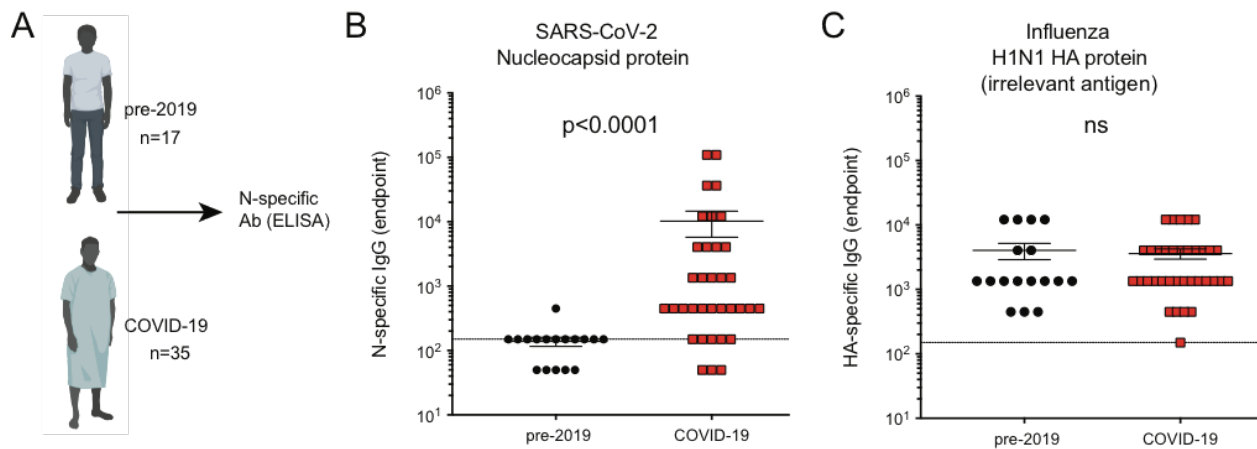


Figure 1. SARS-CoV-2 nucleocapsid specific antibody after SARS-CoV-2 infection in a cohort of patients admitted to Northwestern University Hospital. (A) Human pre-2019 plasma from healthy individuals were used as control. Data shown are from an ongoing study, in which participants were infected on different dates, hence the heterogeneity in the nucleocapsid-specific antibody responses. SARS-CoV-2 infection was confirmed by RT-PCR. Antibody responses were evaluated by ELISA. **(B)** Summary of SARS-CoV-2 nucleocapsid-specific antibodies in sera. **(C)** Summary of influenza HA-specific antibodies in sera (used as irrelevant antigen control). Dashed lines represent the LOD. Indicated P-value was calculated using Mann Whitney test. Error bars represent SEM.

Figure 2

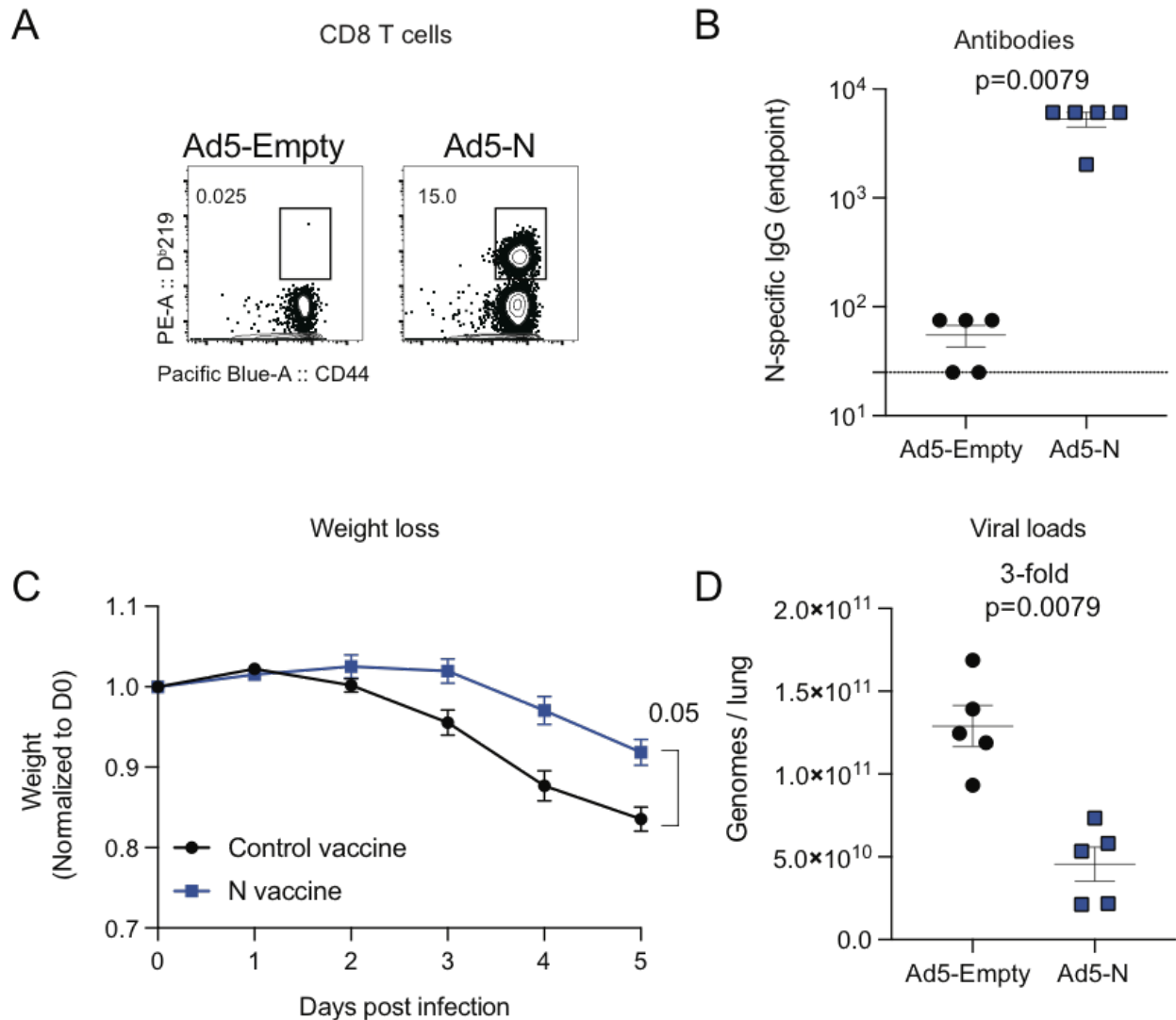


Figure 2. A nucleocapsid vaccine improves the control of SARS-CoV-2 infection.

(A) Representative FACS plots showing the frequencies of SARS-CoV-2 nucleocapsid-specific CD8 T cells (D^bN219+) in PBMCs. **(B)** Summary of SARS-CoV-2 nucleocapsid-specific antibodies in sera. **(C)** Weight loss. **(D)** Viral loads in lungs at day 5 post-infection. K18-hACE2 mice were primed intramuscularly with Ad5 expressing nucleocapsid (Ad5-N), and after 2 weeks, adaptive immune responses were evaluated,

and mice were then challenged intranasally with 5×10^4 PFU of SARS-CoV-2. Weight loss was recorded daily, and RNA was harvested from the lungs at day 5 post-infection. Viral RNA was quantified by RT-qPCR. Challenges were performed with a total of 5 mice per group in Biosafety level 3 (BSL-3) facilities. Indicated P-values were calculated using Mann Whitney test.

Figure 3

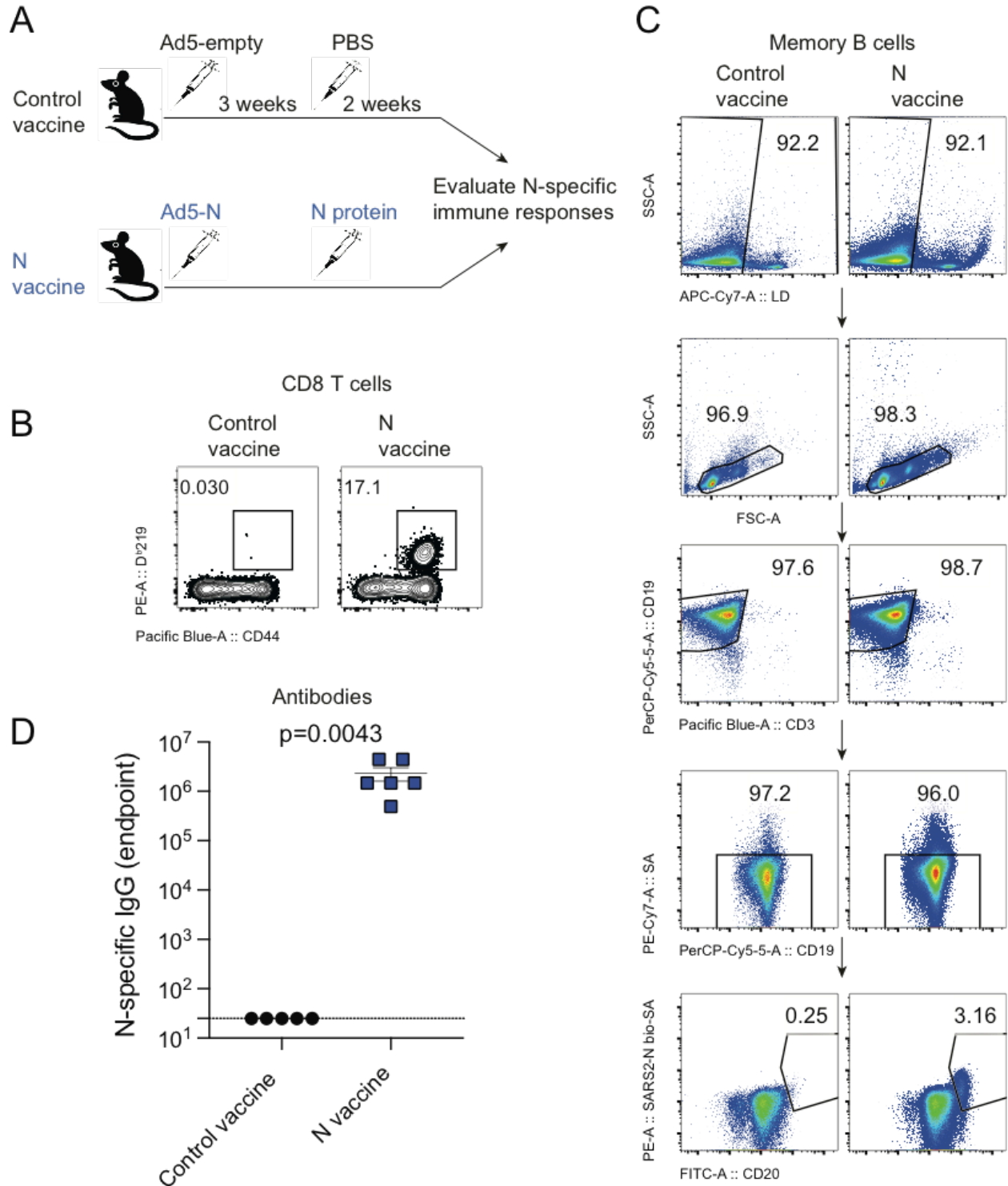


Figure 3. Immunogenicity of a SARS-CoV-2 nucleocapsid vaccine regimen. (A)

Experimental approach for evaluating immune responses after nucleocapsid vaccination. **(B)** Representative FACS plots showing the frequencies of SARS-CoV-2 nucleocapsid-specific CD8 T cells (D^bN219+) in PBMCs. **(C)** Representative FACS plots showing the frequencies of SARS-CoV-2 nucleocapsid-specific memory B cells in spleen. Splenocytes were MACS-purified by negative selection to enrich for B cells, facilitating visualization of nucleocapsid-specific B cells. **(D)** Summary of SARS-CoV-2 nucleocapsid-specific antibodies in sera. Data are from week 2 post-boost, and from an experiment with n=5-6 per group. Experiment was performed twice with similar results. Indicated P-values were calculated using Mann Whitney test.

Figure 4

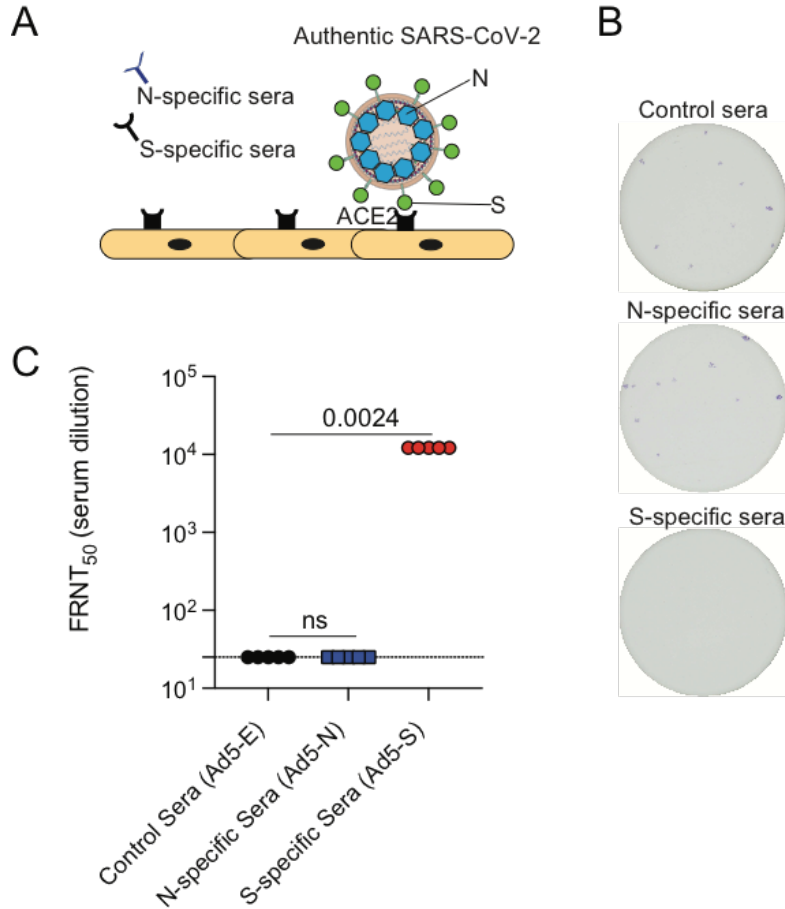


Figure 4. Nucleocapsid-specific humoral responses do not prevent SARS-CoV-2 infection. **(A)** Experimental approach for performing focus reduction neutralization titer (FRNT) assays on Vero cells using live SARS-CoV-2 (USA-WA1/2020). See Materials and Methods for technical information. **(B)** Representative wells showing the frequencies of SARS-CoV-2+ cells (1:450 sera dilution). **(C)** Summary of FRNT₅₀ titers in sera. Data are from week 3 post-vaccination. Data are from an experiment with n=5 per group. Experiment was performed twice with similar results. Indicated P-values were calculated using Kruskal-Wallis test (multiple comparisons).

Figure 5

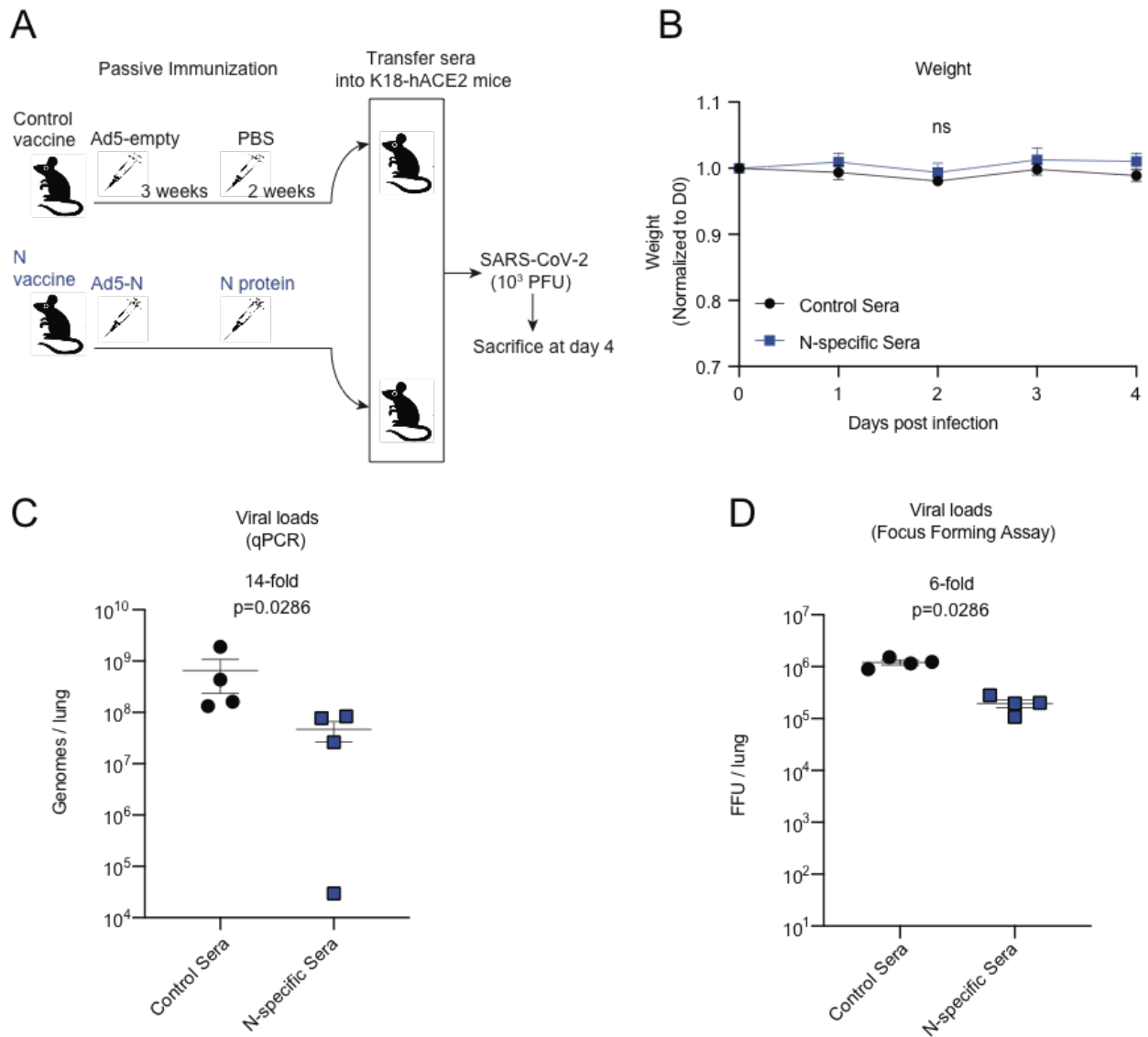


Figure 5. Nucleocapsid-specific humoral responses improve the control of SARS-CoV-2 infection. (A) Experimental approach for evaluating viral control after passive immunization with nucleocapsid-specific sera. (B) Weight loss. (C) Viral loads in lungs by RT-pPCR. (D) Viral loads in lungs by focus forming assays. C57BL/6 mice were primed intramuscularly with Ad5 expressing nucleocapsid (Ad5-N), and after 3 weeks they were boosted with soluble nucleocapsid (N) protein. After 2 weeks post-boost, sera

from these mice were pooled and 500 μ L of these sera were adoptively transferred into naïve K18-hACE2 recipient mice. On the following day, the K18-hACE2 mice were challenged intranasally with 10^3 PFU of SARS-CoV-2. RNA was harvested from the lungs at day 4 post-infection, and viral RNA was quantified. Challenges were performed with a total of 4 mice per group in Biosafety level 3 (BSL-3) facilities. Indicated P-values were calculated using Mann Whitney test.

Figure 6

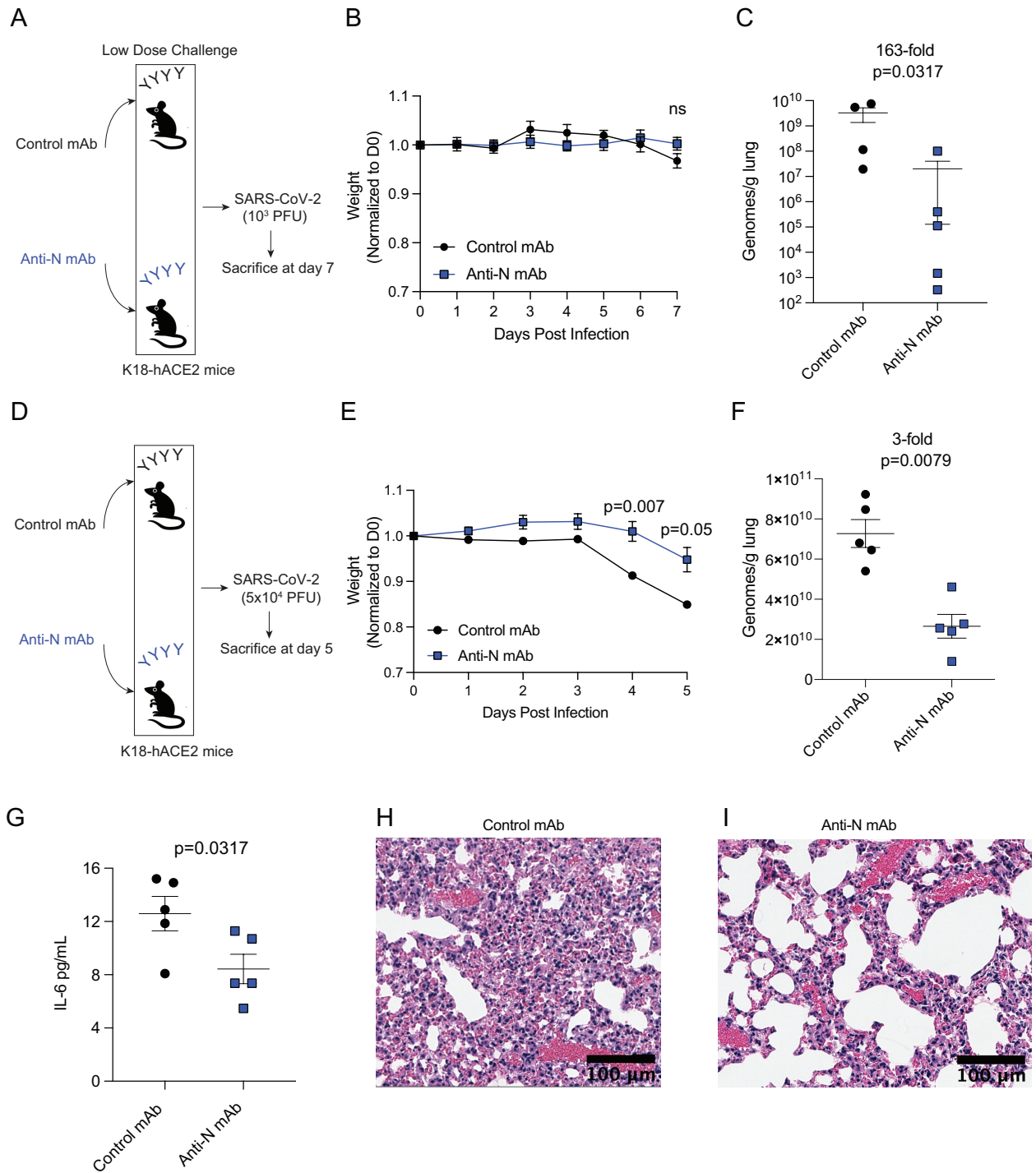


Figure 6. Nucleocapsid-specific monoclonal antibody (mAb) improves the control of SARS-CoV-2 infection. (A) Experimental approach for evaluating viral control after

treatment with nucleocapsid-specific mAb during a low dose viral challenge. 800 μg of mAb (IgG control or anti-N) were injected intraperitoneally into naïve K18-hACE2 recipient mice. On the following day, the K18-hACE2 mice were challenged intranasally with 10^3 PFU of SARS-CoV-2. **(B)** Weight loss. **(C)** Viral loads in lungs by RT-qPCR. RNA was harvested from the lungs at day 7 post-infection, and viral RNA was quantified. **(D)** Experimental approach for evaluating viral control after treatment with nucleocapsid-specific mAb during a high dose viral challenge. 800 μg of mAb (IgG control or anti-N) were injected intraperitoneally into naïve K18-hACE2 recipient mice. On the following day, the K18-hACE2 mice were challenged intranasally with 5×10^4 PFU of SARS-CoV-2. **(E)** Weight loss. **(F)** Viral loads in lungs by RT-qPCR. RNA was harvested from the lungs at day 5 post-infection, and viral RNA was quantified. **(G)** IL-6 levels in sera. **(H-I)** H&E stains of lung. Panels A-C are from low dose viral challenges; panels D-I are from high dose viral challenges. Challenges were performed with a total of 4-5 mice per group in Biosafety level 3 (BSL-3) facilities. Indicated P-values were calculated using Mann Whitney test.

Figure 7

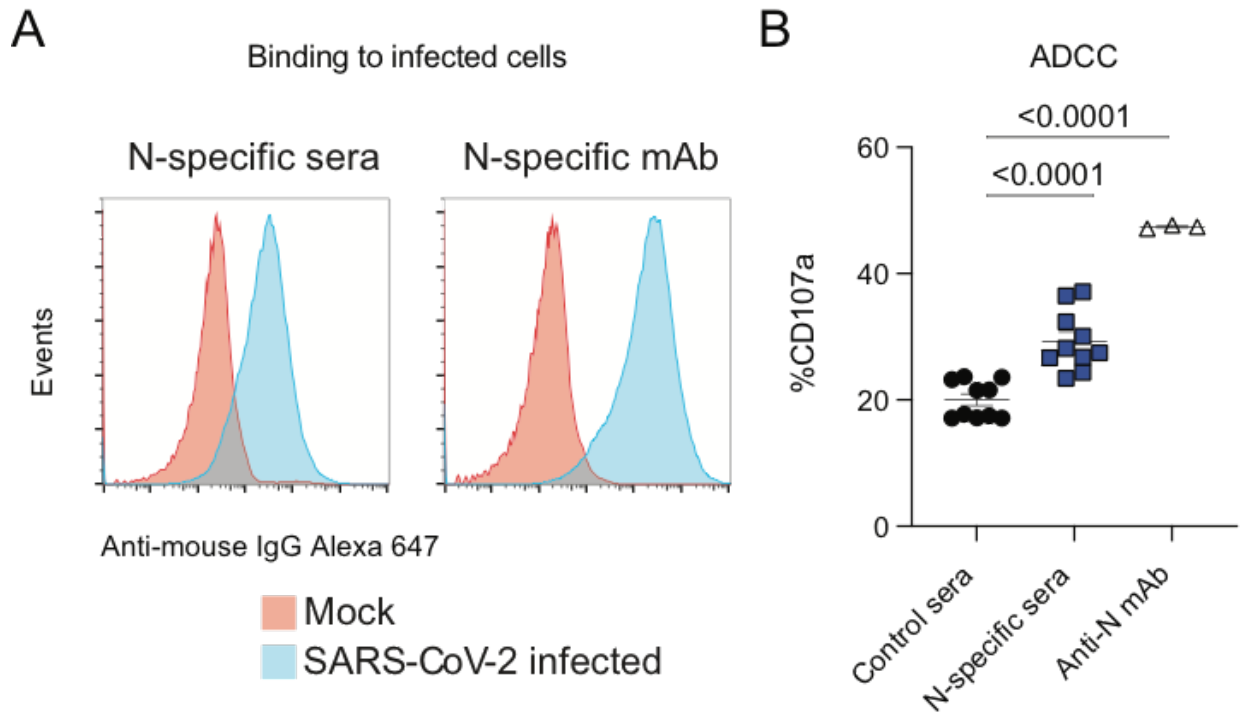


Figure 7. N-specific antibodies bind to infected cells and trigger ADCC. (A) Representative FACS plots showing that N-specific sera and n-specific mAb bind to SARS-CoV-2 infected cells. 293-ACE2 cells were infected with SARS-CoV-2 and binding to N-specific antibody was assessed by flow cytometry using a secondary antibody bound to Alexa 647. Control cells were mock infected. **(B)** N-specific antibodies trigger ADCC. 293-ACE2 cells were infected with SARS-CoV-2 and co-cultured with effectors (NKL-mCD16 cells), together with N-specific immune sera or anti-N mAb (clone 1C7). CD107a expression on NKL-mCD16 cells was quantified by flow cytometry after 5 hr. Data are from 1:30 dilution. Data are from two experiments, each with n=5 per group. All data are shown. Indicated P-values were calculated using two-way ANOVA test (Dunnett's multiple comparisons test).