

1 **Ultrapotent miniproteins targeting the receptor-binding domain protect against**
2 **SARS-CoV-2 infection and disease in mice**

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27

28 **SUMMARY**

29 Despite the introduction of public health measures and spike protein-based vaccines to
30 mitigate the COVID-19 pandemic, SARS-CoV-2 infections and deaths continue to rise.
31 Previously, we used a structural design approach to develop picomolar range miniproteins
32 targeting the SARS-CoV-2 receptor binding domain. Here, we investigated the capacity of
33 modified versions of one lead binder, LCB1, to protect against SARS-CoV-2-mediated lung
34 disease in human ACE2-expressing transgenic mice. Systemic administration of LCB1-Fc
35 reduced viral burden, diminished immune cell infiltration and inflammation, and completely
36 prevented lung disease and pathology. A single intranasal dose of LCB1v1.3 reduced SARS-
37 CoV-2 infection in the lung even when given as many as five days before or two days after virus
38 inoculation. Importantly, LCB1v1.3 protected *in vivo* against a historical strain (WA1/2020), an
39 emerging B.1.1.7 strain, and a strain encoding key E484K and N501Y spike protein
40 substitutions. These data support development of LCB1v1.3 for prevention or treatment of
41 SARS-CoV-2 infection.

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44 INTRODUCTION

45 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the cause of the
46 Coronavirus Disease 2019 (COVID-19) pandemic, has resulted in global disease, suffering, and
47 economic hardship. Despite implementation of public health measures, SARS-CoV-2
48 transmission persists principally through human-to-human spread (Day, 2020; Li et al., 2020;
49 Standl et al., 2020). SARS-CoV-2-induced clinical manifestations range from asymptomatic
50 infection to severe pneumonia, multi-organ failure, and death. Although the underlying
51 mechanisms that dictate disease severity are poorly understood, the immunocompromised, the
52 elderly, and those with specific comorbidities (*e.g.*, history of cardiovascular disease, diabetes,
53 or obesity) are at increased risk for poor outcome (Zhou et al., 2020).

54 SARS-CoV-2 entry into target cells is facilitated by the spike glycoprotein through
55 binding to its principal receptor, angiotensin converting enzyme 2 (ACE2) (Hoffmann et al.,
56 2020; Letko et al., 2020). Once the virus is attached to the cell-surface, the spike protein is
57 cleaved by the cell membrane-associated protease, TMPRSS2, resulting in membrane fusion
58 and release of the viral RNA genome into the host cell cytoplasm (Hoffmann et al., 2020;
59 Matsuyama et al., 2020). As the dominant antigen on the surface of the virion, the spike protein
60 is the primary target of antibody-based countermeasures (Jeyanathan et al., 2020; Krammer,
61 2020). At present, a small number of antibody therapies and vaccines have been granted
62 emergency use authorization (EUA) by the United States Food and Drug Administration to
63 prevent or treat SARS-CoV-2 infection and disease. Nonetheless, viral evolution and the
64 emergence of SARS-CoV-2 variants in the United Kingdom (B.1.1.7), South Africa (B.1.351),
65 Brazil (B.1.1.248), and elsewhere jeopardize these countermeasures through potential loss-of-
66 binding and diminished neutralization (Galloway et al., 2021; Leung et al., 2021; Tegally et al.,
67 2020; Voloch et al., 2020).

68 We recently generated a panel of short, 56-amino acid miniproteins that bind the SARS-
69 CoV-2 receptor-binding domain (RBD) with high affinity and potently neutralize authentic virus in

70 cell culture with half-maximal effective concentration (EC_{50}) values < 30 pM (Cao et al., 2020).
71 Here, using a stringent model of SARS-CoV-2 disease pathogenesis in human ACE2 (hACE2)-
72 expressing transgenic mice (Golden et al., 2020; Winkler et al., 2020a), we evaluated the
73 efficacy *in vivo* of one of these miniprotein binders, LCB1. For our *in vivo* experiments, we
74 evaluated two versions of LCB1: (a) an Fc-modified bivalent form, LCB1-hIgG-Fc9 (LCB1-Fc)
75 that should extend half-life *in vivo* and engage effector arms of the immune system; and (b) a
76 further optimized, monomeric form of LCB1 lacking an Fc domain, LCB1v1.3. Intraperitoneal
77 administration of LCB1-Fc at one day pre- or post SARS-CoV-2 exposure conferred substantial
78 protection including an absence of weight loss, reductions in viral burden approaching the limit
79 of detection, and inhibition of lung inflammation and pathology. Intranasal delivery of LCB1v1.3
80 conferred protection as many as five days before or two days after SARS-CoV-2 inoculation.
81 Dosing experiments revealed that LCB1v1.3 retained efficacy at pharmacologically attainable
82 concentrations and was weakly immunogenic. Most importantly, LCB1v1.3 protected animals
83 against the currently emerging B.1.1.7 United Kingdom variant and a SARS-CoV-2 strain
84 encoding key spike substitutions E484K and N501Y present in both the South Africa (B.1.351)
85 and Brazil (B.1.1.248) variants of concern. Overall, these studies establish LCB1-Fc and
86 LCB1v1.3 as possible treatments to prevent or mitigate SARS-CoV-2 disease.

87

88 RESULTS

89 **LCB1v1.3 prophylaxis limits viral burden and clinical disease.** Using computational
90 design and functional screens, we previously identified LCB1 as a potent miniprotein inhibitor of
91 SARS-CoV-2 infection (Cao et al., 2020). We modified LCB1 to generate two versions for *in vivo*
92 testing: (a) we introduced polar mutations into LCB1 to increase expression yield and solubility
93 without altering RBD binding (LCB1v1.3) and (b) we modified LCB1 by fusing it to a human
94 IgG1 Fc domain (LCB1-Fc) to enhance bioavailability. LCB1v1.3 and LCB1-Fc bound avidly to a
95 single RBD within the S trimer (**Fig 1A**) with dissociation constants (K_D) of less than 625 and
96 156 pM, respectively (**Fig 1B**). LCB1v1.3 and LCB1-Fc also potently neutralized an authentic
97 SARS-CoV-2 isolate (2019n-CoV/USA_WA1/2020 [WA1/2020]) (EC_{50} of 14.4 and 71.8 pM,
98 respectively; **Fig 1C**).

99 To determine the protective potential of these miniproteins against SARS-CoV-2, we
100 utilized K18 human hACE2-expressing transgenic mice, which develop severe lung infection
101 and disease after intranasal inoculation of SARS-CoV-2 (Golden et al., 2020; Winkler et al.,
102 2020a). In prophylaxis studies, a single 250 μ g (10 mg/kg) dose of LCB1-Fc administered by
103 intraperitoneal injection (i.p.) one day prior to intranasal (i.n.) inoculation with 10^3 PFU of SARS-
104 CoV-2 WA1/2020 prevented weight loss compared to animals given a control protein (influenza
105 A virus hemagglutinin minibinder) designed using similar computational methods (**Fig 1D**). After
106 LCB1-Fc prophylaxis, infectious virus was not detected in the lungs at 4- or 7-days post-
107 infection (dpi), whereas high levels were observed in animals administered control protein (**Fig**
108 **1E, top and bottom**). Similarly, viral RNA levels in the lung, heart, spleen, and brain of LCB1-Fc
109 treated animals were at or near the limit of detection of the assay at 4 or 7 dpi (**Fig 1F-I**). LCB1-
110 Fc treatment had no effect on viral RNA levels in nasal wash samples obtained at 4 dpi (**Fig 1J**),
111 results that are similar to a recent study of a neutralizing human antibody in hamsters (Zhou et
112 al., 2021). However, viral RNA levels were reduced at 7 dpi, suggesting that LCB1-Fc treatment
113 accelerated viral clearance or prevented spread in the upper respiratory tract.

114 Diffuse alveolar damage, inflammation, and pneumonia are manifestations of COVID-19
115 lung disease, culminating in respiratory failure and a requirement for mechanical ventilation
116 (Johnson et al., 2020; Kordzadeh-Kermani et al., 2020). We evaluated the capacity of LCB1-Fc
117 to prevent the compromised lung function seen after SARS-CoV-2 infection of K18-hACE2 mice
118 (Winkler et al., 2020a). At 7 dpi, mechanical ventilation tests of lung biomechanics in animals
119 treated with LCB1-Fc showed no difference from naïve animals (**Fig 2A**), whereas mice
120 receiving the control binder protein showed decreased inspiratory capacity and lung compliance
121 as well as increased pulmonary resistance, elastance, and tissue damping, all consistent with
122 compromised lung function. These biophysical properties resulted in disparate pressure-volume
123 loops between control binder and LCB1-Fc treated or naïve animals. We also assessed the
124 effect of LCB1-Fc treatment on SARS-CoV-2-induced lung pathology. Lung sections of animals
125 collected at 7 dpi with SARS-CoV-2 showed widespread inflammation characterized by a
126 cellular infiltrate and airspace consolidation in control protein-treated but not LCB1-Fc treated or
127 naïve mice (**Fig 2B**). At 4 dpi, inflammatory cytokine and chemokine RNA signatures in the lung
128 were absent in LCB1-Fc treated but not control binder treated animals, suggesting that LCB1-Fc
129 treatment prevents virus infection and inflammation in the lung (**Fig 2C and S1**).

130 **Post-exposure therapy with anti-RBD binders reduces viral burden.** To evaluate its
131 efficacy in a post-exposure setting, we administered LCB1-Fc by i.p. injection at 1 dpi. Therapy
132 with LCB1-Fc prevented weight loss (**Fig 3A**) and reduced viral burden in all tested tissues at 4
133 and 7 dpi (**Fig 3B-G**). Infectious virus was not recovered from the lungs of LCB1-Fc treated
134 animals collected at either timepoint. Lung sections confirmed that therapy with LCB1-Fc
135 improved pathological outcome (**Fig 3H**). At 7 dpi, immune cell infiltrates were absent in the
136 lung sections of LCB1-Fc treated but not control binder-treated animals.

137 We next tested the efficacy of LCB1v1.3 as an i.n.-delivered post-exposure therapy. I.n.
138 delivery, might enable self-administration of an anti-SARS-CoV-2 biological drug. Indeed,
139 miniprotein inhibitors against influenza virus have shown efficacy as a nasal mist (Chevalier et

140 al., 2017). For these studies, we used LCB1v1.3 because it can bind an increased number of
141 RBD molecules for a given mass dose, resulting in increased neutralization activity (**Fig 1C**).
142 Whereas high levels of SARS-CoV-2 RNA were detected in the lungs and other peripheral
143 tissues of control binder-treated animals at 7 dpi, infection was reduced in animals receiving
144 LCB1v1.3 by i.n. administration at D+1 or D+2 after inoculation with SARS-CoV-2 (**Fig 3I and**
145 **S2**). Levels of viral RNA were reduced in the nasal washes of animals receiving LCB1v1.3 after
146 treatment at D+1 but not D+2 compared to control binder-treated animals (**Fig 3J**).

147 **Intranasal delivery of LCB1v1.3 confers protection against SARS-CoV-2 when**
148 **administered up to 5 days before infection.** We next evaluated the durability of LCB1v1.3
149 administered via i.n. prophylaxis. At 5 days, 3 days, 1 day, or 6 hours prior to inoculation with
150 10^3 PFU of SARS-CoV-2, K18-hACE2 transgenic mice received a single 50 μ g i.n. dose of
151 LCB1v1.3 or the control binder. At 4 or 7 dpi, viral burden in tissues was determined by RT-
152 qPCR. As expected, protection by LCB1v1.3 was better when administered closer to the time of
153 SARS-CoV-2 exposure, as reflected by greater reductions in viral load and weight loss (**Fig 4A-**
154 **D and S3**). However, even mice receiving LCB1v1.3 five days prior to inoculation and collected
155 at 7 dpi showed reduced viral RNA levels in the lung compared to control binder treated
156 animals. Regardless of the collection timepoint, lung viral RNA levels were reduced in animals
157 receiving LCB1v1.3 three days prior to inoculation with SARS-CoV-2.

158 An important consideration for our binders as a potential therapy is scalable production
159 and feasible dosing. To begin to address this issue, we tested a range of i.n. doses LCB1v1.3
160 for efficacy (**Fig 4E-J**). Treatment with as little as 2 μ g (0.1 mg/kg) of LCB1v1.3 prevented
161 SARS-CoV-2-induced weight loss. Doses between 2 and 10 μ g (0.1 to 0.5 mg/kg) of LCB1v1.3
162 reduced viral RNA levels in the lung, heart, and spleen at 7 dpi relative to control binder-treated
163 animals. Moreover, animals receiving a 50 μ g dose of LCB1v1.3 showed minimal, if any, lung
164 inflammation (**Fig 4K**). Collectively, these results indicate that even low doses of LCB1v1.3,

165 when administered via an i.n. route prior to exposure, can limit SARS-CoV-2 infection and
166 disease in the stringent K18-hACE transgenic mouse model of pathogenesis.

167 **LCB1v1.3 is weakly immunogenic and retains protective activity after repeated**
168 **dosing.** One concern for biological drugs is their potential immunogenicity, which could limit
169 bioavailability and efficacy. To begin to address this concern, we treated K18-hACE2 transgenic
170 mice with 50 µg of control binder or LCB1v1.3 every three days for a total of 18 days (**Fig 5A**).
171 At this time, we collected sera and assessed the presence of anti-LCB1v1.3 antibodies. Only 1
172 of 10 mice developed IgG antibodies against LCB1v1.3 (**Fig 5B**). To determine if repeated
173 dosing affected LCB1v1.3-mediated protection, we challenged the cohort with 10³ PFU of
174 SARS-CoV-2. Again, substantial protection against weight loss (**Fig 5C**) and viral infection in the
175 lung and other organs was observed in all animals receiving LCB1v1.3 (**Fig 5D-H**).

176 **LCB1v1.3 protects against emerging SARS-CoV-2 variants.** The emergence of
177 variant strains harboring possible escape mutations is of great concern for antibody-based
178 countermeasures that were designed against historical SARS-CoV-2 spike proteins (Chen et al.,
179 2021; Wang et al., 2021a; Wang et al., 2021b; Wibmer et al., 2021). Accordingly, we evaluated
180 the activity of LCB1v1.3 against a B.1.1.7 isolate containing deletions at 69-70 and 144-145,
181 and substitutions at N501Y, A570D, D614G, and P681H, and against a recombinant WA1/2020
182 strain containing key substitutions present in the B.1.351 and B.1.248 variant strains at residues
183 E484K, N501Y, and D614G (Xie et al., 2021a). Although the neutralizing activity of LCB1v1.3
184 against the B.1.1.7 and E484K/N501Y/D614G strains was approximately 45 to 50-fold lower
185 than for the WA1/2020 strain, the EC₅₀ values still were ~800 pM and 667 pM, respectively (**Fig**
186 **6A**). To determine whether LCB1v1.3 could protect *in vivo* against SARS-CoV-2 strains with
187 concerning spike protein substitutions, we treated K18-hACE2 transgenic mice with a single i.n.
188 50 µg dose of LCB1v1.3 or control binder one day prior to inoculation with 10³ PFU of B.1.1.7 or
189 E484K/N501/D614G SARS-CoV-2. Notably, LCB1v1.3 treatment before challenge with either

190 variant strain protected against weight loss (**Fig 6B and 6H**) and viral infection in all tissues
191 collected at 6 dpi (**Fig 6C-G and 6I-M**). Thus, LCB1v1.3 is effective against both circulating and
192 emerging strains of SARS-CoV-2.
193

194 **DISCUSSION**

195 Here, using the stringent K18-hACE2 mouse model of SARS-CoV-2 pathogenesis, we
196 show that LCB1-Fc, an Fc-containing version of a previously reported SARS-CoV-2 RBD
197 binding miniprotein, LCB1 (Cao et al., 2020), prevented SARS-CoV-2 infection and disease
198 when administered one day before or after virus inoculation. Lung biomechanics of mice treated
199 with LCB1-Fc mirrored those of naïve animals in all parameters tested. Although formal
200 pharmacokinetics studies are needed to establish half-life and bioavailability of our miniprotein,
201 we expect the addition of the Fc moiety improves systemic levels. While our protection studies
202 with LCB1v1.3, which lacks an Fc domain, suggest that our miniprotein binders confer
203 protection through their neutralizing activity, engagement of complement and FcγRs could
204 augment the therapeutic activity of LCB1-Fc, as described recently with anti-SARS-CoV-2
205 monoclonal antibodies (Schafer et al., 2021; Winkler et al., 2020b). These functions could be
206 explored using loss-of-Fc function (e.g., LALA-PG) variants of LCB1-Fc. Depending on the
207 outcome, Fc effector functions could be optimized further through glycan modification or Fc
208 mutation (Kang and Jung, 2019).

209 We also evaluated the efficacy of LCB1v1.3, an optimized, monomeric form of LCB1
210 without an Fc domain. A single i.n. dose of LCB1v1.3 reduced viral burden when administered
211 as many as five days before or two days after SARS-CoV-2 infection. While several antibody-
212 based intravenous therapies have been developed against SARS-CoV-2, our i.n. delivery
213 approach is unique. I.n. therapy of SARS-CoV-2 has been reported only with type I interferon in
214 a hamster model of disease (Hoagland et al., 2021) and efficacy was limited. The K18-hACE2
215 mouse model recapitulates several aspects of severe COVID-19, including lung inflammation
216 and reduced pulmonary function (Golden et al., 2020; Winkler et al., 2020a). Importantly,
217 LCB1v1.3 binder treatment before or after infection limited immune cell infiltration and lung
218 inflammation, which prevented tissue damage and compromise of respiratory function. Since
219 K18-hACE2 mice are highly vulnerable to infection, the therapeutic window of treatment is

220 limited (Winkler et al., 2020b). While we observed reductions in lung viral burden from a single
221 i.n. dose of LCB1v1.3 given two days after virus inoculation, it will be important to improve upon
222 this result. Possible ways to achieve this include higher dosing, repeated dosing, or extended
223 half-life engineering. As part of our proof-of-principle studies for a nasal prophylaxis, we
224 observed little immunogenicity of LCB1v1.3, suggesting that repeated dosing may be possible.
225 Evaluation of miniprotein binders in hamsters and NHPs is needed to extend the efficacy data
226 and provide further rationale for human clinical trials.

227 Although several antibody-based therapies demonstrate promise against SARS-CoV-2,
228 and a few have been granted EUA status, viral evolution could jeopardize these interventions as
229 evidenced by the emerging variants in the United Kingdom (B.1.1.7), South Africa (B.1.351),
230 Brazil (B.1.248), and elsewhere. Indeed, we and others have observed that many monoclonal
231 and polyclonal antibodies showed reduced neutralization activity against several of these variant
232 strains (Chen et al., 2021; Wang et al., 2021a; Wang et al., 2021b; Wibmer et al., 2021; Xie et
233 al., 2021b). In comparison, LCB1v1.3 showed efficacy against historical (WA1/2020) and
234 emerging (B.1.1.7 and E484K/N501Y/D614G) SARS-CoV-2 strains. Based on the cryo-EM
235 structure of the parent LCB1 binder in complex with SARS-CoV-2 RBD (Cao et al., 2020), only
236 the N501Y mutation is expected to affect binding. While we observed a decrease in the
237 neutralizing activity of LCB1v1.3 against the emerging variants, EC_{50} values were still less than
238 800 pM, suggesting substantial potency was retained. Additional optimization of LCB1-Fc- and
239 LCB1v1.3-RBD binding interactions, through computational design and functional validation,
240 could reduce the effects of variant mutations on neutralizing activity. Moreover, the development
241 of binder combinations that target different regions of the spike protein of multiple emerging
242 SARS-CoV-2 variants is planned.

243 Compared to other potential SARS-CoV-2 antibody-based treatments, miniproteins have
244 several benefits: (a) due to their smaller size, they can bind each protomer of a single trimeric
245 spike, resulting in greater potency for a given dose; (b) they can be manufactured cost-

246 effectively; (c) if warranted, they can be refined to overcome escape by new SARS-CoV-2
247 variants; and (d) they can be mixed using linker proteins to generate multimerized constructs
248 that limit resistance. In summary, our data highlight the promise of rational protein antiviral
249 design and support the development of LCB1v1.3 and LCB1-Fc as potent SARS-CoV-2-specific
250 countermeasures.
251

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274

275 **AUTHOR CONTRIBUTIONS**

276 J.B.C., L.S., D.B., and M.S.D. designed the research. J.B.C., R.E.C., E.S.W., S.S., and
277 N.M.K. performed mouse experiments and clinical analyses. J.B.C. and B.Y. performed viral

278 burden analysis. J.B.C. and E.S.W. performed pulmonary mechanics analysis. A.L.B. analyzed
279 the tissue sections for histopathology. J.B.C and R.E.C. performed neutralization analysis.
280 L.Cao optimized protein designs, generated computational models, and performed BLI analysis.
281 L. Car. and R.R. purified and prepared the miniproteins. I.G. and L.S. developed and performed
282 ELISA analysis. X.X. and P.Y.S. provided the recombinant virus strain. J.B.C. and M.S.D. wrote
283 the initial draft, with other authors providing editorial comments and helpful discussions about
284 the research.

285

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287 M.S.D. is a consultant for Inbios, Vir Biotechnology, NGM Biopharmaceuticals, and
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291 coinventors on a provisional patent application that incorporates discoveries described in this
292 manuscript. D.B. is a cofounder of Neoleukin Therapeutics.

293

294 **FIGURE LEGENDS**

295 **Figure 1. LCB1-Fc prophylaxis protects against SARS-CoV-2 infection. (A)**
296 Molecular surface representation of three LCB1v1.3 miniproteins bound to individual protomers
297 of the SARS-CoV-2 spike protein trimer (left: side view; right: top view). **(B)** Binding curves of
298 purified LCB1v1.3 and LCB1-Fc to SARS-CoV-2 RBD as monitored by biolayer interferometry
299 (one experiment performed in technical duplicate). **(C)** Neutralization curves of LCB1v1.3,
300 LCB1-Fc, or control binder against a SARS-CoV-2 WA1/2020 isolate (EC_{50} values: 14.4 pM,
301 71.8 pM, and >10,000 nM respectively; average of two experiments, each performed in
302 duplicate). **(D-J)** 7 to 8-week-old female and male K18-hACE2 transgenic mice received 250 μ g
303 of LCB1-Fc or control binder by i.p. injection one day prior to i.n. inoculation with 10^3 PFU of
304 SARS-CoV-2. Tissues were collected at 4 and 7 dpi. **(D)** Weight change following LCB1-Fc
305 administration (mean \pm SEM; $n = 8$, two experiments: two-way ANOVA with Sidak's post-test:
306 *** $P < 0.001$, **** $P < 0.0001$). **(E)** Infectious virus measured by plaque assay at 4 or 7 dpi in
307 the lung ($n = 8$, two experiments: Mann-Whitney test; *** $P < 0.001$). **(F-J)** Viral RNA levels at 4
308 or 7 dpi in the lung, heart, spleen, brain, or nasal wash ($n = 8$, two experiments: Mann-Whitney
309 test: ns, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

310 **Figure 2. LCB1-Fc prophylaxis prevents SARS-CoV-2-mediated lung disease. (A)**
311 Respiratory mechanics parameters: inspiratory capacity, resistance, elastance tissue damping,
312 quasi-static compliance, and pressure-volume loops measured at 7 dpi ($n = 3-6$, two
313 experiments: two-way ANOVA with Tukey's post-test: ns, not significant, * $P < 0.05$, ** $P < 0.01$,
314 *** $P < 0.001$ between indicated groups). **(B)** Hematoxylin and eosin staining of lung sections
315 from mice treated at D-1 and collected at 7 dpi with SARS-CoV-2. Images show low (left) and
316 high (right; boxed region from left) magnification. Scale bars for all images, 100 μ m.
317 Representative images from $n = 3$ mice per group. **(C)** Heat-map of cytokine mRNA levels from
318 lung tissues of SARS-CoV-2 infected mice at 4 dpi. For each cytokine, the fold-change was

319 calculated relative to age-matched naïve control animals after normalization to *Gapdh* and the
320 Log_2 (fold change) was plotted (n = 8 mice/group relative to n = 3 naïve controls).

321 **Figure 3. Post-exposure delivery of anti-RBD binders reduces SARS-CoV-2**
322 **burden. (A-G)** 7 to 8-week-old female and male K18-hACE2 transgenic mice received 250 μg
323 of LCB1-Fc or control binder by i.p. injection one day after i.n. inoculation with 10^3 PFU of
324 SARS-CoV-2. Tissues were collected at 4 or 7 dpi. **(A)** Weight change following LCB1-Fc
325 administration (mean \pm SEM; n = 6, two experiments: two-way ANOVA with Sidak's post-test: **
326 $P < 0.01$, **** $P < 0.0001$). **(B)** Infectious virus in the lung measured by plaque assay at 4 or 7
327 dpi in the lung (n = 6, two experiments: ** $P < 0.01$). **(C-G)** Viral RNA levels at 4 or 7 dpi in the
328 lung, heart, spleen, brain, or nasal wash (n = 6, two experiments: Mann-Whitney test: ns, not
329 significant, * $P < 0.05$, ** $P < 0.01$). **(H)** Hematoxylin and eosin staining of lung sections from
330 mice treated at D+1 and collected at 7 dpi with SARS-CoV-2. Images show low (left) and high
331 (right; boxed region from left) magnification. Scale bars for all images, 100 μm . Representative
332 images from n = 3 mice per group. **(I-J)** 7 to 8-week-old male K18-hACE2 transgenic mice
333 received a single 50 μg i.n. dose of LCB1v1.3 or control binder at one- or two-days post-
334 inoculation with 10^3 PFU of SARS-CoV-2. Viral RNA levels at 7 dpi in the lung **(I)** or nasal wash
335 **(J)** (n = 6, two experiments: one-way ANOVA: ns, not significant, * $P < 0.05$, **** $P < 0.0001$).

336 **Figure 4. Intranasal administration of LCB1v1.3 reduces viral infection even when**
337 **given 5 days prior to SARS-CoV-2 exposure. (A-D)** 7 to 8-week-old female K18-hACE2
338 transgenic mice received a single i.n. 50 μg dose of LCB1v1.3 or control binder at the indicated
339 time prior to i.n. inoculation with 10^3 PFU of SARS-CoV-2. Tissues were collected at 4 or 7 dpi
340 and viral RNA levels were determined (n = 5-6 animals per group, two-experiments: two-way
341 ANOVA with Sidak's post-test: ns, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P <$
342 0.0001). **(E-J)** 7 to 8-week-old female K18-hACE2 transgenic mice received the indicated i.n.
343 dose of LCB1v1.3 or control binder at one day prior to i.n. inoculation with 10^3 PFU of SARS-

344 CoV-2. **(E)** Weight change following LCB1v1.3 or control binder administration (mean \pm SEM; n
345 = 6, two experiments: two-way ANOVA with Sidak's post-test compared to the control binder
346 treated group: ** $P < 0.01$, **** $P < 0.0001$). **(F-J)** Viral RNA levels at 7 dpi in the lung, heart,
347 spleen, brain, or nasal wash (n = 6, two experiments: Kruskal-Wallis ANOVA with Dunn's post-
348 test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). **(K)** Hematoxylin and eosin staining of lung sections
349 from mice treated with a single i.n. 50 μ g dose of LCB1v1.3 or control binder at D-1 and
350 collected at 7 dpi with SARS-CoV-2. Images show low (left) and high (right; boxed region from
351 left) magnification. Scale bars for all images, 100 μ m. Representative images from n = 3 mice
352 per group.

353 **Figure 5. Immunogenicity of LCB1v1.3 and protection from challenge.** **(A)** Scheme
354 of experimental details. K18-hACE2 transgenic mice (n = 10 to 12 per group) were treated every
355 3 days with 50 μ g of LCB1v1.3 or control binder by i.n. administration. On day 18 post-
356 treatment, animals were bled and anti-LCB1v1.3 antibodies were measured. The following day,
357 animals were challenged with 10^3 PFU of SARS-CoV-2, and tissues were collected at 7 dpi. **(B)**
358 Binding of serum antibodies to LCB1v1.3 as measured by ELISA (three experiments). Dashed
359 line indicated limit of detection of the assay. **(C)** Weight change following LCB1v1.3 or control
360 binder administration (mean \pm SEM; two experiments: two-way ANOVA with Sidak's post-test:
361 **** $P < 0.0001$). **(D-H)** Viral RNA levels at 7 dpi in the lung, heart, spleen, brain, or nasal wash
362 (two experiments: Mann-Whitney test: * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

363 **Figure 6. LCB1v1.3 protects mice against B.1.1.7 variant and WA1/2020**
364 **E484K/N501Y/D614G strains.** **(A)** Neutralization of LCB1v1.3 against B.1.1.7 or WA1/2020
365 E484K/N501Y/D614G SARS-CoV-2 (EC₅₀ values: 802 pM and 667 pM, respectively; mean of
366 two experiments, each performed in duplicate). **(B-G)** 7 to 8-week-old female K18-hACE2
367 transgenic mice were treated with a single 50 μ g i.n. dose of LCB1v1.3 or control binder at 1
368 day prior to i.n. inoculation with 10^3 PFU of B.1.1.7. **(B)** Weight change following LCB1v1.3 or

369 control binder administration (mean \pm SEM; $n = 6$, two experiments: two-way ANOVA with
370 Sidak's post-test: *** $P < 0.001$, **** $P < 0.0001$). **(C-G)** Viral RNA levels at 6 dpi in the lung,
371 heart, spleen, nasal wash, or brain ($n = 6$, two experiments: Mann-Whitney test: * $P < 0.05$, ** P
372 < 0.01). **(H-M)** 8-week-old male K18-hACE2 transgenic mice were treated with a single 50 μg
373 i.n. dose of LCB1v1.3 or control binder at 1 day prior to i.n. inoculation with 10^3 PFU of
374 WA1/2020 E484K/N501Y/D614G. **(H)** Weight change following LCB1v1.3 or control binder
375 administration (mean \pm SEM; $n = 6$, two experiments: two-way ANOVA with Sidak's post-test: *
376 $P < 0.05$, **** $P < 0.0001$). **(I-M)** Viral RNA levels at 6 dpi in the lung, heart, spleen, nasal wash,
377 or brain ($n = 6$, two experiments: Mann-Whitney test: * $P < 0.05$, ** $P < 0.01$).

378

379 SUPPLEMENTAL FIGURE LEGENDS

380 **Figure S1. Cytokine and chemokine induction following SARS-CoV-2 infection,**
381 **Related to Fig 2C.** Individual plots for cytokine and chemokine RNA levels in the lungs of
382 SARS-CoV-2 infected mice at 4 dpi following treatment with control or LCB1-Fc binders ($n = 8$
383 per group, two experiments: Mann-Whitney test: ns, not significant, * $P < 0.05$, ** $P < 0.01$, *** P
384 < 0.001). Data were used to generate the heat-map in **Figure 2C**.

385 **Figure S2. Intranasal delivery of LCB1v1.3 at 1 or 2 days post-SARS-CoV-2**
386 **infection reduces viral burden, Related to Fig 3. (A-C)** 7 to 8-week-old male K18-hACE2
387 transgenic mice received a single 50 μg i.n. dose of LCB1v1.3 or control binder at one- or two-
388 days post-inoculation with 10^3 PFU of SARS-CoV-2. Viral RNA levels at 7 dpi in the heart **(A)**,
389 spleen **(B)**, or brain **(C)** ($n = 6$, two experiments: one-way ANOVA: * $P < 0.05$, ** $P < 0.01$).

390 **Figure S3. Intranasal prophylaxis of LCB1v1.3 reduces weight loss, Related to Fig**
391 **4.** 7 to 8-week-old female K18-hACE2 transgenic mice received a single 50 μg i.n. dose of
392 LCB1v1.3 or control binder at the indicated time prior to i.n. inoculation with 10^3 PFU of SARS-

393 CoV-2. Weight change was recorded daily (mean \pm SEM; n = 6, two experiments: two-way
394 ANOVA with Sidak's post-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

395 **STAR METHODS**

396 **RESOURCE AVAILABILITY**

397 **Lead Contact.** Further information and requests for resources and reagents should be
398 directed to the Lead Contact, Michael S. Diamond (diamond@wusm.wustl.edu).

399 **Materials Availability.** All requests for resources and reagents should be directed to the
400 Lead Contact author. This includes mice, antibodies, protein minibinders, and viruses. All
401 reagents will be made available on request after completion of a Materials Transfer Agreement.

402 **Data and code availability.** All data supporting the findings of this study are available
403 within the paper and are available from the corresponding author upon request.

404

405 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

406 **Cells and viruses.** Vero E6 (CRL-1586, American Type Culture Collection (ATCC),
407 Vero CCL81 (ATCC), Vero-furin (Mukherjee et al., 2016), and Vero-hACE2-TMPRSS2 (a gift of
408 A. Creanga and B. Graham, NIH) were cultured at 37°C in Dulbecco's Modified Eagle medium
409 (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 μ M HEPES pH 7.3, 1 μ M
410 sodium pyruvate, 1 \times non-essential amino acids, and 100 μ U/ml of penicillin–streptomycin.
411 Additionally, Vero-hACE2-TMPRSS2 cells were cultured in the presence of 5 μ g/mL puromycin.
412 The WA1/202 (2019n-CoV/USA_WA1/2020) isolate of SARS-CoV-2 was obtained from the US
413 Centers for Disease Control (CDC). The B.1.1.7 and WA1/2020 E484K/N501Y/D614G viruses
414 have been described previously (Chen et al., 2021; Xie et al., 2021a). Infectious stocks were
415 propagated by inoculating Vero CCL81 or Vero-hACE2-TMPRSS2 cells. Supernatant was
416 collected, aliquoted, and stored at -80°C. All work with infectious SARS-CoV-2 was performed in
417 Institutional Biosafety Committee-approved BSL3 and A-BSL3 facilities at Washington
418 University School of Medicine using positive pressure air respirators and protective equipment.

419 **Mouse experiments.** Animal studies were carried out in accordance with the
420 recommendations in the Guide for the Care and Use of Laboratory Animals of the National
421 Institutes of Health. The protocols were approved by the Institutional Animal Care and Use
422 Committee at the Washington University School of Medicine (assurance number A3381–01).
423 Virus inoculations were performed under anesthesia that was induced and maintained with
424 ketamine hydrochloride and xylazine, and all efforts were made to minimize animal suffering.

425 Heterozygous K18-hACE c57BL/6J mice (strain: 2B6.Cg-Tg(K18-ACE2)2PrImn/J) were
426 obtained from The Jackson Laboratory. Animals were housed in groups and fed standard chow
427 diets. Mice of different ages and both sexes were administered 10^3 PFU of SARS-CoV-2 via
428 intranasal administration.

429

430 **METHOD DETAILS**

431 **Miniprotein production.** LCB1-Fc was synthesized and cloned by GenScript into
432 pCMVR plasmid, with kanamycin resistance. Plasmids were transformed into the NEB 5-alpha
433 strain of *E. coli* (New England Biolabs) to recover DNA for transient transfection into Expi293F
434 mammalian cells. Expi293F cells were grown in suspension using Expi293F expression medium
435 (Life Technologies) at 33°C, 70% humidity, and 8% CO₂ rotating at 150 rpm. The cultures were
436 transfected using PEI-MAX (Polyscience) with cells grown to a density of 3×10^6 cells per mL
437 and cultivated for 3 days. Supernatants were clarified by centrifugation (5 min at 4000 x *g*,
438 addition of PDADMAC solution to a final concentration of 0.0375% (Sigma Aldrich, #409014),
439 and a second spin (5 min at 4000 x *g*). Clarified supernatants were purified using a MabSelect
440 PrismA 2.6x5 cm column (Cytiva) on an AKTA Avant150 FPLC (Cytiva). Bound protein was
441 washed with five column volumes of 20 mM NaPO₄ and 150 mM NaCl pH 7.2, then five column
442 volumes of 20 mM NaPO₄ and 1 M NaCl pH 7.4, and eluted with three column volumes of 100
443 mM glycine at pH 3.0. The eluate was neutralized with 2 M Tris base to a final concentration of

444 50 mM. SDS-PAGE was used to assess protein purity. The protein was passed through a 0.22
445 μm filter and stored at 4°C until use.

446 LCB1v1.3 with polar mutations (4N, 14K, 15T, 17E, 18Q, 27Q, 38Q) relative to the
447 original LCB1 was cloned into a pet29b vector. LCB1v1.3 was expressed in Lemo21(DE3)
448 (NEB) in terrific broth media and grown in 2 L baffled shake flasks. Bacteria were propagated at
449 37°C to an O.D.₆₀₀ of ~0.8, and then induced with 1 mM IPTG. Expression temperature was
450 reduced to 18°C, and the cells were shaken for ~16 h. The cells were harvested and lysed using
451 heat treatment and incubated at 80°C for 10 min with stirring. Lysates were clarified by
452 centrifugation at 24,000 $\times g$ for 30 min and applied to a 2.6 \times 10 cm Ni Sepharose 6 FF column
453 (Cytiva) for purification by IMAC on an AKTA Avant150 FPLC system (Cytiva). Proteins were
454 eluted over a linear gradient of 30 mM to 500 mM imidazole in a buffer of 50 mM Tris pH 8.0
455 and 500 mM NaCl. Peak fractions were pooled, concentrated in 10 kDa MWCO centrifugal
456 filters (Millipore), sterile filtered (0.22 μm) and applied to either a Superdex 200 Increase 10/300,
457 or HiLoad S200 pg GL SEC column (Cytiva) using 50 mM phosphate pH 7.4, 150 mM NaCl
458 buffer. After size exclusion chromatography, bacterial-derived components were tested to
459 confirm low levels of endotoxin.

460 **Biolayer interferometry.** Biolayer interferometry data were collected using an Octet
461 RED96 (ForteBio) and processed using the instrument's integrated software. Briefly, biotinylated
462 RBD (Acro Biosystems) was loaded onto streptavidin-coated biosensors (SA ForteBio) at 20 nM
463 in binding buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.05% surfactant P20,
464 and 0.5% non-fat dry milk) for 360 s. Analyte proteins (LCB1v1.3 or LCB1-Fc) were diluted from
465 concentrated stocks into binding buffer. After baseline measurement in the binding buffer alone,
466 the binding kinetics were monitored by dipping the biosensors in wells containing the target
467 protein at the indicated concentration (association step) for 3,600 s and then dipping the
468 sensors back into baseline/buffer (dissociation) for 7,200 s.

469 **Plaque assay.** Vero-furin cells (Mukherjee et al., 2016) were seeded at a density of
470 2.5×10⁵ cells per well in flat-bottom 12-well tissue culture plates. The following day, medium
471 was removed and replaced with 200 µL of 10-fold serial dilutions of the material to be titrated,
472 diluted in DMEM+2% FBS, and plates incubated at 37°C with rocking at regular intervals. One
473 hour later, 1 mL of methylcellulose overlay was added. Plates were incubated at 37°C for 72 h,
474 then fixed with 4% paraformaldehyde (final concentration) in PBS for 20 min. Fixed cell
475 monolayers were stained with 0.05% (w/v) crystal violet in 20% methanol and washed twice with
476 distilled, deionized water.

477 **Measurement of viral burden.** Tissues were weighed and homogenized with zirconia
478 beads in a MagNA Lyser instrument (Roche Life Science) in 1,000 µL of DMEM media
479 supplemented with 2% heat-inactivated FBS. Tissue homogenates were clarified by
480 centrifugation at 10,000 rpm for 5 min and stored at –80°C. RNA was extracted using the
481 MagMax mirVana Total RNA isolation kit (Thermo Scientific) on a Kingfisher Flex extraction
482 robot (Thermo Scientific). RNA was reverse transcribed and amplified using the TaqMan RNA-
483 to-CT 1-Step Kit (ThermoFisher). Reverse transcription was carried out at 48°C for 15 min
484 followed by 2 min at 95°C. Amplification was accomplished over 50 cycles as follows: 95°C for
485 15 s and 60°C for 1 min. Copies of SARS-CoV-2 N gene RNA in samples were determined
486 using a previously published assay (Case et al., 2020; Hassan et al., 2020). Briefly, a TaqMan
487 assay was designed to target a highly conserved region of the N gene (Forward primer:
488 ATGCTGCAATCGTGCTACAA; Reverse primer: GACTGCCGCCTCTGCTC; Probe: /56-
489 FAM/TCAAGGAAC/ZEN/AACATTGCCAA/3IABkFQ/). This region was included in an RNA
490 standard to allow for copy number determination down to 10 copies per reaction. The reaction
491 mixture contained final concentrations of primers and probe of 500 and 100 nM, respectively.

492 **Cytokine and chemokine mRNA measurements.** RNA was isolated from lung
493 homogenates as described above. cDNA was synthesized from DNase-treated RNA using the
494 High-Capacity cDNA Reverse Transcription kit (Thermo Scientific) with the addition of RNase

495 inhibitor following the manufacturer's protocol. Cytokine and chemokine expression was
496 determined using TaqMan Fast Universal PCR master mix (Thermo Scientific) with commercial
497 primers/probe sets specific for *IFN-g* (IDT: Mm.PT.58.41769240), *IL-6* (Mm.PT.58.10005566),
498 *IL-1b* (Mm.PT.58.41616450), *Tnfa* (Mm.PT.58.12575861), *CXCL10* (Mm.PT.58.43575827),
499 *CCL2* (Mm.PT.58.42151692), *CCL5* (Mm.PT.58.43548565), *CXCL11* (Mm.PT.58.10773148.g),
500 *Ifnb* (Mm.PT.58.30132453.g), *CXCL1* (Mm.PT.58.42076891) and results were normalized to
501 *GAPDH* (Mm.PT.39a.1) levels. Fold change was determined using the $2^{-\Delta\Delta Ct}$ method comparing
502 treated mice to naïve controls.

503 **Lung Pathology.** Animals were euthanized before harvest and fixation of tissues. The
504 left lung was first tied off at the left main bronchus and collected for viral RNA analysis. The right
505 lung was inflated with approximately 1.2 mL of 10% neutral buffered formalin using a 3-mL
506 syringe and catheter inserted into the trachea. Tissues were embedded in paraffin, and sections
507 were stained with hematoxylin and eosin. Slides were scanned using a Hamamatsu
508 NanoZoomer slide scanning system, and images were viewed using NDP view software
509 (ver.1.2.46).

510 **Respiratory mechanics.** Mice were anesthetized with ketamine/xylazine (100 mg/kg
511 and 10 mg/kg, i.p., respectively). The trachea was isolated via dissection of the neck area and
512 cannulated using an 18-gauge blunt metal cannula (typical resistance of 0.18 cmH₂O.s/mL),
513 which was secured in place with a nylon suture. The mouse then was connected to the flexiVent
514 computer-controlled piston ventilator (SCIREQ Inc.) via the cannula, which was attached to the
515 FX adaptor Y-tubing. Mechanical ventilation was initiated, and mice were given an additional
516 100 mg/kg of ketamine and 0.1 mg/mouse of the paralytic pancuronium bromide via
517 intraperitoneal route to prevent breathing against the ventilator and during measurements. Mice
518 were ventilated using default settings for mice, which consisted in a positive end expiratory
519 pressure at 3 cm H₂O, a 10 mL/kg tidal volume (V_t), a respiratory rate at 150 breaths per minute
520 (bpm), and a fraction of inspired oxygen (FiO₂) of 0.21 (*i.e.*, room air). Respiratory mechanics

521 were assessed using the forced oscillation technique, as previously described (McGovern et al.,
522 2013), using the latest version of the flexiVent operating software (flexiWare v8.1.3). Pressure-
523 volume loops and measurements of inspiratory capacity also were performed.

524 **Neutralization assay.** Serial dilutions of binder proteins were incubated with 10^2 focus-
525 forming units (FFU) of SARS-CoV-2 for 1 h at 37°C. Binder-virus complexes were added to
526 Vero E6 (WA1/2020) or Vero-hACE2-TMPRSS2 (B.1.1.7 and WA1/2020 E484K/N501Y/D614G)
527 cell monolayers in 96-well plates and incubated at 37°C for 1 h. Subsequently, cells were
528 overlaid with 1% (w/v) methylcellulose in MEM supplemented with 2% FBS. Plates were
529 harvested 24-30 h later by removing overlays and fixed with 4% PFA in PBS for 20 min at room
530 temperature. Plates were washed and sequentially incubated with an oligoclonal pool of
531 SARS2-2, SARS2-11, SARS2-16, SARS2-31, SARS2-38, SARS2-57, and SARS2-71 anti-spike
532 protein antibodies (Zhou et al., 2021) and HRP-conjugated goat anti-mouse IgG in PBS
533 supplemented with 0.1% saponin and 0.1% bovine serum albumin. SARS-CoV-2-infected cell
534 foci were visualized using TrueBlue peroxidase substrate (KPL) and quantitated on an
535 ImmunoSpot microanalyzer (Cellular Technologies). Data were processed using Prism software
536 (GraphPad Prism 8.0).

537 **ELISA.** C-terminal biotinylated LCB1.1v3 was immobilized on streptavidin-coated plates
538 (RayBiotech #7C-SCP-1) at 2.5 µg/mL in 100 µL total volume per well and incubated at 4°C
539 overnight. Plates were washed with wash buffer (TBS + 0.1% (w/v) BSA + 0.05% (v/v)
540 Tween20) and blocked with 200 µL/well blocking buffer (TBS + 2% (w/v) BSA + 0.05% (v/v)
541 Tween20) for 1 h at room temperature. Plates were rinsed with wash buffer using 200 µL/well,
542 and 100 µL of 1:100 diluted sera samples in blocking buffer were added to respective wells. For
543 a positive control, Fc-RBD was serially diluted 1:5 starting at 240 ng/mL in 100 µL of blocking
544 buffer. All samples were incubated for 1 h at room temperature. Plates were washed using 200
545 µL/well of wash buffer. For the serum samples, HRP-conjugated horse anti-mouse IgG antibody
546 (Vector Laboratories #PI-2000-1) was diluted 1:200 in blocking buffer, and 100 µL was

547 incubated in each well at room temperature for 30 min. For the positive control, HRP-
548 conjugated mouse anti-human IgG antibody (Invitrogen #05-4220) was diluted 1:500 in blocking
549 buffer, and 100 μ L was incubated in each well at room temperature for 30 min. Plates were
550 rinsed with wash buffer, and 100 μ L of TMB (SeraCare) was added to each well for 2 min. The
551 reaction was quenched by adding 100 μ L of 1N HCl. Optical densities were determined at
552 450nm on a Synergy Neo2 plate reader (BioTek Instruments).

553

554 **QUANTIFICATION AND STATISTICAL ANALYSIS**

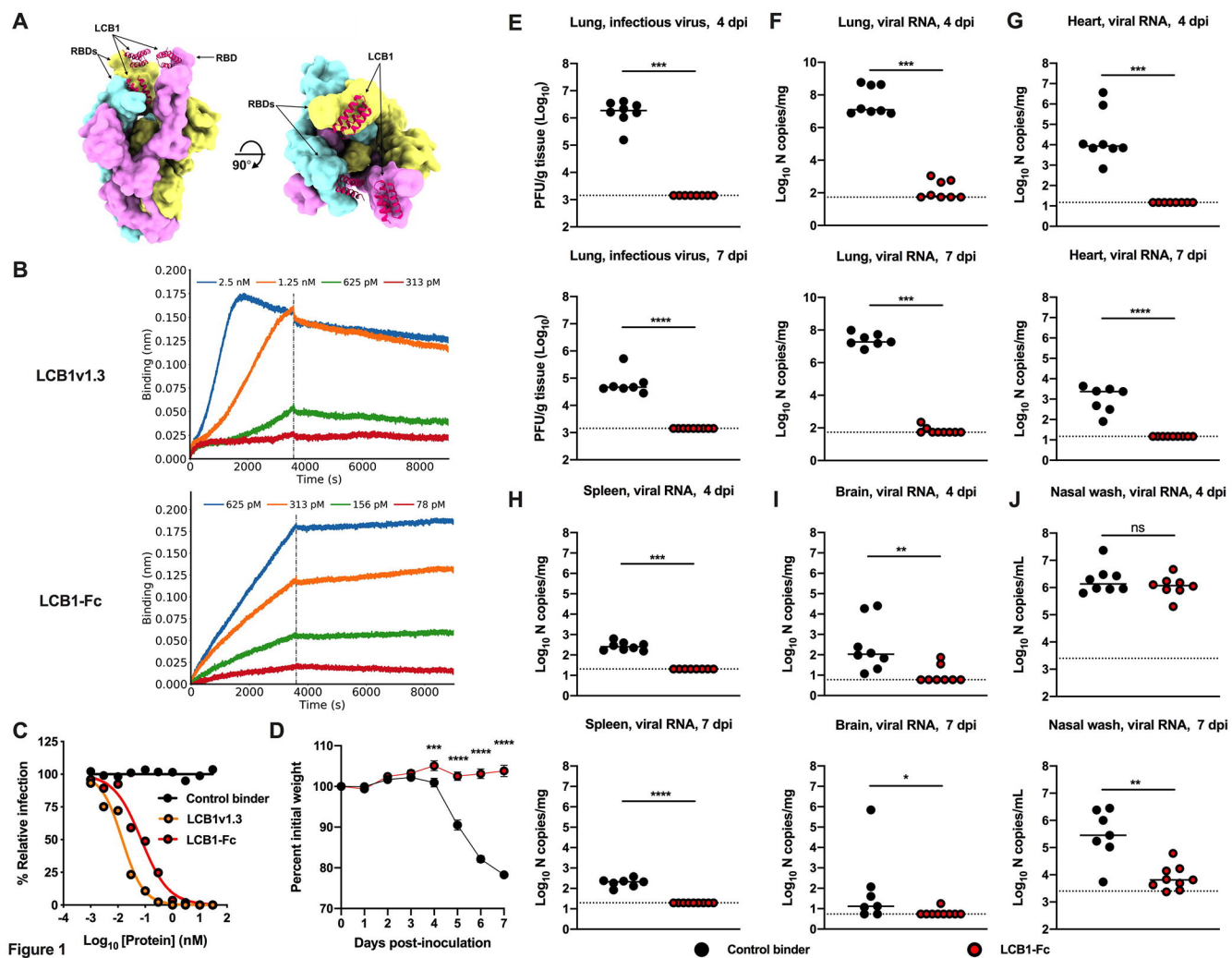
555 Statistical significance was assigned when P values were < 0.05 using Prism Version 8
556 (GraphPad). Tests, number of animals, median values, and statistical comparison groups are
557 indicated in each of the Figure legends. Analysis of weight change was determined by two-way
558 ANOVA. Changes in functional parameters or immune parameters were compared to control
559 binder-treated animals and analyzed by one-way ANOVA with multiple comparisons tests.
560 Statistical analyses of viral burden between two groups were determined by Mann-Whitney test.

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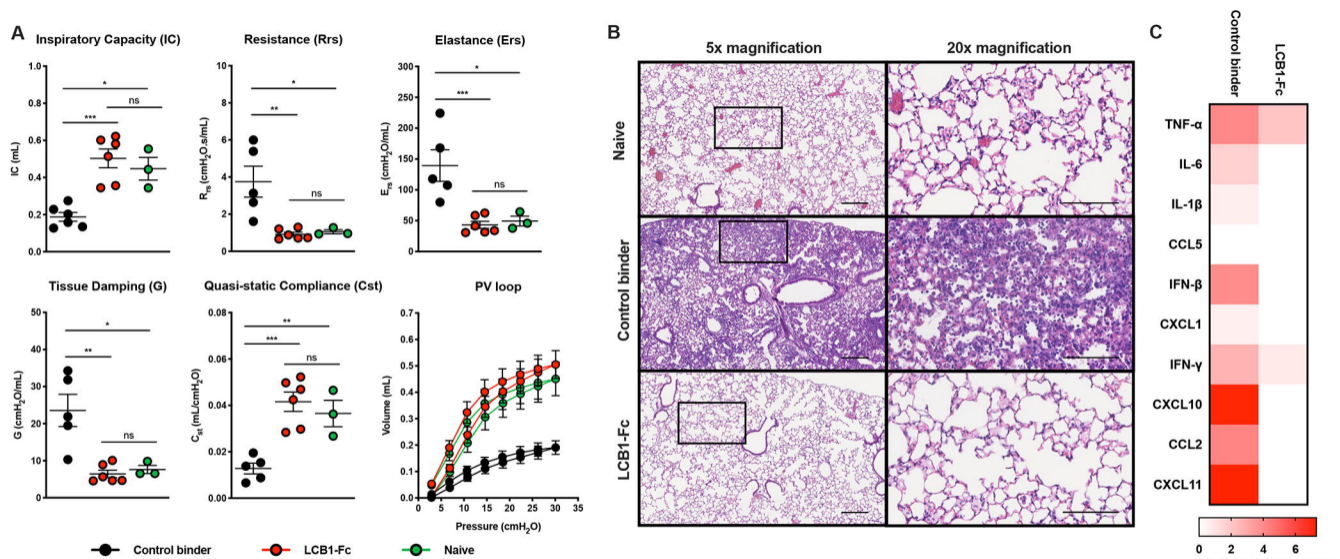
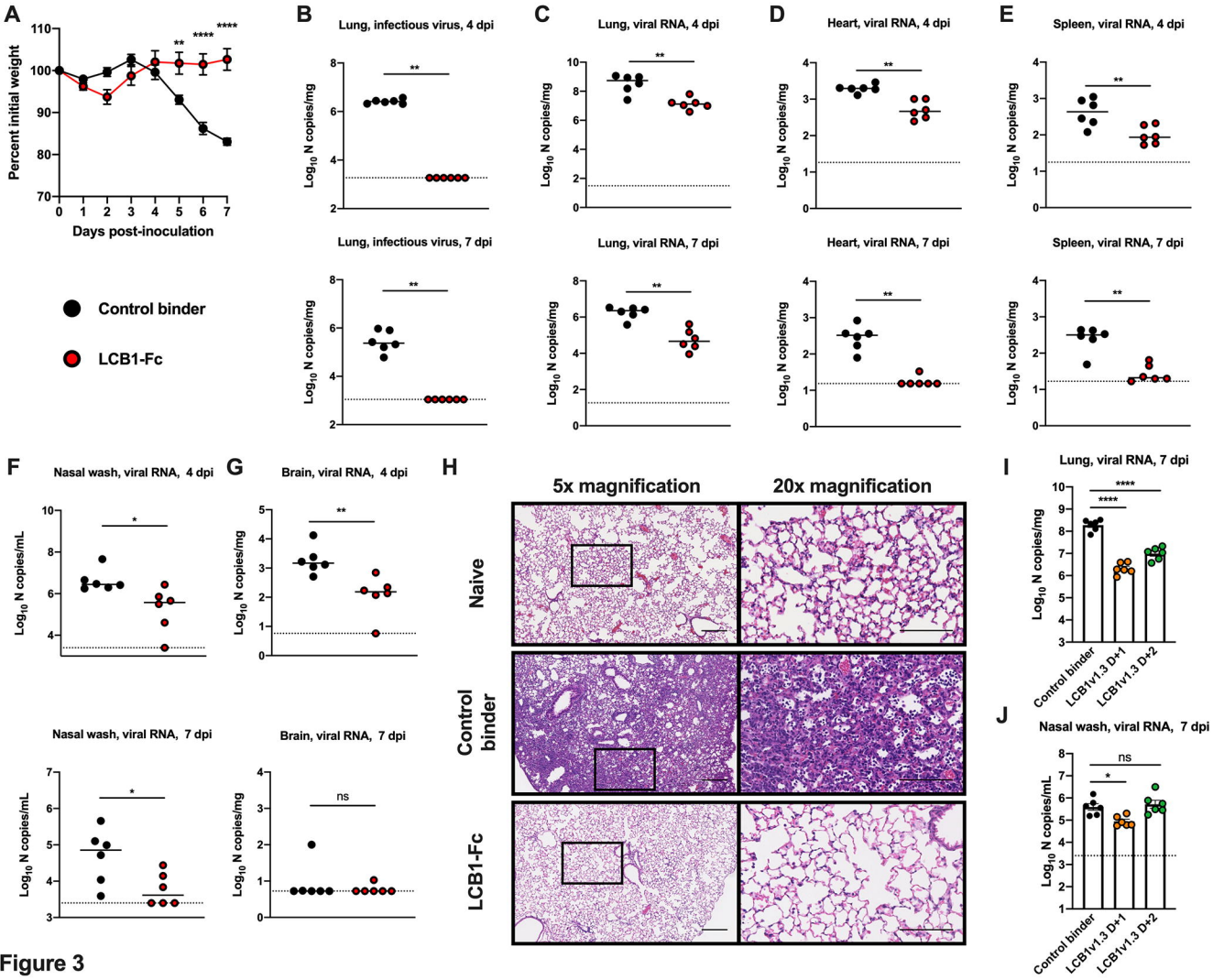


Figure 2



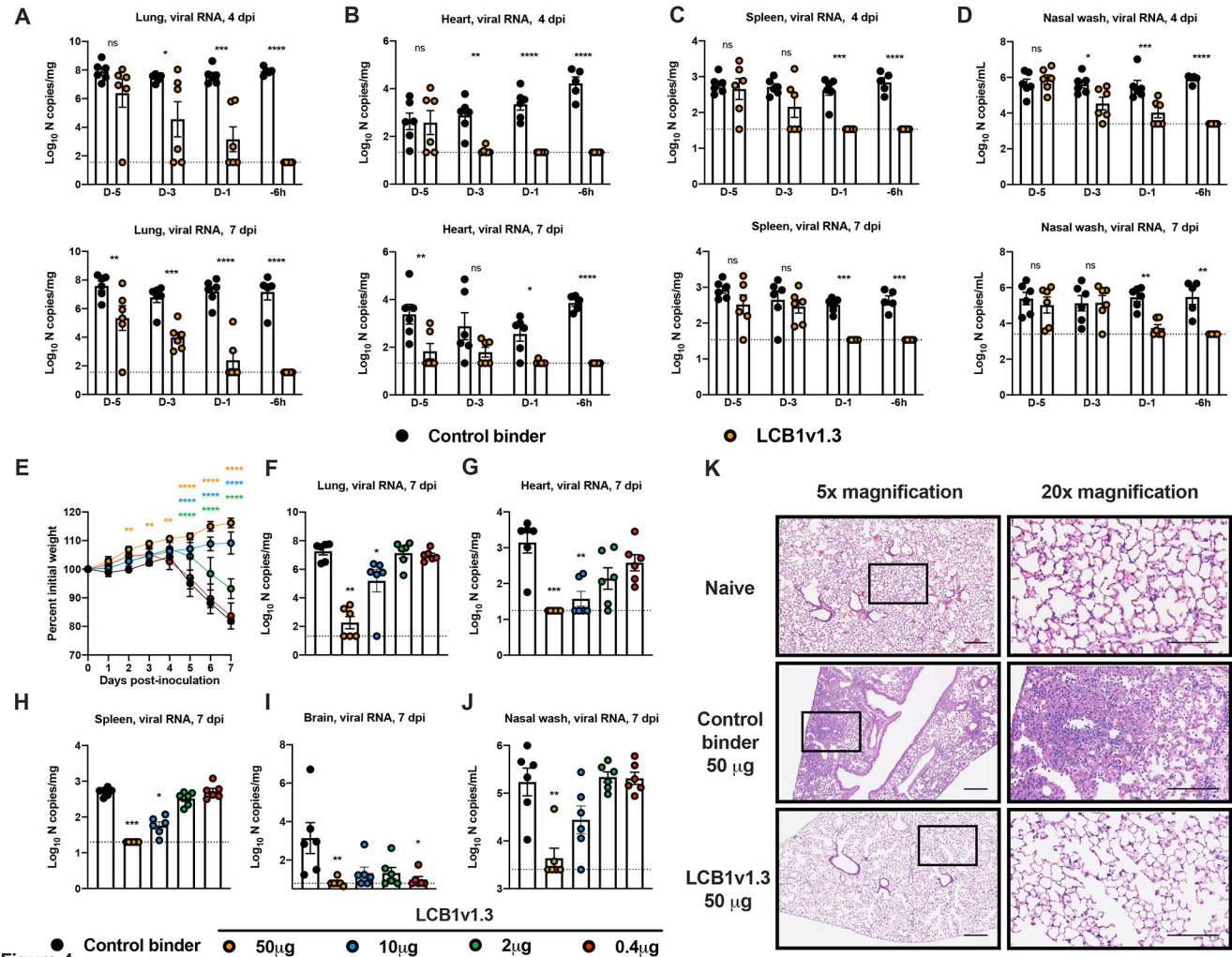


Figure 4

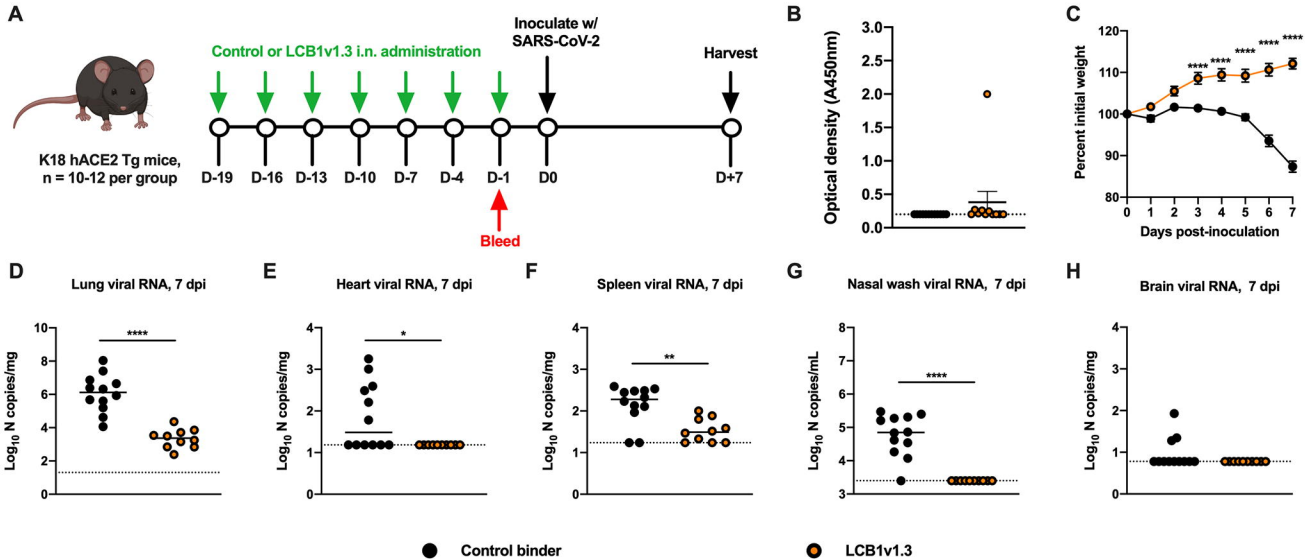
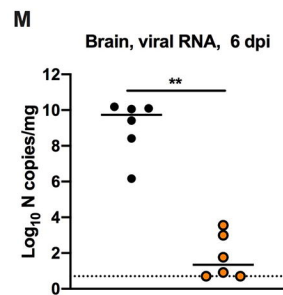
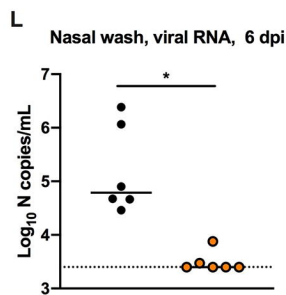
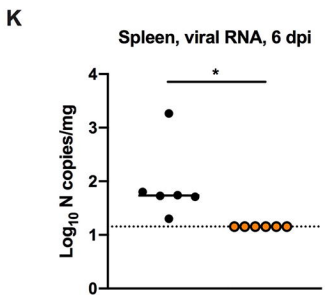
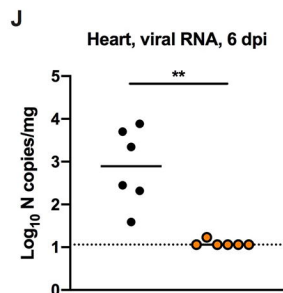
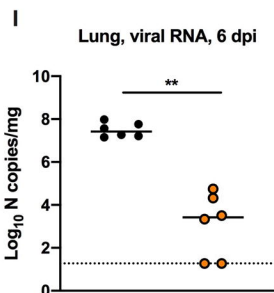
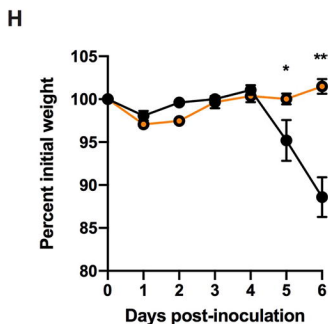
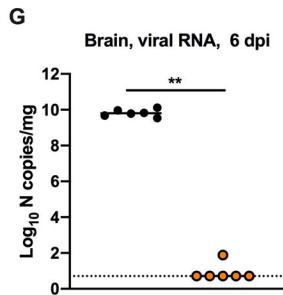
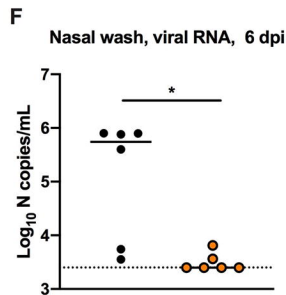
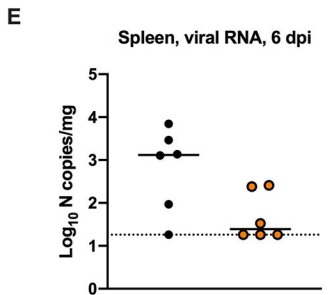
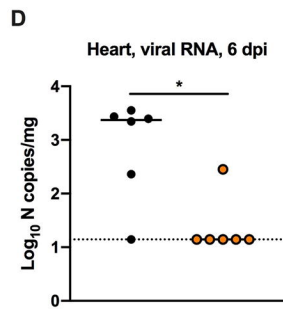
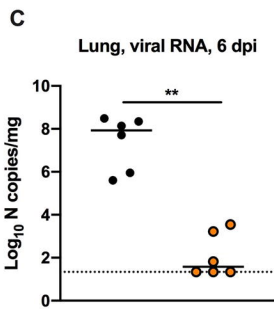
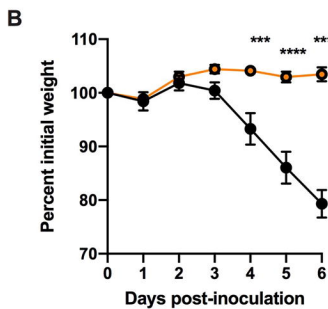
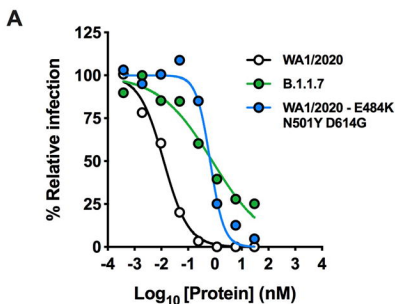


Figure 5

Figure 6



B.1.1.7 isolate

WA1/2020 - E484K/N501Y/D614G