

# A COVID-19 antibody curbs SARS-CoV-2 nucleocapsid protein-induced complement hyperactivation

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1 **A SARS-CoV-2 antibody curbs viral N protein-induced**  
2 **complement hyperactivation**

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## 30 **Summary**

31 **Although human antibodies elicited by the severe acute respiratory**  
32 **syndrome coronavirus 2 (SARS-CoV-2) nucleocapsid (N) protein are**  
33 **profoundly boosted upon infection, little is known about the function of N-**  
34 **reactive antibodies. Herein, we isolated and profiled a panel of 32 N**  
35 **protein-specific monoclonal antibodies (mAbs) from a quick recovery**  
36 **coronavirus disease-19 (COVID-19) convalescent patient who had dominant**  
37 **antibody responses to the SARS-CoV-2 N protein rather than to the SARS-**  
38 **CoV-2 spike (S) protein. The complex structure of the N protein RNA**  
39 **binding domain with the mAb with the highest binding affinity (nCoV396)**  
40 **revealed changes in the epitopes and antigen's allosteric regulation.**  
41 **Functionally, a virus-free complement hyperactivation analysis**  
42 **demonstrated that nCoV396 specifically compromises the N protein-**  
43 **induced complement hyperactivation, which is a risk factor for the**  
44 **morbidity and mortality of COVID-19 patients, thus laying the foundation**  
45 **for the identification of functional anti-N protein mAbs.**

46 **Keywords:** human monoclonal antibody, COVID-19, SARS-CoV-2, nucleocapsid  
47 protein, crystal structure, complement hyperactivation, viral protein targeting  
48 therapy, MASP-2

## 49 **Main**

50 The fatality rate of critical condition coronavirus disease 2019 (COVID-19)  
51 patients is exceptionally high (40% - 49%)<sup>1,2</sup>. Acute respiratory failure and  
52 generalized coagulopathy are significant aspects associated with morbidity and  
53 mortality<sup>3-5</sup>. A subset of severe COVID-19 patients has distinct clinical features  
54 compared to classic acute respiratory distress sndrome (ARDS), with delayed  
55 onset of respiratory distress<sup>6</sup> and relatively well-preserved lung mechanics  
56 despite the severity of hypoxemia<sup>7</sup>. It has been reported that complement-  
57 mediated thrombotic microvascular injury in the lung may contribute to atypical  
58 ARDS features of COVID-19, accompanied by extensive deposition of the  
59 alternative pathway (AP) and lectin pathway (LP) complement components<sup>8</sup>.  
60 Indeed, complement activation is found in multiple organs of severe COVID-19  
61 patients in several other studies<sup>9,10</sup>, as well as in patients with severe acute  
62 respiratory sndrome (SARS)<sup>11,12</sup>. A recent retrospective observational study of  
63 11,116 patients revealed that complement disorder was associated with the  
64 morbidity and mortality of COVID-19<sup>13</sup>.

65 The nucleocapsid (N) protein of severe acute respiratory sndrome coronavirus  
66 2(SARS-CoV-2), the etiology agent of COVID-19, is one of the most abundant  
67 viral structural proteins with multiple functions inside the viral particles, the host  
68 cellular environment, and in *ex vivo* experiments<sup>14-20</sup>. Among these functions, a  
69 recent preprint study found that the SARS-CoV-2 N protein bound to MBL  
70 (mannan-binding lectin)-associated serine protease 2 (MASP-2) and resulted in

71 complement hyperactivation and aggravated inflammatory lung injury<sup>19</sup>.  
72 Consistently, the highly pathogenic SARS-CoV N protein was also found to bind  
73 with MAP19, an alternative product of MASP-2<sup>21</sup>.

74 Although systemic activation of complement plays a pivotal role in protective  
75 immunity against pathogens, hyperactivation of complement may lead to  
76 collateral tissue injury. Thus, how to precisely regulate virus-induced  
77 dysfunctional complement activation in COVID-19 patients remains to be  
78 elucidated. The SARS-CoV-2 N protein is a highly immunopathogenic viral  
79 protein that elicits high titers of binding antibodies in humoral immune  
80 responses<sup>22-24</sup>. Several studies have reported the isolation of human monoclonal  
81 antibodies (mAbs) targeting the SARS-CoV-2 spike (S) protein, helping explain  
82 the possible developing therapeutic interventions for COVID-19<sup>22,25-29</sup>. However,  
83 little is known about the potential therapeutic applications of N protein-targeting  
84 mAbs in the convalescent B cell repertoire. Herein, we report a human mAb  
85 derived from the COVID-19 convalescent patient that specifically targets the  
86 SARS-CoV-2 N protein and functionally compromises complement  
87 hyperactivation *ex vivo*.

#### 88 **Isolation of N protein-reactive mAbs**

89 To profile the antibody response to the SARS-CoV-2 N protein in patients during  
90 the early recovery phase, we collected blood samples from six convalescent  
91 patients seven to 25 days after the onset of the disease symptoms. All patients  
92 recovered from COVID-19 during the outbreak in Zhuhai, Guangdong Province,

93 China, with ages ranging from 23 to 66 years (**Extended Data Table 1**). Our  
94 work and use of patients' samples is in accordance with the declaration of  
95 Helsinki, medical ethics standards and China's laws. Our study was approved by  
96 the Ethics Committee of The Fifth Affiliated Hospital, Sun Yat-sen University, and  
97 all patients signed informed consent forms. SARS-CoV-2 nasal swabs reverse  
98 transcription polymerase chain reaction (RT-PCR) tests were used to confirm  
99 that all 6 COVID-19 patients were negative for SARS-CoV-2 at the time of blood  
100 collection. Plasma samples and peripheral blood mononuclear cells (PBMCs)  
101 were isolated for serological analysis and antibody isolation. Serum antibody  
102 titers to SARS-CoV-2 S and N proteins were measured by enzyme-linked  
103 immunosorbent assay (ELISA) (**Figure 1a, 1b, and Extended Data Table 1**).  
104 Serologic analysis demonstrated that serum antibody titers to the N protein were  
105 substantially higher than those to the S protein in most of the patients. For  
106 example, ZD004 and ZD006 had only minimal levels of antibody response to the  
107 S protein, while they had much higher antibody titers to the N protein. Notably,  
108 the time from disease onset to complete recovery from clinical symptoms of  
109 COVID-19 patient ZD006 was only 9 days (**Extended Data Table 1**).

110 To take advantage of patient ZD006, who was still in the early recovery phase  
111 with a high possibility of a high percentage of antigen-specific plasma cells,  
112 single plasma cells (**Figure 1c**) with the phenotype of CD3<sup>-</sup>/CD14<sup>-</sup>/CD16<sup>-</sup>  
113 /CD235a<sup>-</sup>/CD19<sup>+</sup>/CD20<sup>low-neg</sup>/CD27<sup>hi</sup>/CD38<sup>hi</sup> as well as antigen-specific memory B  
114 cells with the phenotype of CD19<sup>+</sup>/CD27<sup>+</sup> (**Figure 1d**) were sorted from PBMCs  
115 of patient ZD006 by fluorescence-activated cell sorting (FACS). To ensure an

116 unbiased assessment, the sorting of antigen-specific memory B cells was carried  
117 out with combined probes for both fluorophore-labeled S and N recombinant  
118 proteins. Variable regions of immunoglobulin (Ig) heavy and light chain gene  
119 segment ( $V_H$  and  $V_L$ , respectively) pairs from the sorted single cells were  
120 amplified by RT-PCR, sequenced, annotated and expressed as recombinant  
121 mAbs using the methods described previously<sup>30</sup>. Recombinant mAbs were  
122 screened against SARS-CoV-2 S and N proteins. In total, we identified 32 mAbs  
123 that reacted with the SARS-CoV-2 N protein, including 20 mAbs from plasma  
124 cells and 12 mAbs from memory B cells (**Extended Data Table 2**). We found that  
125 IgG1 is the predominant isotype at 46.9%, followed by IgG3 (25.0%), IgA (18.8%),  
126 IgG2 (6.3%) and IgM (3.1) (**Figure 1e**).  $V_H$  gene family usage in SARS-CoV-2 N  
127 protein-reactive antibodies was 18.8% for  $V_{H1}$ , 62.5% for  $V_{H3}$ , 9.4% for  $V_{H4}$ ,  
128 6.2% for  $V_{H5}$  and 3.1% for  $V_{H7}$ , respectively (**Figure 1f**), which was similar to the  
129 distribution of  $V_H$  families collected in the NCBI database. Nine of 32 SARS-CoV-  
130 2 N protein-reactive antibodies had no mutations from their germline  $V_H$  and  $V_H$   
131 gene segments (**Figure 1f, Extended Data Table 2**). The average mutation  
132 frequency of the remaining mutated antibodies was 5.3% (+/-3.6%) in  $V_H$  and  
133 3.5% (+/-2.7%) in  $V_L$ .

134 Consistent with the lower serum antibody titers to the SARS-CoV-2 S protein, we  
135 identified only eight SARS-CoV-2 S protein-reactive mAbs, including 5 antibodies  
136 from plasma cells and three antibodies from memory B cells. The  $V_H$  gene  
137 segment of the S protein-reactive antibodies had either no (6/8) or minimal  
138 (1/300) mutations (**Figure 1g**). There were no significant differences in

139 complementarity-determining region 3 (CDR3) length in amino acid residues  
140 between the N- (**Figure 1h**) and S-reactive antibodies (**Figure 1i**).

141 Approximately a quarter of antibodies directed to the N protein (**Figure 1f**) and  
142 almost all of antibodies to the S protein that had no or minimal mutations from  
143 their germlines (**Figure 1g**) had a primary antibody response similar to other  
144 typical primary viral infections. However, relatively high  $V_H$  mutation frequencies  
145 (mean of 5.7%) of the majority of antibodies to the N proteins were more similar  
146 to the mutation frequencies of antibodies from the secondary responses to the  
147 influenza vaccination reported previously<sup>31</sup>. Although patient ZD006 was  
148 hospitalized for only nine days after the onset of COVID-19 symptoms, the  
149 patient had high serum antibody titers, and the majority of the isolated N-reactive  
150 antibodies had a high mutation frequency, whereas the S-reactive antibodies had  
151 no or minimal mutations. These results reflect a much stronger antigen  
152 stimulation to the host driven by the SARS-CoV-2 N protein than by the S protein.

### 153 **Binding characterizations of anti-N mAbs**

154 To determine the antigenic targets by the N-reactive antibodies, we next  
155 analyzed the binding activities by ELISA with variant constructs of the N protein  
156 (full length N protein (N-FL): 1-400; N protein N-terminal domain (N-NTD): 41-  
157 174; and N protein C-terminal domain (N-CTD): 250-364) (**Figure 2a**). Among  
158 the 32 mAbs that bound to N-FL, 13 antibodies bound to N-NTD, and one  
159 antibody bound to N-CTD (**Figure 2b**). A total of nine antibodies, including one  
160 antibody (nCoV400) that bound to N-CTD, seven mAbs (nCoV396, nCoV416,

161 nCoV424, nCoV425, nCoV433, nCoV454, and nCoV457) that bound to N-NTD  
162 and one mAb (nCoV402) that bound only to N-FL but not to the other variant N  
163 proteins, were chosen as representative antibodies for further studies. Purified  
164 antibodies were confirmed to bind the N-FL protein by ELISA (**Figure 2c**). The  
165 affinity of these antibodies to the N-FL protein was measured by surface plasmon  
166 resonance (SPR) (**Figure 2d**). In an effort to further characterize the functional  
167 and structural relationships, three antibodies, nCoV396, nCOV416 and nCOV457,  
168 were selected for the production of recombinant fragment antigen-binding (Fab)  
169 antibodies based on their unique characteristics. The mAb nCoV396 has a  $V_H$   
170 mutation frequency of 2.8% but a high binding affinity with a  $K_D$  of 1.02 nM  
171 (**Figure 2d**) to the N protein. The mAbs nCOV416 and nCOV457 have high  $V_H$   
172 mutations at 11.1% and 8.7%, respectively and have a binding affinity to the N  
173 protein with  $K_D$  values of 7.26 nM and 12.6 nM, respectively (**Figure 2d**,  
174 **Extended Data Table 3**).

### 175 **Complex structure of mAb with N-NTD**

176 To investigate the molecular interaction mechanism of the mAb nCoV396 with  
177 the N protein, we next solved the complex structure of the SARS-CoV-2 N-NTD  
178 with the nCoV396 Fab nCoV396Fab at a 2.1 Å resolution by X-ray  
179 crystallography. The final structure was fitted with the visible electron density  
180 spanning residues 49-173 (SARS-CoV-2 N-NTD), 1-220 (nCoV396Fab, the  
181 heavy chain of the Fab), and 1-213 (nCoV396Fab, the light chain of Fab, except  
182 for the residues ranging from 136-141). The complete statistics for the data  
183 collection, phasing, and refinement are presented in **Extended Data Table 4**.

184 With the help of the high-resolution structure, we were able to designate all  
185 complementarity-determining regions (CDRs) in nCoV396Fab as the following:  
186 light chain CDR1, residues 23-32 (L-CDR1), light chain CDR2, residues 51-54 (L-  
187 CDR2), light chain CDR3, residues 94-100 (L-CDR3), heavy chain CDR1,  
188 residues 26-33 (H-CDR1), heavy chain CDR2, residues 51-57 (H-CDR2), and  
189 heavy chain CDR3, residues 99-108 (H-CDR3). Among them, we identified the  
190 interaction interface between N-NTD and L-CDR1, L-CDR3, H-CDR1, H-CDR2,  
191 and H-CDR3 of nCoV396Fab with an unambiguous electron density map (**Figure**  
192 **3a, Extended Data Figure 1a**).

193 The interacting CDRs pinch the CT tail of the SARS-CoV-2 N-NTD (residues  
194 ranging from 159 to 172), with extensive binding contacts and a buried surface  
195 area of 1,079 Å<sup>2</sup> (**Extended Data Table 5**). Light chain L-CDR1 and L-CDR3 of  
196 nCoV396Fab interact with residues ranging from 159-163 of N-NTD via  
197 numerous hydrophilic and hydrophobic contacts (**Figure 3b, Extended Data**  
198 **Figure 1b**). Notably, the SARS-CoV-2 N-NTD residue Q163 is recognized by the  
199 L-CDR3 residue T95 via a hydrogen bond and simultaneously stacks with the L-  
200 CDR3 residue W96 and the L-CDR1 residue Y31 (**Figure 3c**). In addition, a  
201 network of interactions from the heavy chain H-CDR2 and H-CDR3 of  
202 nCoV396Fab to residues 165-172 of N-NTD suggests that the conserved residue  
203 K169 of SARS-CoV-2 N-NTD has a critical role in nCoV396 antibody binding.  
204 K169 is recognized via hydrogen bonds with the E99 δ-carboxyl group and the  
205 T100, D102, S105 main-chain carbonyl groups inside the H-CDR3 of  
206 nCoV396Fab (**Figure 3d**). In addition, SARS-CoV-2 N-NTD L167 also interacts

207 with I33, V50, N57, and A59 of H-CDR1 and H-CDR2 of nCoV396Fab through  
208 hydrophobic interactions (**Figure 3e**). Interestingly, all three residues (Q163,  
209 L167, and K169) of SARS-CoV-2 N-NTD are relatively conserved in the highly  
210 pathogenic betacoronavirus N protein (**Extended Data Figure 2a**), which implies  
211 that nCoV396 may cross-interact with the SARS-CoV N protein or the Middle  
212 East respiratory syndrome coronavirus (MERS-CoV) N protein. Indeed, the  
213 binding affinities measured by SPR analysis demonstrate that nCoV396 interacts  
214 with the SARS-CoV N protein and the MERS-CoV N protein with a  $K_D$  of 7.4 nM  
215 (**Extended Data Figure 2b - c**).

216 To discover the conformational changes between the SARS-CoV-2 N-NTD apo-  
217 state and the antibody-bound state, we next superimposed the complex structure  
218 with the N-NTD structure (PDB ID: 6M3M)<sup>14</sup>. The superimposition results suggest  
219 that the CT tail of SARS-CoV-2 N-NTD unfolds from the basic palm region upon  
220 nCoV396Fab binding (**Figure 3f**), which likely contributes to the allosteric  
221 regulation of the normal full-length N protein function. Additionally, nCoV396Fab  
222 binding results in a 7.4 Å movement of the  $\beta$ -finger region outward from the RNA  
223 binding pocket, which may enlarge the RNA binding pocket of the N protein  
224 (**Figure 3f**).

225 In summary, our crystal structural data demonstrated that the human mAb  
226 nCoV396 recognizes the SARS-CoV-2 N protein via a pinching model, resulting  
227 in a dramatic conformational change in residues 159 to 172, which is the linker  
228 region of N-NTD that is connected with other domains.

## 229 **MAb curbs N-induced complement activation**

230 Although a recent study suggests that the complement cascade is hyperactive by  
231 the N protein in the lungs of COVID-19 patients *via* the lectin pathway<sup>19</sup>, it is  
232 unclear how to develop a virus-free and an effective system for analyzing the role  
233 of the SARS-CoV-2 N protein on complement hyperactivation. To this end, we  
234 developed a clinical autoimmune disease serum-based protease enzymatic  
235 approach to assess complement activation levels in the presence of the SARS-  
236 CoV-2 N protein. Since complement activation initiated by the lectin pathway  
237 features MASP-2 proteases by specific activity for cleaving complement  
238 components 2 and 4 (C2 and C4)<sup>32</sup>, we designed a C2 internal quenched  
239 fluorescent peptide-based analysis route for *ex vivo* complement hyperactivation  
240 (**Figure 4a**). Briefly, serum was collected from the peripheral blood of volunteers  
241 with an autoimmune disease, as their serum contains the necessary components  
242 for complement activation characterized by elevated C3 levels (**Extended Data**  
243 **Table 6**). Next, we collected the fluorescence signal from cleaved C2 synthetic  
244 peptide substrates (2Abz-SLGRKIQLys(Dnp)-NH<sub>2</sub>) in reaction mixtures  
245 containing autoimmune disease serum in the absence or presence of the SARS-  
246 CoV-2 N protein with or without the mAb nCoV396. The initial reaction rate ( $v_0$ )  
247 was estimated at a single concentration of individual sera from duplicate  
248 measurements over a range of substrate concentrations. The steady-state  
249 reaction constants maximal velocity ( $V_{max}$ ) and Michaelis constant ( $K_m$ ) were  
250 determined for comparisons (**Figure 4a**).

251 As shown in **Figure 4b**, the calculated  $V_{\max}$  of reactions without any other  
252 exogenous proteins is 1.49 response units (RU)·s<sup>-1</sup>. Additions of the SARS-CoV-  
253 2 N protein (concentrations ranging from 0.5 μM to 10 μM) in the reactions  
254 remarkably elevate the  $V_{\max}$  up to 2-fold, ranging from 2.37 ~ 3.02 RU·s<sup>-1</sup>.  
255 Similarly, additions of the SARS-CoV-2 N protein led to an approximate 1.8-fold  
256 increase in the  $V_{\max}/K_m$  values, which suggested that the specificity constant  
257 ( $K_{\text{cat}}/K_m$ ) of MASP-2 to substrates is increased in the presence of the viral N  
258 protein as the enzyme concentrations are equivalent among the reactions  
259 **(Extended Data Table 7 - 8)**. To confirm the kinetic analyses, Hanes plots ( $[S]/V$   
260 versus  $[S]$ ) were also drawn and found to be linear **(Figure 4c)**. Therefore, the  
261 addition of the SARS-CoV-2 N protein does not change the single substrate  
262 binding site characterization of the enzymatic reactions. To assess the  
263 suppression ability of nCoV396 to the SARS-CoV-2 N protein-induced  
264 complement hyperactivation function, we next conducted complement  
265 hyperactivation analyses at various N protein: nCoV396 ratios. As shown in  
266 **Figure 4d**, the addition of the N protein elevates the  $V_{\max}$  value up to 40-fold (1:0  
267 ratio), whereas the addition of the antibody nCoV396 decreases the  $V_{\max}$  in a  
268 dose-dependent manner **(Extended Data Table 9)**. To further validate the  
269 function of nCoV396, we next performed complement hyperactivation analyses in  
270 five other serum samples from autoimmune disease donors. Consistently, the  
271  $V_{\max}$  of reactions was boosted in the presence of the N protein in all samples but  
272 declined in the presence of both the mAb nCoV396 and the N protein **(Figure 4e)**.  
273 In conclusion, these results demonstrate that the SARS-CoV-2 N protein is

274 capable of inducing complement hyperactivation *ex vivo*, not only by facilitating  
275 the  $V_{\max}$  of MASP-2 catalytic activity but also by enhancing the substrate binding  
276 specificity in the reactions. The N-reactive mAb nCoV396 specifically  
277 compromises the SARS-CoV-2 N protein-induced complement hyperactivation  
278 within clinical serum samples.

## 279 **Discussion**

280 From a quickly recovered COVID-19 patient, we isolated 32 mAbs specifically  
281 targeting the SARS-CoV-2 N protein. The binding affinity of mAbs ranged from 1  
282 nM to 25 nM, which is comparable with the binding affinity of mature S protein-  
283 reactive antibodies<sup>22,25-29</sup> and the other mature antibodies identified during acute  
284 infections<sup>33,34</sup>. The characteristics of the isolated N-reactive mAbs are different  
285 from those of the isolated S-reactive mAbs in COVID-19 patients during the early  
286 recovery phase, suggesting that sampling time is pivotal for identifying differential  
287 immune responses to different SARS-CoV-2 viral proteins.

288 The crystal structure of nCoV396 bound to SARS-CoV-2 N-NTD elucidates the  
289 interaction mechanism of the complex between the first reported N protein-  
290 reactive human mAb and its targeted N protein. Three conserved amino acids  
291 (Q163, L167, and K169) in the N protein are responsible for nCoV396 recognition,  
292 which provides evidence of cross-reactivity of nCoV396 to the N protein of  
293 SARS-CoV or MERS-CoV. Intriguingly, the nCoV396 binding of SARS-CoV-2 N-  
294 NTD undergoes several conformational changes, resulting in an enlargement of  
295 the N-NTD RNA binding pocket enlargement and partial unfolding of the basic

296 palm region. More importantly, this conformational change occurs in the CT tail of  
297 the N-NTD, which may alter the positioning of individual domains in the context of  
298 the full-length protein and lead to a potential allosteric effect for protein functions.

299 Complement is one of the first lines of defense in innate immunity and is  
300 essential for cellular integrity and tissue homeostasis and for modifying the  
301 adaptive immune response<sup>35</sup>. Emerging evidence suggests that the complement  
302 system plays a vital role in a subset of critical COVID-19 patients, with features of  
303 atypical acute respiratory distress syndrome, disseminated intravascular  
304 coagulation, and multiple organ failure<sup>9,10,36</sup>. A few pieces of evidence show that  
305 the N protein of highly pathogenic coronaviruses (SARS-CoV-2 and SARS-CoV)  
306 is involved in the initiation of MASP-2-dependent complement activation<sup>19,21</sup>.  
307 Encouragingly, critical COVID-19 patients treated with complement inhibitors,  
308 including small molecules to the complement component C3 and an antibody  
309 targeting the complement component C5, show remarkable therapeutic  
310 outcomes<sup>19</sup>. Currently, there are 11 clinical trials related to targeting the  
311 complement pathway (<https://clinicaltrials.gov>). To avoid adverse effects of  
312 human complement component-targeting therapy, a viral protein-specific  
313 approach is warranted. The antibody nCoV396 isolated from COVID-19  
314 convalescent patients is an excellent potential candidate with a high binding  
315 affinity to the N protein and high potency to inhibit complement hyperactivation.  
316 As revealed by atomic structural information, the binding may allosterically  
317 change the full-length N protein conformation. To determine the role of nCoV396  
318 in the suppression of complement hyperactivation, we monitored MASP-2

319 protease activity based on its specific fluorescence-quenched C2 substrate in  
320 sera from autoimmune disease patients. The complete complement components  
321 in the sera of patients with autoimmune disorders allow us to monitor the  
322 activating effects of the SARS-CoV-2 N protein and its specific mAbs. Although  
323 we cannot calculate the other steady-state enzymatic reaction constants as the  
324 precise concentration of MASP-2 in serum is unknown, we identified the  $V_{max}$  of  
325 the specific C2 substrate for the enzymatic reaction. We demonstrated that the  
326 SARS-CoV-2 N protein elevated the  $V_{max}$  of the reaction, up to 40-fold, in the  
327 sera of all 7 individuals tested, while nCoV396 effectively suppressed the  $V_{max}$  of  
328 the reaction mixtures. These results indicated that serum-based complement  
329 activation analysis of autoimmune disease patients is a virus-free and an  
330 effective method for examining complement activation mediated by the SARS-  
331 CoV-2 N protein.

332 Although the precise interaction of the SARS-CoV-2 N protein with MASP-2  
333 remains to be elucidated, our work defined the region on the SARS-CoV-2 N  
334 protein recognized by the mAb nCoV396 that plays an important role in  
335 complement hyperactivation and indicates that human mAbs from convalescents  
336 could be a promising potential therapeutic candidate for the treatment of COVID-  
337 19.

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- 437

## 438 **Methods**

### 439 **Recombinant SARS-CoV-2 S-ECD and N proteins.**

440 Recombinant SARS-CoV-2 S protein (extracellular domain of the S protein (ECD)  
441 with His and FLAG Tags, Z03481) was purchased from GenScript. SARS-CoV-2  
442 (formerly known as 2019-nCoV, recombinant full-length N protein with a CT 6x  
443 His tag (His tag, 40588-V08B) was purchased from Sino Biological. The SARS-  
444 CoV-2 N protein expression plasmid (SARS-CoV N-FL) was a gift from the  
445 Guangdong Medical Laboratory Animal Center. SARS-CoV and MERS-CoV N-  
446 FL were purchased from RuiBiotech. SARS-CoV-2 N-FL (residues 1 to 419),  
447 SARS-CoV-2 N-NTD domain (residues 41 to 174), SARS-CoV-2 N-CTD domain  
448 (residues 250 to 364), SARS-CoV N-FL and MERS-CoV N-FL were cloned into  
449 the pET-28a vector and expressed in the Rosetta *E. coli* strain. Expression of  
450 SARS-CoV-2 N-FL and variants in *E. coli* was induced with 0.1 mM isopropylthio-  
451  $\beta$ -galactoside (IPTG) and cultured overnight at 16 °C in Terrific Broth media.  
452 Expressed recombinant N proteins were initially purified by using nickel column  
453 chromatography and further purified via size-exclusion chromatography.

### 454 **PBMCs from COVID-19 patients and sorting of single plasma cells and** 455 **memory B cells by FACS**

456 Blood samples were collected 9 - 25 days after the onset of the disease from  
457 patients who had recovered from COVID-19 infection. PBMCs and plasma were  
458 isolated from blood samples by Ficoll-Paque PLUS (GE, 17-1440-02) density  
459 gradient centrifugation. All work related to human subjects was carried out in

460 compliance with Institutional Review Board protocols approved by the  
461 Institutional Review Board of the Fifth Affiliated Hospital of Sun Yat-sen  
462 University. Single plasma cells with the surface markers CD3<sup>-</sup>, CD14<sup>-</sup>, CD16<sup>-</sup>,  
463 CD235a<sup>-</sup>, CD19<sup>+</sup>, CD20<sup>low-neg</sup>, CD27<sup>hi</sup> and CD38<sup>hi</sup> and memory B cells with the  
464 surface markers CD3<sup>-</sup>, CD14<sup>-</sup>, CD16<sup>-</sup>, CD235a<sup>-</sup>, CD20<sup>-</sup>, CD19<sup>+</sup>, CD27<sup>+</sup>, SARS-  
465 CoV-2 S<sup>+</sup> and SARS-CoV-2 N<sup>+</sup> (BD Biosciences and Invitrogen) were sorted into  
466 individual wells in 96-well microtiter plates containing cell lysis and RT buffer for  
467 Ig gene amplification by fluorescence activated cell sorting (FACS) as previously  
468 described<sup>37</sup> on a BD FACS Aria SORP. Data were analyzed using BD FACS  
469 Diva 8.0.1 software.

#### 470 **Isolation and expression of Ig V<sub>H</sub> and V<sub>L</sub> genes**

471 Genes encoding V<sub>H</sub> and V<sub>L</sub> were amplified by reverse transcription (RT) and  
472 nested primer chain reaction (PCR) and nested PCR using the method  
473 previously described<sup>38</sup>. PCR products of Ig V<sub>H</sub> and V<sub>L</sub> genes were purified using  
474 a PCR purification kit (QIAGEN), sequenced in forward and reverse directions  
475 (Thermo Fisher scientific) and annotated by using IMGT/V-QUEST  
476 ([www.imgt.org/IMGT\\_vquest](http://www.imgt.org/IMGT_vquest)). Functional V<sub>H</sub> and V<sub>L</sub> genes were used for  
477 assembling full-length Ig heavy and light chain linear expression cassettes by  
478 one-step overlapping PCR<sup>38</sup>. HEK-293T cells in 12-well plates were transfected  
479 with the assembled Ig heavy and light chain pairs derived from the same single  
480 individual plasma cells using Effectene (QIAGEN) as the transfection reagent<sup>38</sup>.

#### 481 **Production of recombinant IgG and Fab antibodies**

482 For the production of purified full-length IgG1 antibodies, the V<sub>H</sub> and V<sub>L</sub> genes  
483 were cloned into the pCDNA3.1<sup>+</sup> (Invitrogen)mammalian expression vector  
484 containing either the human IgG1 constant region gene, the human kappa light  
485 chain constant region gene or the lambda light chain constant region gene using  
486 standard recombinant DNA technology<sup>38</sup>. For the production of the purified  
487 nCoV396Fab antibody, a stop codon TGA was introduced after the sequence (5'-  
488 TCTTGTGACAAA-3'), which encodes the amino acid residues SCDK, just before  
489 the hinge of the human IgG1 constant region<sup>39</sup>. Recombinant IgG1 antibodies  
490 and the nCoV396Fab antibody were produced in 293F cells cultured in serum-  
491 free medium by cotransfection with the generated IgG1 full-length or Fab heavy-  
492 and light chain gene expression plasmid pairs using polyethylenimine<sup>40</sup>. Full-  
493 length IgG1 antibodies were purified by using Protein A column chromatography  
494 as described previously<sup>38</sup>. The nCoV396Fab antibody used for the crystal  
495 structure was purified by Lambda FabSelect, an affinity resin designed for the  
496 purification of human Fab with a lambda light chain (GE Healthcare)<sup>39</sup>.

#### 497 **Analysis of the binding of plasma antibodies and isolated mAbs to the S** 498 **and N proteins by ELISA**

499 We collected plasma from 6 patients and measured serum antibody titers using  
500 recombinant SARS-CoV-2 S and N proteins as antigens to coat ELISA plates.  
501 Antibodies in the supernatant of the transfected 293T cultures harvested 3 days  
502 after transfection were screened by ELISA as described previously<sup>38</sup>. The binding  
503 of purified antibodies to the N or S protein was also analyzed by ELISA. Briefly,  
504 all protein antigens (S, N-FL, N-NTD (41-174), and N-CTD (250-364)) were used

505 at 200 ng/well to coat 96-well high-binding ELISA plates (Nunc 442404) using  
506 carbonate-bicarbonate buffer at pH 9.6. Plates were incubated overnight at 4 °C  
507 and blocked at room temperature for 2 h with PBS blocking buffer containing 5%  
508 w/v goat serum and 0.05% Tween-20. Plasma or supernatant of transfected  
509 293T cell cultures or purified mAbs in serial dilutions in PBS were incubated at  
510 37 °C for 1 h. Goat anti-human IgG-horseradish peroxidase (HRP, 1:10,000  
511 dilution) (Promega, W4031) as the secondary antibody diluted in blocking buffer  
512 was added and incubated at 37 °C for 1 h. These plates were then washed 5  
513 times with PBS and developed with 100 µL of 3,3',5,5'-Tetramethylbenzidine  
514 (TMB) substrate/well (Solarbio PR1200). The reaction of the plates was stopped  
515 with 50 µL of 2 M H<sub>2</sub>SO<sub>4</sub>/well and read at a wavelength of 450 nm by an ELISA  
516 reader. The relative affinity of mAbs to the N protein antigen was determined as  
517 the effective concentration (EC<sub>50</sub>) of the antibody resulting in half maximal  
518 binding to the antigen by curve fitting with GraphPad Prism software.

### 519 **Affinity and kinetic measurements by SPR**

520 The binding affinity ( $K_D$ ), association rate ( $k_a$ ) and dissociation rate ( $k_d$ ) of purified  
521 mAbs to the N protein were determined by SPR using a Biacore X100 System.  
522 Anti-human Fc IgG antibody was first immobilized on a CM5-chip to  
523 approximately 6,000 RU by covalent amine coupling using a human antibody  
524 capture kit (GE Healthcare). Purified mAbs were captured on channel 2 of the  
525 CM5 chip to approximately 200 RU. Five 2-fold serial dilutions of the N protein  
526 starting at 40 µg/ml, 20 µg/ml, 10 µg/ml or 4 µg/ml were injected at a rate of 30  
527 µL/min for 90 s with a 600 s dissociation. The chip was regenerated by injection

528 of 3 M MgCl<sub>2</sub> for 30 s. All experiments were performed at room temperature, and  
529 data were analyzed using Biacore X100 Evaluation Software (version: 2.0.1).  
530 Curves were fitted to a 1:1 binding model to determine the kinetic rate constants  
531 ( $k_a$  and  $k_d$ ).  $K_D$  values were calculated from these rate constants.

### 532 **Crystallization and data collection**

533 SARS-CoV-2 N-NTD (41–174) was cloned into the pRSF-Duet-1 vector with an  
534 NT 6x His-SUMO tag, recombinantly expressed in *E. coli* and purified as an NT  
535 6x His-Sumo-tagged protein. After Ni column chromatography followed by ulp1  
536 digestion for tag removal, the SARS-CoV-2 N-NTD (41–174) protein was further  
537 purified via size-exclusion chromatography. Prior to crystallization, the SARS-  
538 CoV-2 N-NTD (41–174) sample was mixed with nCoV396Fab at a 1:1.5 molar  
539 ratio for approximately half an hour and then further purified via size-exclusion  
540 chromatography. Crystals were grown by the sitting drop method using a  
541 Mosquito LCP crystallization robot with 0.3  $\mu$ L of protein (6 mg/ml) mixed with 0.3  
542  $\mu$ L of well solution at 16 °C. Better crystals were obtained in 0.01 M calcium  
543 chloride dihydrate, 0.05 M sodium cacodylate trihydrate (pH 7.2), 1.675 M  
544 ammonium sulfate and 0.5 mM spermine. The crystals were harvested after 3  
545 days. Crystals were frozen in liquid nitrogen in the reservoir solution  
546 supplemented with 25% glycerol (v/v) as a cryoprotectant. X-ray diffraction data  
547 were collected at the Shanghai Synchrotron Radiation Facility BL18U at a  
548 wavelength of 0.979 Å and a temperature of 100 K. The complex structure of  
549 SARS-CoV-2 N-NTD with the mAb nCoV396 was determined by Phenix  
550 molecular replacement using the SARS-CoV-2 N-NTD structure (PDB ID: 6M3M)

551 and the monoclonal antibody omalizumab Fab (PDB ID: 6TCN) as the search  
552 models. The X-ray diffraction and structure refinement statistics are summarized  
553 in Extended Data Table 4. The final Ramachandran statistics are 97.1% favored,  
554 2.9% allowed and 0.0% outliers.

### 555 **Fluorescence-quenched substrate assays**

556 The fluorescence-quenched substrate (FQS) [C2 P4–P4' (2Abz-SLGRKIQL-  
557 Lys(Dnp)-NH<sub>2</sub>)] was synthesized and purified in greater than 90% purity by  
558 Sangon Biotech (Shanghai, China). The FQS was solubilized in 50% (v/v)  
559 dimethylformamide (DMF). Assays were carried out in fluorescence assay buffer  
560 (FAB) (0.05 M Tris–HCl, 0.15 M NaCl, 0.2% (w/v) PEG 8,000, and pH 7.4) at  
561 37 °C using final substrate concentrations in the range of 2.8125–90 μM. A range  
562 of substrate dilutions, performed in triplicate, were prepared in FAB and were  
563 added to the wells at 100 μl/well in the assay plates. The plates were incubated  
564 at 37 °C for 10 minutes. Five microliters of serum from autoimmune patients was  
565 added to the mixture of the SARS-CoV-2 N protein with nCoV396 at a 1:1.2  
566 molar ratio in the assay plates. Fluorescence intensity was measured on an  
567 EnVision 2015 Multimode plate reader (PerkinElmer) at an excitation wavelength  
568 of 320 nm and an emission wavelength of 420 nm for the FQS. The initial  
569 reaction rate was estimated at a single concentration of enzyme from duplicate  
570 measurements over a range of substrate concentrations. To determine steady-  
571 state reaction constants ( $V_{max}$ , half saturation constant ( $K_{0.5}$ ) and Hill  
572 coefficient( $h$ )), the experimental results were fitted using GraphPad Prism  
573 Version 8.0 (GraphPad Software, San Diego, CA) to an equation describing

574 positive cooperativity:  $Y = V_{max} \times \frac{X^h}{K_{0.5^h} + X^h}$ , where 0.5 defines the relationship  
575 between the reaction rate (V) and the substrate concentration ([S]) when more  
576 than one binding site applies.

## 577 **Methods references**

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## 601 **Author Contributions**

602 S. C., H. S., F. X. and H-X. L. contributed the conception of the study and  
603 established the construction of the article. S. C. and H-X. L. designed the  
604 experiments and wrote the manuscript. S. K., M. Y., S. H. contributed to protein  
605 purification and crystallization, *in vitro* protein-protein interaction analysis, and  
606 complement activation analysis. Y. W. contributed to mAbs isolation, *in vitro*  
607 protein-protein interaction analysis. S. C., S. K. M. Y., and S. H. performed  
608 structural determination and validation. S. C., S. K., Y. W. drew figures. X. C., Y.  
609 C., Q. C., Z. Z., Z. Z., Z. H., X. H., H. S., W. Z., and H. H. contributed to

610 interpretation of data. Z. H., J. L., G. J., and F. X. contributed to clinical samples  
611 collections. S.K., M.Y., S. H., Y.W. contributed equally to this work.

## 612 **Conflict of Interest**

613 The authors declare no conflict of interest.

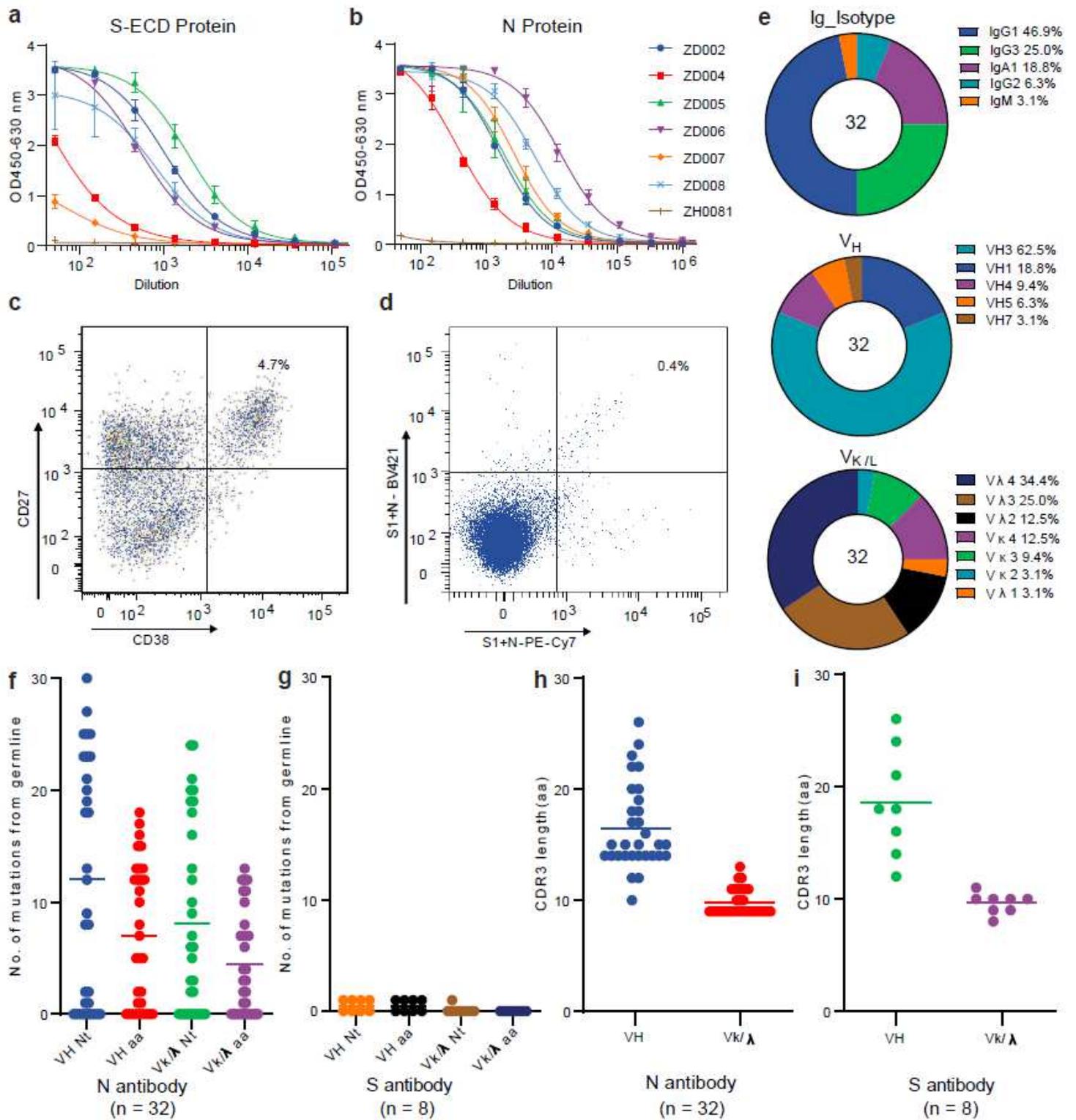
## 614 **Data availability statement**

615 The structure in this paper is deposited to the Protein Data Bank with 7CR5  
616 access code.

## 617 **Additional Information**

618 Correspondence and requests for materials should be addressed to Shoudeng  
619 Chen ([chenshd5@mail.sysu.edu.cn](mailto:chenshd5@mail.sysu.edu.cn)) and/or Hua-Xin Liao ([tliao805@jnu.edu.cn](mailto:tliao805@jnu.edu.cn)).

# Figures



**Figure 1**

Acquisition and characterization of antibodies. Serum antibody titers of six SARS-CoV-2 convalescent patients to the SARS-CoV-2 S (a) and N (b) proteins measured by ELISA. Sorting of single plasma cells (c) with CD38 and CD27 double-positive B cells and single N and S proteinspecific memory B cells (d) by

FACS. (e) Percentage of different isotypes, VH and VL gene families of 32 isolated N-reactive antibodies. (f) Number of mutations in nucleotides and amino acids in VH and VL ( $V_k$  and  $V_\lambda$ ) of 32 N-reactive antibodies and eight S-reactive antibodies (g). Length of the 32 Nreactive antibodies (h) and eight S-reactive antibodies (i) in H-CDR3.

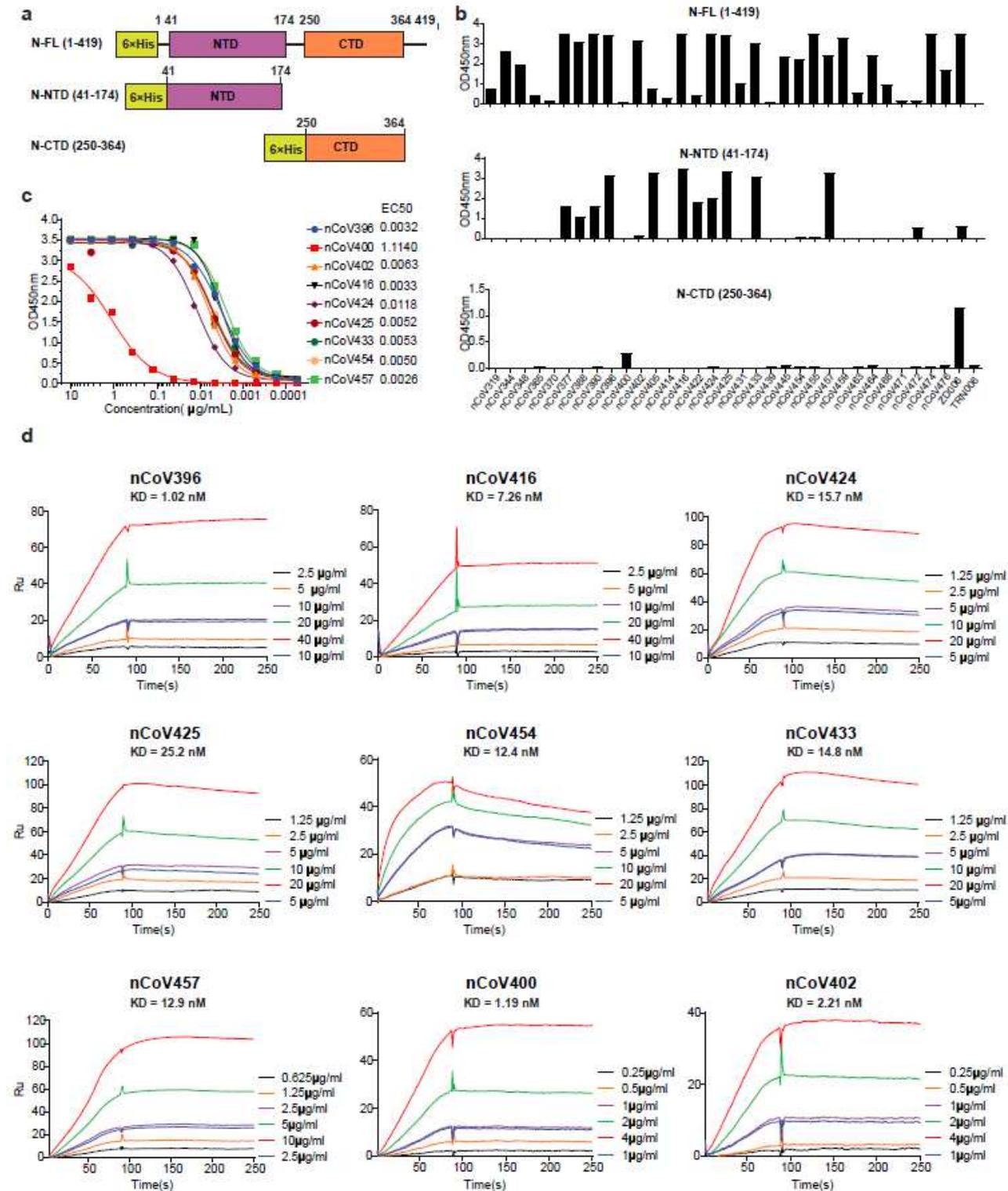


Figure 2

Reactivity and affinity of the isolated antibodies to the N protein antigens. (a) Schematic presentation of the SARS-CoV-2 N protein and two variants. (b) Antibodies expressed in transfected 293 cells were evaluated for binding to N-FL, N-NTD and N-CTD by ELISA. Plasma from patient ZD006 and an irrelevant mAb TRN006 were used as the positive control and negative control, respectively. (c) The ability of nine purified antibodies to the N-FL protein was determined by ELISA. (d) Binding affinity of nine selected antibodies to the N protein was measured by SPR. KD values are shown above the individual plots.

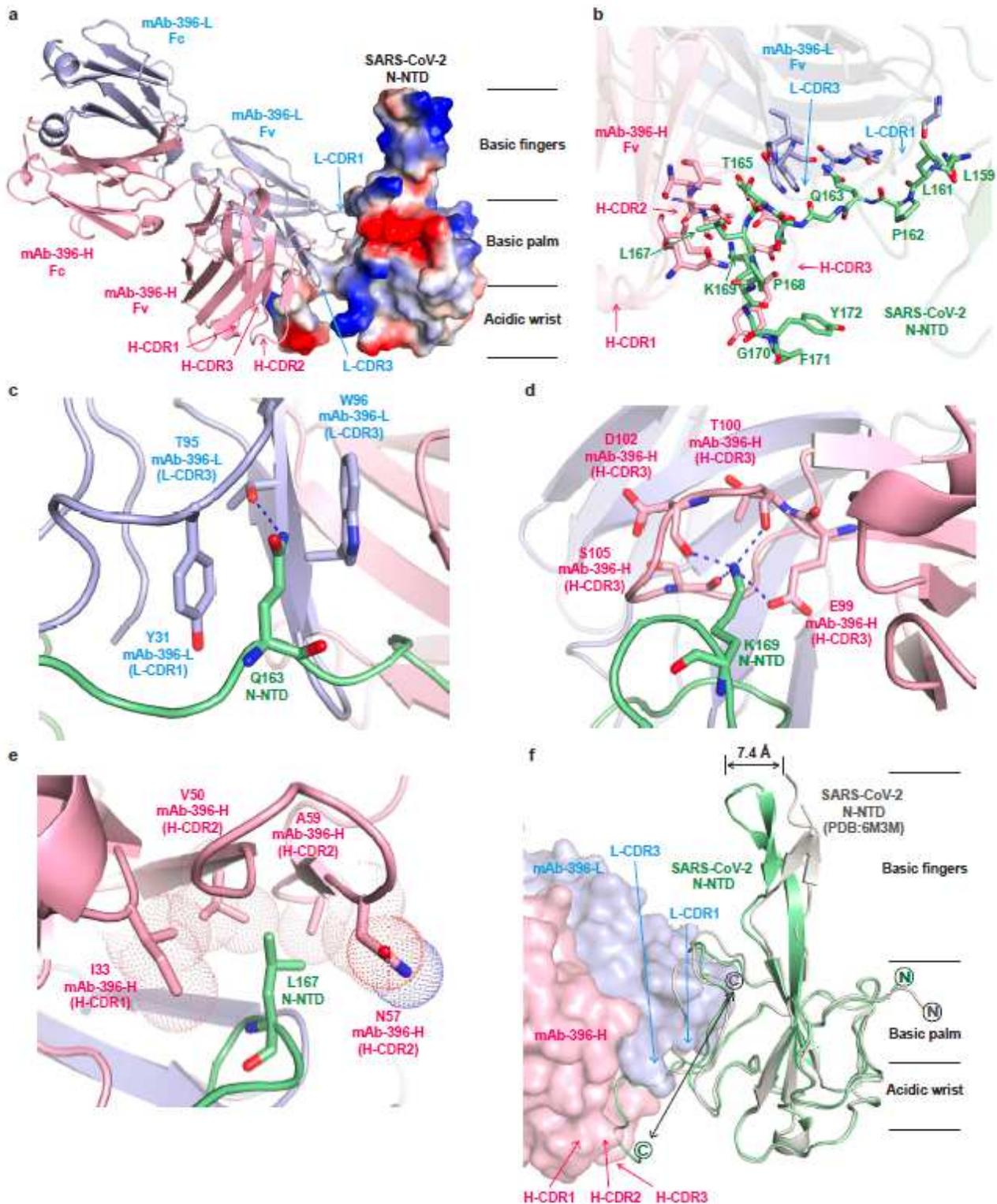


Figure 3

Complex structure of mAb nCoV396 with SARS-CoV-2 N-NTD(a) Overall structure of the mAb SARS-CoV-2 N-NTD complex. The light chain (pink) and heavy chain (blue) of mAb nCoV396 are illustrated with the ribbon representation. SARS-CoV-2 N-NTD is illustrated with an electrostatic surface, in which blue denotes a positive charge potential, while red indicates a negative charge potential. (b) The N-NTD epitope recognized by mAb nCoV396. The interacting residues of N-NTD and nCoV396 are highlighted with the stick representation. Recognition of Q163 (c), K169 (d) and L167 (e) in N-NTD by mAb nCoV396. The dashed blue line represents hydrogen bonds. Hydrophobic interactions are illustrated with the dot representation. (f) Conformational changes in N-NTD upon mAb nCoV396 binding. The apo structure of N-NTD is colored gray. Antibody-bound N-NTD is colored green. The N-terminus and C-terminus of the N-NTD are labeled with circles. mAb nCoV396 is illustrated with surface representation. All figures were prepared by PyMol.

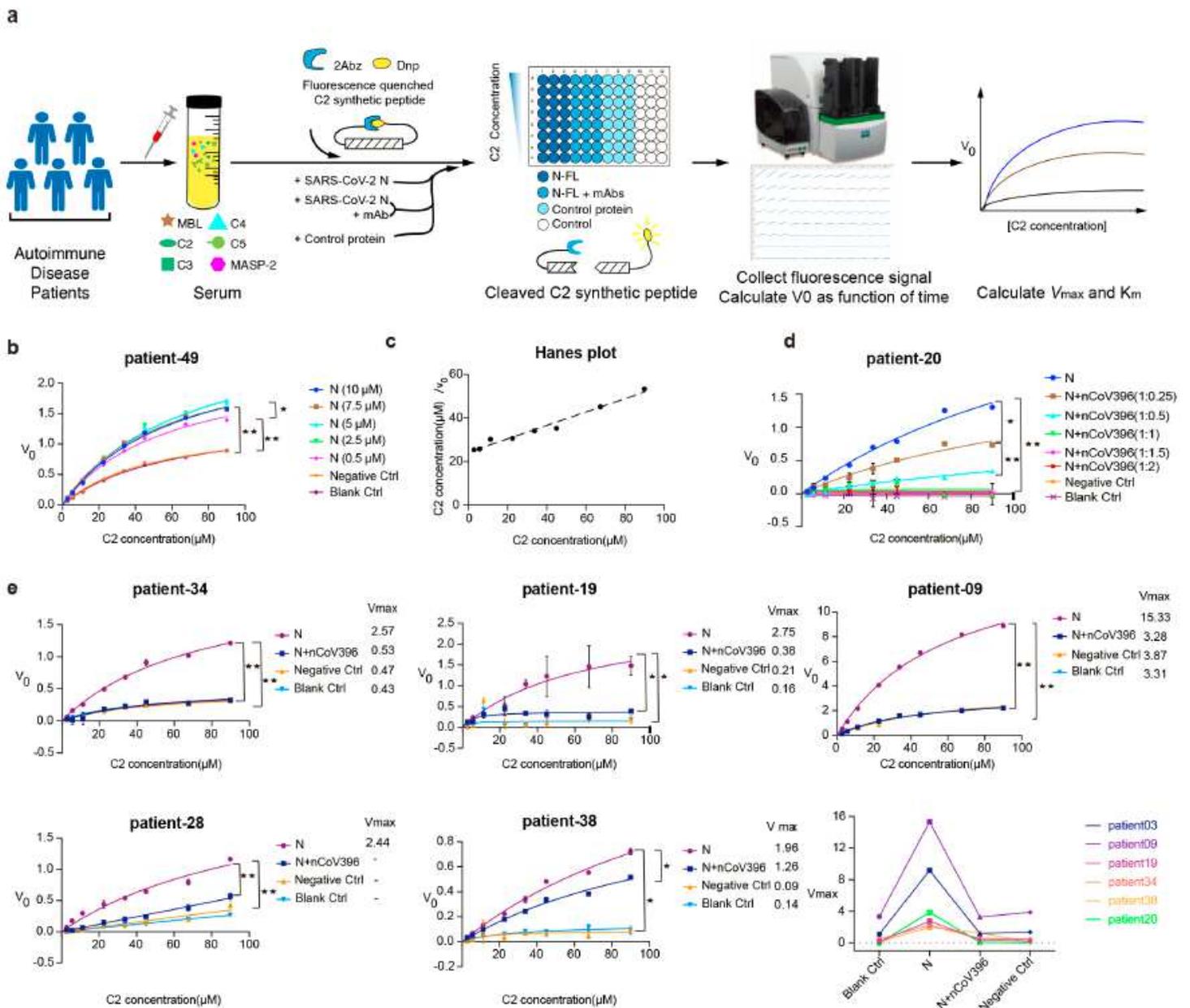


Figure 4

Antibody nCoV396 compromises SARS-CoV-2 N protein-induced complement hyperactivation. (a) Flow scheme of the SARS-CoV-2 N protein and nCoV396 influencing the protease activity of MASP-2 in the serum of autoimmune disease patients. The Michaelis-Menten curve shows the effect of increasing the N protein concentration (b) and antibody concentration (d) on the substrate C2 cleavage of MASP-2 in the serum of patient 49 and patient 20. (c) A Hanes plot where C2 concentrationN0 is plotted against C2 concentration with the addition of 5  $\mu$ M N protein. (e) The mAb nCoV396 inhibits the N protein-induced excessive cleavage of C2 in the serum of six autoimmune disease patients, and the last panel shows a summary of Vmax for all patients. Negative control (Negative Ctrl) and blank control (Blank Ctrl) represent reactions containing bovine serum albumin (BSA) instead of N or N+mAb and without exogenous protein, respectively. The mean and standard deviation (SD) values of three technical replicates are shown. P values: \*P < 0.05; \*\*P < 0.01; "-" indicates that the experimental kinetics did not conform to Michaelis-Menten kinetics.

## Supplementary Files

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