

1 **Title:** Combined Molnupiravir and Nirmatrelvir Treatment Improves the Inhibitory Effect on
2 SARS-CoV-2 in Rhesus Macaques

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17 **Short Title:** Efficacy of Molnupiravir and Nirmatrelvir in SARS-CoV-2 Rhesus Macaque Model

18

19 **One Sentence Summary:** The combination of molnupiravir and nirmatrelvir inhibits SARS-
20 CoV-2 replication and shedding more effectively than individual treatments in the rhesus
21 macaque model.

22

23

24 **Abstract**

25 The periodic emergence of SARS-CoV-2 variants of concern (VOCs) with unpredictable clinical
26 severity and ability to escape preexisting immunity emphasizes the continued need for antiviral
27 interventions. Two small molecule inhibitors, molnupiravir (MK-4482), a nucleoside analog, and
28 nirmatrelvir (PF-07321332), a 3C-like protease inhibitor, have each recently been approved as
29 monotherapy for use in high risk COVID-19 patients. As preclinical data are only available for
30 rodent and ferret models, we originally assessed the efficacy of MK-4482 and PF-07321332
31 alone and then in combination Against infection with the SARS-CoV-2 Delta VOC in the rhesus
32 macaque COVID-19 model. Notably, use of MK-4482 and PF-07321332 in combination
33 improved the individual inhibitory effect of both drugs. Combined treatment resulted in milder
34 disease progression, stronger reduction of virus shedding from mucosal tissues of the upper
35 respiratory tract, stronger reduction of viral replication in the lower respiratory tract, and reduced
36 lung pathology. Our data strongly indicate superiority of combined MK-4482 and PF-07321332
37 treatment of SARS-CoV-2 infections as demonstrated here in the closest COVID-19 surrogate
38 model.

39

40 **Introduction**

41 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of
42 coronavirus disease 2019 (COVID-19) [1], was initially reported in China in December 2019 [2]
43 and has subsequently spread through the rest of Asia and around the World. As of writing
44 (August 2022), the pandemic accounts for over 599 million confirmed SARS-CoV-2 infections
45 and 6.47 million deaths. Following an initial relatively stable phase, the pandemic is now being
46 driven by regional and global waves of newly emerging SARS-CoV-2 variants. These variants
47 emerge on a steady but unpredictable basis. To date, 13 variants of interest (VOIs) or concern
48 (VOCs) associated with mutations that alter key aspects of transmissibility, immune evasion or
49 severity of disease have emerged, excluding the 7 Omicron subvariants currently circulating [3,
50 4]. Mutations associated with VOCs are most frequently located within the spike protein where
51 they affect functions such as receptor binding affinity and antibody neutralization capacity but
52 can be located throughout the entire SARS-CoV-2 genome [3-9].

53 Molnupiravir (MK-4482) and nirmatrelvir (PF-07321332) are orally administered antivirals that
54 have recently been approved for use to treat high risk COVID-19 patients [10-13]. These
55 compounds target distinct stages of the SARS-CoV-2 replication cycle, but neither directly
56 targets stages involving the viral spike protein [14, 15]. MK-4482 is a nucleoside analogue
57 affecting the SARS-CoV-2 polymerase fidelity resulting in a catastrophic rate of mutation that
58 ultimately reduces progeny virus infectivity [15]. PF-07321332 is a 3C-like protease inhibitor
59 that prevents cleavage of the SARS-CoV-2 polyprotein, again ultimately inhibiting viral
60 replication [14]. PF-07321332 is currently used in combination with low dose ritonavir, an
61 inhibitor of cytochrome P450-3A4, where ritonavir increases the PF-07321332 serum half-life

62 allowing for increased potency [14]. MK-4482 is used under the trade name Lagevrio™. PF-
63 07321332 together with ritonavir comprises the commercial COVID-19 treatment, Paxlovid™.
64 Results from recent studies in tissue culture and rodent and ferret models suggested that both
65 MK-4482 and PF-07321332 retain activity when used as monotherapies against emerging VOCs
66 including Omicron [16-18]. Herein, we originally investigated the efficacy of MK-4482 and PF-
67 07321332, both as individual monotherapies and in combination, against the SARS-CoV-2 Delta
68 VOC in the rhesus macaque COVID-19 model [19]. Notably, use of MK-4482 and PF-07321332
69 in combination improved the individual inhibitory effect of both drugs on clinical outcome, viral
70 RNA and infectious viral loads in the upper and lower respiratory tract of infected animals. Our
71 study demonstrates efficacy of MK-4482 and PF-07321332 as monotherapy, but with improved
72 efficacy when used in combination, against SARS-CoV-2 in the closest COVID-19 surrogate
73 model.

74

75 **Results**

76 **Study design.** To assess the efficacy of the two treatments individually and in combination,
77 rhesus macaques were randomly divided into vehicle- or MK-4482-, PF-07321332- or
78 combination treatment groups (n=5 per group). Animals were infected with 2×10^6 TCID₅₀ of
79 SARS-CoV-2 Delta VOC by combined intranasal and intratracheal routes (1×10^6 TCID₅₀ each
80 route). Drug treatments began 12 hours post-infection (12hpi) with animals receiving either
81 vehicle or 130mg/kg MK-4482 (260mg/kg/day) or 20mg/kg PF-07321332 + 6.5mg/kg ritonavir
82 (40mg/kg/day, 13mg/kg/day) or a combination of all 3 compounds consisting of the same doses
83 as for individual treatments. Treatments were administered by oral gavage every 12h (7

84 treatments in total). The study ended 4 days post-infection (dpi) (12h following last treatment), at
85 which time animals were euthanized for tissue collection and analysis (Fig. 1A).

86 **Combination treatment results in a significant reduction in clinical disease.** Animals were
87 scored for signs of disease daily by the same person blinded to the study groups using a
88 previously established scoring sheet [19]. A score (0–15) was assigned for each of the following:
89 general appearance, skin and fur, nose/mouth/eyes/head, respiration, feces and urine, food intake,
90 and locomotor activity. All scoring was performed prior to anesthesia for treatments and
91 examinations. Groups scored evenly in the morning prior to inoculation (0dpi). Animals in the
92 vehicle treated group showed the highest scores throughout the experiment peaking at 2dpi.
93 Animals in the group receiving the combination treatment scored the lowest throughout (Fig.
94 1B). Animals in the groups treated with either MK-4482 or PF-07321332 alone scored in
95 between (Fig. 1B). Although differences between groups were not statistically significant on any
96 single day of the study, AUC analysis showed a significant difference between the vehicle
97 treated group and the group receiving the combination therapy (Fig 1C).

98 **Combination treatment results in a significantly greater reduction of SARS-CoV-2**
99 **shedding than monotherapy alone.** Nasal and oral swabs were collected from rhesus macaques
100 during examinations performed on 1, 2 and 4dpi. Subgenomic E (sgE)-based RT-PCR was used
101 as an initial measure of active SARS-CoV-2 replication [20]. Compared to the vehicle treated
102 group, sgE viral RNA loads in the nasal swabs were lower in all three treatment groups and
103 remained significantly lower in the combination therapy group for the duration of the study. The
104 PF-07321332, but not MK-4482, monotherapy treatment group was also significantly lower at
105 4dpi (Fig 2A). These differences were further amplified in the infectious titers recovered from
106 the nasal swabs. At 1dpi, all treated animals had significantly lower infectious titers. By 2dpi, the

107 differences between treated and untreated animals were further amplified and a larger subset of
108 animals in each treatment group had no detectable infectious virus (Fig 2B). There were no
109 significant differences in the levels of viral RNA detected in the oral swabs at any timepoint
110 examined (Fig 2C), but AUC analysis performed for the entirety of the study did reveal a
111 significant difference between the combination therapy and the vehicle control (Fig S1a).
112 Infectious virus was significantly lower at 1dpi in all treated groups when compared to the
113 vehicle control group, and treated groups remained significantly lower over the entirety of the
114 study (Fig 1D).

115 **Combination treatment results in a significantly greater reduction of SARS-CoV-2**
116 **replication in the lower respiratory tract.** Bronchoalveolar lavages (BALs) were collected
117 during each clinical examination at 1, 2 and 4dpi. As shown in Fig 3A, sgE RNA loads were
118 lower in PF-07321332 and combination therapy groups at 1dpi and 2dpi, compared to the vehicle
119 controls. The differences were more pronounced between groups at 2dpi but at no time was the
120 sgE significantly different between groups (Fig 3A). AUC analysis of the BALs did reveal a
121 significant difference between the vehicle treated and combination treated groups (Fig S1b).
122 Infectious virus in the BAL samples were lower in all treatment groups at 1dpi and 2dpi as
123 compared to the vehicle control group. The combination therapy was significantly lower than the
124 vehicle controls at 1dpi (Fig. 3B). Although only one animal in the MK-4482 group and no
125 animals in the combination therapy groups had detectable infectious virus at 2dpi, these results
126 were not statistically significant. By 4dpi all groups had a single animal with detectable levels of
127 infectious virus with all other animals being negative (Fig 3B).

128 Tissue samples from each lung lobe were collected at 4dpi for viral load analysis. RNA was
129 isolated from each sample for PCR analysis. Each lobe value was then pooled for total lung

130 comparisons between groups. Animals receiving PF-07321332 and the combination therapy had
131 lower levels of sgE RNA viral loads in the lungs than the vehicle treated group; the combination
132 therapy group had significantly less detectable sgE viral loads than either the vehicle and MK-
133 4482 treated groups (Fig 3C). Infectious virus loads in lungs were significantly reduced in all
134 treatment groups by study end at 4dpi (Fig 3D).

135 **Treated animals had less pathology and viral antigen in the upper and lower respiratory**
136 **tissues.** Histological analysis of samples removed from each lung lobe revealed treatments
137 decreased lung pathology. The vehicle controls developed minimal-to-marked pathology in four
138 of five animals. These lesions were characterized as multifocal, mild to marked interstitial
139 pneumonia characterized by thickening of alveolar septa by edema fluid and fibrin and small to
140 moderate numbers of macrophages and fewer neutrophils. Alveoli contained increased numbers
141 of pulmonary macrophages and neutrophils. Multifocal perivascular infiltrates of small to
142 moderate numbers of lymphocytes forming perivascular cuffs were also observed. There was
143 minimal type II pneumocyte hyperplasia consistent with the early SARS-CoV-2 disease
144 progression (4dpi) (Fig. 4A). All treatment groups exhibited a decreased overall presence of
145 interstitial pneumonia compared to the vehicle controls. MK-4482 treatments resulted in a
146 reduction in observable interstitial pneumonia to 3 out of 5 animals and those lesions were
147 minimal (Fig. 4B). Treatment with PF-07321332 reduced the number of effected animals to 2 of
148 5, with both scoring minimal and mild in one lung lobe each (Fig. 4C). Treatment with the drug
149 combination reduced pneumonia even further with only 1 of 5 animals displaying minimal
150 lesions in two lung lobes (Fig. 4D). Immunohistochemistry (IHC) of the same lung samples
151 revealed a reduction in observable viral antigen between the vehicle treated group and all 3
152 treated groups (Fig 4E-H). Nasal turbinates were also collected at the time of euthanasia with no

153 observable pathology in either the respiratory or olfactory epithelium (Fig S2). Although there
154 was an absence of pathology, IHC analysis did show scattered immunoreactivity in the vehicle
155 treated animals and little to no observable viral antigen in treated groups (Fig S2).

156 **Bioavailable drugs were detected in both sera and lung samples.** To assess circulating drug
157 levels across each treatment regimen to ensure bioavailability in the pulmonary compartment,
158 levels of MK-4482, PF-07321332, and ritonavir were quantified in plasma collected prior to drug
159 administration at each clinical exam time point and in clarified lung homogenates collected at the
160 time of necropsy (Table 1). As previously described [21], EIDD-1931, the active nucleobase
161 metabolite of the MK-4482 pro-drug, was used as a surrogate for MK-4482; MK-4482 signals
162 were analyzed in all samples and a standard curve was assessed but no signals above the limit of
163 detection were observed as expected due to its rapid metabolism. As plasma was collected prior
164 to dosing at each exam point, levels of each therapeutic molecule in the plasma reflect the lowest
165 circulating concentrations over the treatment course. At each time point, levels of all 3 drugs
166 were readily detectable, with a mean of 46.47nm for EIDD-1931 and 12.75nm for PF-07321332
167 in plasma. Drug concentrations were lower in lung tissue with a mean of 21.52nmol/g EIDD-
168 1931 and 0.06nmol/g PF-07321332. Combination therapy increased these values in both plasma
169 and lungs to 76.17nM EIDD-1931 and 19.21nM PF-07321332 in plasma and 23.84 nmol/g
170 EIDD-1931 and 0.36 nmol/g PF-07321332 in the lungs. All values agree with the treatment
171 scheme in both plasma and lung homogenate samples, with anticipated slight temporal
172 fluctuations. Lung levels of EIDD-1931 were also in good agreement with previous data
173 examining efficacy of MK-4482 in the Syrian golden hamster model [21].

174 **Discussion**

175 SARS-CoV-2 VOCs, which are now driving the pandemic, are characterized by their altered
176 transmission rate, pathogenicity or evasion from prior immunity in individuals either vaccinated
177 or previously infected [3, 6]. This continued emergence and selection of SARS-CoV-2 variants
178 within populations with preexisting levels of spike-focused immunity remains a threat to the
179 long-term effectiveness of all current commercial SARS-CoV-2 vaccines, which are all spike-
180 based, as well as spike-directed treatment approaches such as antibody therapy. It is therefore
181 important to improve existing drugs/dosing regimens and to continue the development of new
182 drugs that work independently from an effect on the spike protein. Antiviral compounds
183 targeting SARS-CoV-2 replication and processing of the viral polyprotein are obvious
184 candidates. MK-4482 and PF-07321332 are compounds targeting the SARS-CoV-2 polymerase
185 and protease, respectively [14, 15]. Both compounds have shown SARS-CoV-2 antiviral efficacy
186 as single treatments in tissue culture [18, 21] and rodent and ferret disease models [21-25].
187 Molnupiravir (MK-4482) and Paxlovid™ (PF-07321332 and ritonavir) have recently been
188 approved for use in patients considered high risk for developing severe COVID-19 [11, 12].
189 Neither MK-4482 or PF-07321332 have been evaluated for their antiviral efficacy in a SARS-
190 CoV-2 nonhuman primate (NHP) disease model. Using the rhesus macaque model of SARS-
191 CoV-2 infection [19] we assessed the anti-viral activities of both compounds individually as well
192 as in combination against the SARS-CoV-2 Delta VOC. The Delta VOC was selected as this
193 variant has been shown to cause the most severe infection of all VOCs, especially compared to
194 the rather mild infection by Omicron, tested in the rhesus macaque model to date [26].
195 Utilization of the more pathogenic Delta VOC therefore assured use of the most stringent NHP
196 challenge model available to measure treatment efficacy. As shown here, individual treatment
197 with both compounds resulted in significantly reduced SARS-CoV-2 viral load in the upper and

198 lower respiratory tract significantly reducing SARS-CoV-2 shedding and replication, and
199 ultimately led to less severe respiratory disease compared to vehicle treated animals.

200 As the mode of action for MK-4482 and PF-07321332 are distinct, the former being a nucleoside
201 analog and the latter an inhibitor of viral protease inhibitor, combined therapy may provide a
202 potential benefit over monotherapy. This was supported by recent *in vitro* data demonstrating a
203 synergistic antiviral effect of MK-4482 and PF-07321332 against Delta and Omicron VOCs in
204 comparison to individual drug treatment [27, 28], and *in vivo* for a Korean strain isolated early in
205 the pandemic [29] using a transgenic mouse model [30]. In our present study, combined
206 administration of MK-4482 and PF-07321332 treatment in the rhesus macaque model was well
207 tolerated as indicated by clinical observation and blood chemistry/hematology analyses showing
208 no indication for adverse reaction. Compared to individual drug treatments, combined therapy
209 resulted in increased efficacy with decreased SARS-CoV-2 shedding and replication early post
210 infection and milder disease as compared to monotherapy.

211 Dosing for the study was allometrically based on the clinical treatment schedules currently
212 approved for use for COVID-19 treatment. Molnupiravir (MK-4482) is prescribed as an 800mg
213 twice daily oral treatment (1600mg in total) within 5 days of symptom onset [31]. Similarly,
214 nirmatrelvir is an oral treatment prescribed for twice daily oral treatment of 300mg (600mg total)
215 with the addition of 100mg ritonavir (200mg/total) [32]. Each pharmaceutically active compound
216 was assessed in plasma taken at each clinical exam point prior to dosing and in lung
217 homogenates at necropsy to confirm their presence at the desire level and to ensure the absence
218 of any unanticipated drug interaction between the molnupiravir and nirmatrelvir treatments. The
219 levels of each active compound were not negatively affected by the combined treatment regimen.

220 Phase 3 clinical trials of molnupiravir indicate the drug is effective in preventing severe disease,
221 with lower adverse events documented than the placebo group [10]. Paxlovid™ is also an
222 effective treatment for COVID-19 when administered within 5 days of symptom onset [13].
223 However, recent reports of viral recrudescence following the cessation of molnupiravir and
224 Paxlovid™ monotherapy in a sub-set of patients has raised the question of efficacy in relation to
225 treatment length and/or dosage [33]. A recent single patient case study indicates that rebound, at
226 least for Paxlovid™, may not correspond to the development of genetic resistance [34].
227 Combination therapy of MK-4482 and PF-07321332 may counteract the “rebound effect” and
228 thereby enable maintained use of the relatively short 5-day treatment course. Loss of treatment
229 efficacy of a single treatment approach due to viral escape is also always a concern. *In vitro*
230 studies using coronaviruses related to SARS-CoV-2 suggest that molnupiravir may have a high
231 genetic barrier to development of drug resistance [35]. However, recent studies passaging SARS-
232 CoV-2 under suboptimal levels of nirmatrelvir have shown the relatively rapid development of
233 resistance corresponding to defined mutations within the viral protease [36, 37]. The use of a
234 combination therapy as established here would be expected to reduce the possibility for viral
235 escape, as has been established for treatment of other genetically mutable viruses, hepatitis C and
236 human immunodeficiency virus [38, 39] .

237 There are limitations to our study that need to be addressed in future experimental and clinical
238 studies. First, due to the mild-to-moderate level of clinical disease in rhesus macaques following
239 SARS-CoV-2 infection, it was not possible to assess the combination therapy against severe
240 disease. Since SARS-CoV-2 Delta infection likely shows the most severe infection in a NHP
241 model [26], this could be addressed in lethal rodent models or clinical trials. A suggestion of
242 such efficacy against more severe disease is indicated in the recent study using a lethal

243 transgenic COVID-19 mouse model [30]. Second, we only tested the efficacy of the current
244 human dose for monotherapy. Dose reduction might also be achievable in combination therapy
245 which needs to be carefully designed and analyzed. Third, escape mutant development needs to
246 be studied in comparison between mono- and combination therapy, especially given the recent
247 studies suggesting the susceptibility of nirmatrelvir to resistance development *in vitro* [36, 37].
248 Lastly, clinical trials need to ultimately show the benefit of the combination therapy, which if as
249 tolerable as suggested by our present study, could have benefits such as reducing disease severity
250 in the lower and upper respiratory tract, the latter of which could be a game changer by also
251 reducing transmission.

252 In conclusion, MK-4482 and PF-07321332 had potent inhibitory effects on SARS-CoV-2
253 replication, shedding and disease manifestation – now demonstrated in the NHP COVID-19
254 model, the closest surrogate to humans. Notably, the two drugs were most effective when used in
255 combination. Combination therapy could help to reduce the individual drug doses and slow down
256 drug resistance development. Combination therapy might also improve clinical outcomes in
257 those patients prone towards more severe disease development such as immunocompromised and
258 those with other underlying disease. This study strongly supports the use of MK-4482 or PF-
259 07321332 for the treatment of COVID-19 cases and provides the preclinical evidence for
260 combination therapy as an improved therapeutic option.

261

262 **Materials and Methods**

263 **Biosafety and ethics.** SARS-CoV-2 studies were approved by the Institutional Biosafety
264 Committee (IBC) and performed in the BSL-4 laboratories at Rocky Mountain Laboratories

265 (RML), NIAID, NIH. IBC-approved standard operating procedures were used for sample
266 removal from biocontainment. RML is an AALAC accredited facility, and the RML Institutional
267 Animal Care and Use Committee approved the animal studies. Animal studies followed
268 institutional guidelines for animal use, the guidelines and basic principles in the NIH Guide for
269 the Care and Use of Laboratory Animals, the Animal Welfare Act, United States Department of
270 Agriculture and the United States Public Health Service Policy on Humane Care and Use of
271 Laboratory Animals. Rhesus macaques were singly housed in adjacent primate cages to enable
272 social interactions between animals. The animal room was climate-controlled with a fixed 12
273 hours light-dark cycle. Animals were provided commercial monkey chow provided twice daily
274 with vegetables, fruit, and treats to supplement. Water was available *ad libitum*. Human
275 interaction, manipulanda, toys, video and music were provided for enrichment. Animals were
276 monitored for signs of disease at least twice daily throughout the experiment.

277 **Rhesus macaque study design.** Male and female rhesus macaques 2-12 years of age were
278 divided into vehicle (N=5) or treatment (N=5 for MK-4482, N=5 for PF-07321332, and N=5 for
279 the MK-4482/PF-07321332 combination) groups prior to infection. MK-4482 (DC Chemicals),
280 PF-07321332 (DC Chemicals) and ritonavir (DC Chemicals) were first dissolved in DMSO and
281 then resuspended to 5ml total volume in food grade peanut oil for oral delivery. Treated rhesus
282 macaques received either 130mg/kg MK-4482, 20mg/kg PF-07321332 + 6.5mg/kg ritonavir or
283 the combination of all 3 compounds (130mg/kg MK-4482 + 20mg/kg PF-07321332 +6.5mg
284 ritonavir) every 12 hours beginning 12hpi and ending 84hpi. Vehicle control animals received
285 the same treatment volume of peanut oil (5ml) on the same schedule as treatment groups.
286 Animals were infected with a total of 2×10^6 TCID₅₀ of the SARS-CoV-2 Delta variant by two
287 routes, intranasal and intratracheal. Intranasal inoculation consisted of a 0.5ml injection directly

288 into each nare (1.0 ml total) with a Mucosal Atomization Device (MAD) system. Intratracheal
289 inoculations were performed with the use of a bronchoscope for deposition of 4mls of virus
290 directly into the main-stem bronchi. All procedures were performed on anesthetized animals.
291 Animals were monitored twice daily and scored blindly every morning by the same person for
292 signs and progression of disease as previously reported [19] In brief, animals were scored based
293 on general and skin/coat appearance, activity, mucosal discharge, respiration, feces and urine
294 output, and appetite. Clinical exams were performed prior to challenge and at D0, D1, D2 and
295 D4. Oropharyngeal, nasal and rectal swabs, as well as whole blood and serum were collected at
296 every exam. Bronchoalveolar lavage samples were collected at D0, D1 and D2, but not at D4 to
297 avoid complications with pathology at time of autopsy. Animals were euthanized on day 4 post-
298 infection and tissues were collected at necropsy for virological and pathology analysis.

299 **Virus and cells.** SARS-CoV-2 variant hCoV-19/USA/KY-CDC-2-4242084/2021 (B.1.617.2,
300 Delta) was kindly contributed by B. Zhou, N. Thornburg and S. Tong (Centers for Disease
301 Control and Prevention, USA). The viral stock was sequenced via Illumina-based deep
302 sequencing to confirm identity and identify any possible contaminants prior to use. Virus
303 propagation was performed in DMEM (Sigma) supplemented with 2% fetal bovine serum
304 (Gibco), 1 mM L-glutamine (Gibco), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco).
305 Vero E6 cells, kindly provided by R. Baric, University of North Carolina, were maintained in
306 DMEM (Sigma) supplemented with 10% fetal calf serum, 1 mM L-glutamine, 50 U/mL
307 penicillin and 50 µg/mL streptomycin.

308 **Viral genome detection.** A QiaAmp Viral RNA kit was used to extract RNA from swabs or
309 tissue samples (30 mg or less). A Quantifast kit was used according to manufacturer's protocols
310 and one-step real-time RT-PCR was used to quantify subgenomic viral RNA using a region of

311 the E gene [40]. RNA standards counted by droplet digital PCR were used in 10-fold serial
312 dilutions and run in parallel to calculate viral RNA copies.

313 **Virus titration assay.** To calculate infectious virus titers in tissue samples, tissues were
314 homogenized in 1ml DMEM using a TissueLyzer (Qiagen) and clarified by low-speed
315 centrifugation. Vero-E6 cells were inoculated with 10-fold serial dilutions of homogenized lung
316 or swab samples in 100 μ l DMEM (Sigma-Aldrich) supplemented with 2% fetal bovine serum, 1
317 mM L-glutamine, 50 U/ml penicillin and 50 μ g/ml streptomycin. Cells were incubated for six
318 days and then scored for cytopathogenic effects (CPE) and TCID₅₀ was calculated via the Reed-
319 Muench formula [41].

320 **Pharmokinetics.** All solvents and extraction reagents were LCMS grade and purchased from
321 Fisher Scientific. Lung sections were extracted and levels of EIDD-1931 were measured as
322 previously described [21]. Plasma samples were processed via dilution of 100 μ L of plasma into
323 300 μ l of ice-cold methanol. The precipitant was cleared by centrifugation and the supernatant
324 was prepared for sample injection. Multiple reaction monitoring (MRM) ion pair signals were
325 developed and optimized for ritonavir and PF-07321332 (nirmatrelvir) from standards
326 (Supplemental Table 1). All pharmaceutical actives were measured from a single injection on a
327 Sciex ExionLC™ AC system with a Waters XBridge® Amide column (130 \AA , 3.5 μ m,
328 3 mm \times 100 mm) with binary gradient elution from 95% acetonitrile, 0.8% acetic acid,
329 10 mM ammonium acetate to 50% acetonitrile, 0.8% acetic acid, 10 mM ammonium. All
330 signals were detected in positive mode using a Sciex 5500 QTRAP® mass spectrometer
331 equipped with an electrospray ionization source (CUR: 40, CAD: Med, ISV: 2500, Temp: 450,
332 GS1: 50, GS2: 50). All signals were detected under positive mode ionization and compared to an
333 8-point standard curve. All molecules of interested were detected using two MRM pairs and

334 quantified using the higher signal to noise MRM. Limit of quantification was 400 pg/ml for PF-
335 07321332 and 300 pg/ml for ritonavir.

336 **Histopathology:** Tissues samples were collected, placed into cassettes and fixed in 10%
337 formalin for 7 days. Prior to removal from BSL-4, tissues were transferred into fresh 10%
338 formalin and incubated an additional 24 hours. Tissues were then processed with a Sakura VIP-6
339 Tissue Tek, on a 12-hour automated schedule, using a graded series of ethanol, xylene, and
340 PureAffin. Following processing, samples were embedded in Pureaffin paraffin polymer (Cancer
341 Diagnostics, Durham, NC, USA) and sectioned at 5 μ m and dried overnight at 42°C prior to
342 hematoxylin and eosin (H&E) staining. IHC tissues were processed using the Discovery Ultra
343 automated stainer (Ventana Medical Systems) with a ChromoMap DAB kit (Roche Tissue
344 Diagnostics cat#760-159) for immunohistochemistry (IHC) staining. Specific immunoreactivity
345 was detected using a validated GenScript U864YFA140-4/CB2093 NP-1 SARS-CoV-2-specific
346 antiserum at a 1:1000 dilution. The secondary antibody was an anti-rabbit IgG polymer (cat#
347 MP-6401) from Vector Laboratories ImPress VR as previously described [21].

348 **Statistical analyses.** Statistical analysis was performed in Prism 9. Difference in
349 viral load and infectious titers between study groups was assessed by ordinary two-way ANOVA
350 (multiple time points) or by multiple comparisons using the Kruskal-Wallis test (single time-
351 points). AUC was calculated and then analyzed by an ordinary one-way ANOVA.

352

353 **AUTHOR CONTRIBUTIONS**

354 K. Rosenke, M.A. Jarvis and H. Feldmann contributed to the design, data analysis, and writing of
355 the manuscript.

356 K. Rosenke, H. Feldmann, J. Lovaglio, M. Lewis, F. Feldmann, W. Bohler, C. Shaia and G.
357 Saturday contributed to the execution of the study

358 A. Okumura, M.C. Lewis, F. Feldmann, W. Bohler, E. Borhnsen, B. Schwarz, C. Bosio and J.
359 Callison contributed experimental support and data analysis.

360 G. Saturday and C. Shaia contributed to the pathological analysis.

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378 Plymouth.

379

380 **COMPETING INTERESTS**

381 MAJ is employed, in part, by the Vaccine Group Ltd. All authors declare that they have no
382 competing interests.

383

384 **DATA AND MATERIALS AVAILABILITY**

385 All data is presented here. Additional information can be requested through the corresponding
386 authors.

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391

392 **DISCLAIMER**

393 The opinions, conclusions and recommendations in this report are those of the authors and do not
394 necessarily represent the official positions of the National Institute of Allergy and Infectious
395 Diseases (NIAID) at the National Institutes of Health (NIH), The Vaccine Group Ltd or the
396 University of Plymouth.

397

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500

501 **Table 1**

Treatment	Vehicle	MK-4482	PF-07321332	MK-4482 + PF-07321332
Plasma				
EIDD-1931 (nmol)	0	46.47 ± 7.31	0	76.17 ± 15.53
PF-07321332 (nmol)	0	0	12.75 ± 4.41	19.21 ± 7.46
Ritonavir (nmol)	0	0	0.70 ± 0.36	0.31 ± 0.13
Lung				
EIDD-1931 (nmol/g)	0	21.52 ± 7.00	0	23.84 ± 6.58
PF-07321332 (nmol/g)	0	0	0.06 ± 0.03	0.36 ± 0.08
Ritonavir (nmol/g)	0	0	0.03 ± 0.02	0.13 ± 0.06

502

503 **Table 1: Measurements of pharmaceutical active or surrogate pharmaceutical metabolite**

504 **in each treatment group.** Measurements were made in plasma at each clinical examination

505 point prior to dosing and in lung homogenate at the study end point from a single lobe of the

506 lung. The EIDD-1931 metabolite was used as a surrogate for MK-4482 and its associated

507 metabolites due to instability of MK-4482. The mean and standard error on the mean are

508 displayed (plasma: n=15; lung tissue: n=5).

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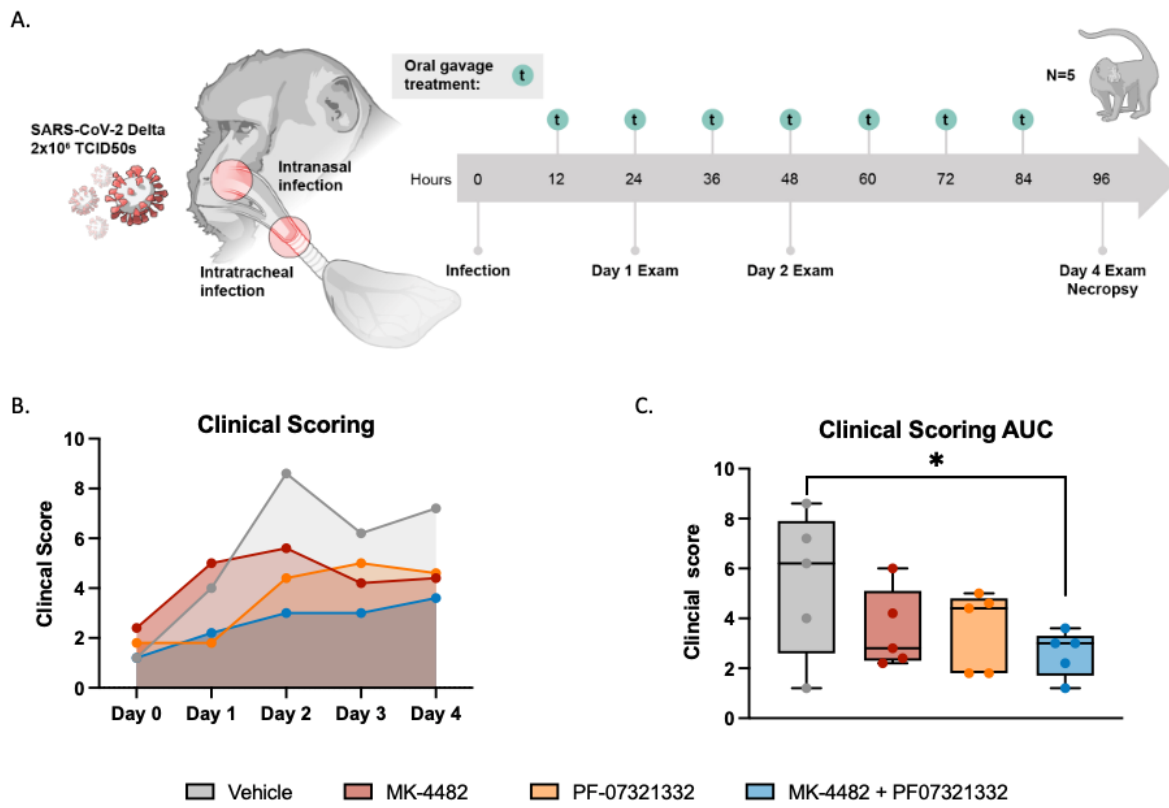
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Figure 1

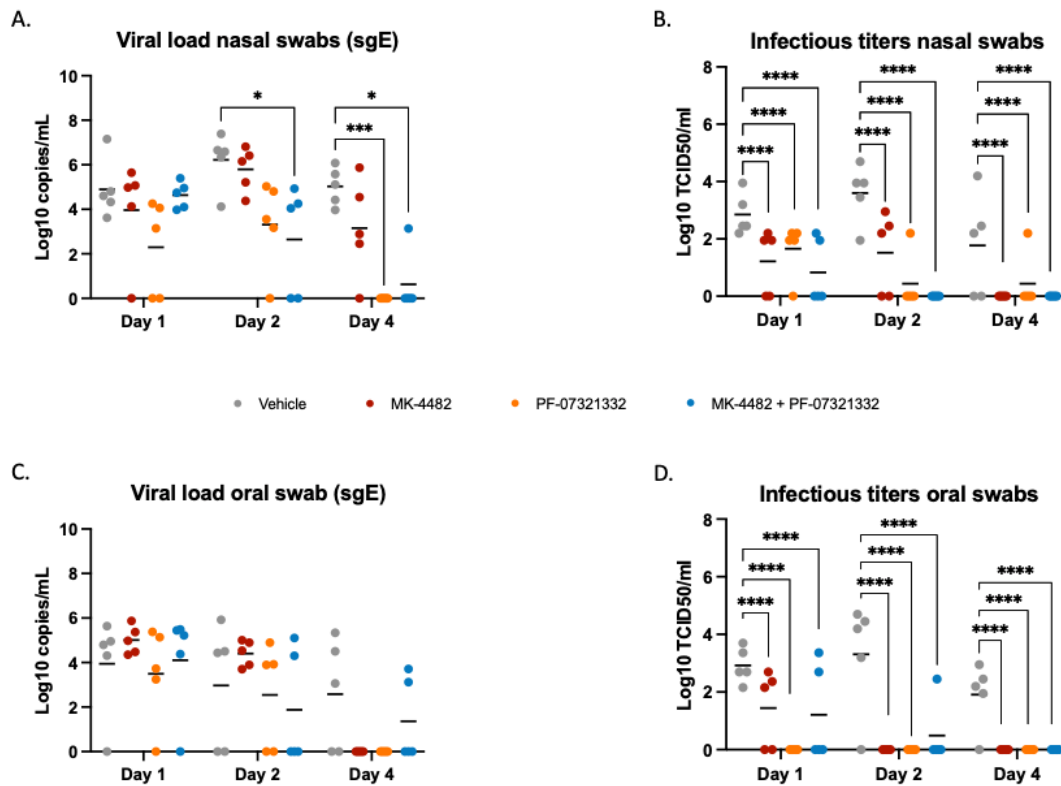


516

517 **Figure 1. Combination therapy of MK-4482 and PF-07321332 reduced clinical scoring in**
518 **rhesus macaques. Experimental design (A).** Rhesus macaques (n = 5) were infected with 2 x
519 10⁶ TCID₅₀ SARS-CoV-2 by combined intranasal and intratracheal routes. Treatments were
520 started 12 hours post-infection, with continued dosing every 12 hours. Clinical exams were
521 conducted on 1, 2 and 4 dpi and animals were euthanized and necropsied on 4dpi. **Clinical**
522 **scoring (B).** Animals were scored daily for clinical signs of disease over the course of the study.
523 **AUC analysis of clinical scoring (C).** Clinical scores were calculated for each animal per day,
524 AUC was then calculated over the course of the study and displayed in minimum-to-maximum
525 boxplot with median displayed. Ordinary one-way ANOVA with multiple comparisons were
526 used to evaluate significance (*P-value = 0.01 to 0.05).

527

Figure 2



528

529 **Figure 2. Combination therapy of MK-4482 and PF-07321332 significantly reduced virus**

530 **replication in the upper respiratory tract of SARS-CoV-2 infected rhesus macaques. Viral**

531 **RNA load in nasal (A) and oral swabs (C).** Nasal and oral swabs were collected on 1, 2 and

532 4dpi and viral RNA loads were determined by quantitative RT-PCR targeting sgE RNA.

533 **Infectious titers in nasal (B) and oral (D) swabs.** Nasal and oral swabs were collected on 1, 2

534 and 4dpi and infectivity was determined by using a tissue culture infectious dose (TCID) assay

535 with virus titers presented as TCID₅₀/ml. Statistical differences in viral load and infectious virus

536 titers in each study arm were assessed by a two-way ANOVA using Tukey's multiple

537 comparisons test (P-values, * = 0.01 to 0.05, ** = 0.001 to 0.01, *** = 0.0001 to 0.001, **** =
538 <0.0001).

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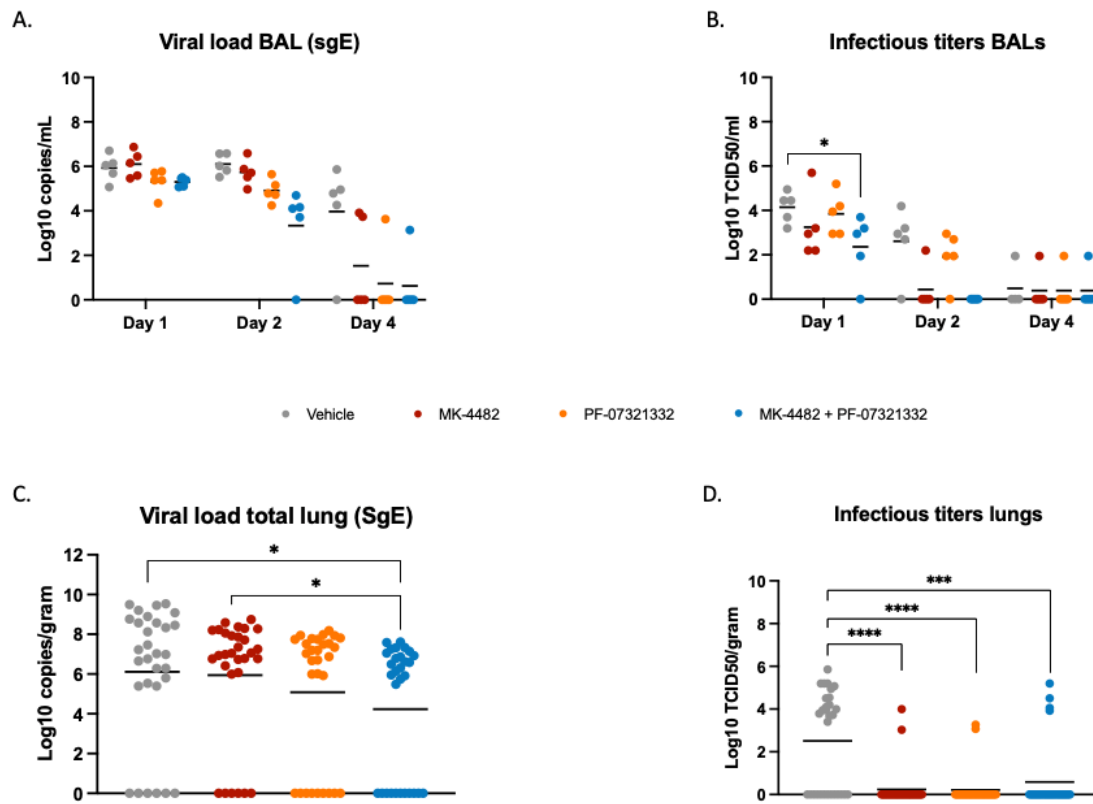
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Figure 3



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547 **Figure 3. Combination therapy of MK-4482 and PF-07321332 significantly reduced viral**
548 **replication in the lower respiratory tract of SARS-CoV-2 infected rhesus macaques. Viral**
549 **RNA load in BAL (A) and total lung lobes (C).** BAL samples were collected on 1, 2 and 4dpi
550 and lung samples were collected following necropsy on 4dpi. Viral RNA loads from BAL and
551 lung samples were determined by quantitative RT-PCR targeting sgE RNA. **Infectious titers in**
552 **BAL (B) and total lung lobes (D).** BAL samples were collected on 1, 2 and 4dpi and lung
553 samples were collected following necropsy on 4dpi. Infectivity was determined using a TCID₅₀
554 assay and are presented as TCID₅₀/ml. Samples from each lung lobe (n=6) of each animal were
555 assessed individually and compiled for analysis (C, D). Statistical differences in viral load and
556 infectious virus titers in the BAL were assessed by two-way ANOVA using Tukey's multiple

557 comparisons test. Statistical differences in total lung lobes were analyzed with Kruskal-Wallis
558 with Dunn's multiple comparisons test (P-values, * = 0.01 to 0.05, ** = 0.001 to 0.01, *** =
559 0.0001 to 0.001, **** = <0.0001).

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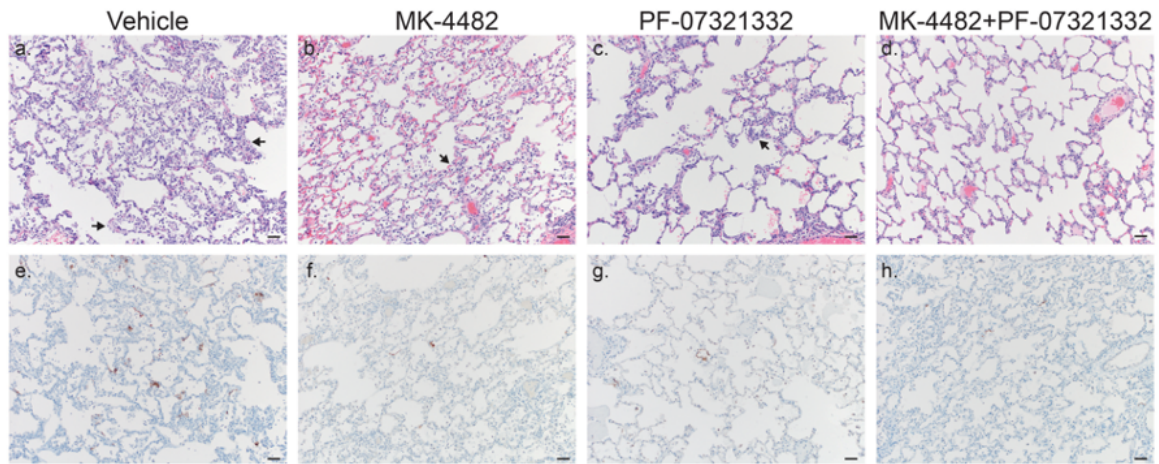
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Figure 4



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570 **Figure 4: Combination therapy reduced lung pathology and SARS-CoV-2 antigen load in**
571 **SARS-CoV-2 infected rhesus macaques.** Lung tissues were collected on 4dpi and stained with
572 either hematoxylin and eosin (H&E) or immunohistochemistry (IHC). **H&E staining of**
573 **representative tissues sections of the lungs (A-D). IHC staining of SARS-CoV-2 antigen in**
574 **corresponding representative lung sections (E-H).** (A) Vehicle control animals showed
575 moderate interstitial pneumonia (arrows) and moderate immunoreactivity of type I pneumocytes
576 in 4 of 5 animals (A & E). MK-4482 treated animals showed mild interstitial pneumonia
577 (arrows) and scattered immunoreactive type I pneumocytes in 3 of 5 animals (B & F). PF-
578 07321332 treated animals showed mild interstitial pneumonia (arrows) and scattered
579 immunoreactive type I pneumocytes in 2 of 5 animals (C & G). Combination treatment animals
580 basically showed no interstitial pneumonia with 1 of 5 animals displaying minimal lesions and
581 scattered immunoreactive type I pneumocytes (D & H). 100X, Bar=100µm

582

583

584 **Supplementary Table 1**

Target	MRM pair (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
PF-07321332*	500.0/110.0	75	10	30	7
PF-07321332	500.0/69.0	180	5	80	30
Ritonavir*	721.0/140.0	185	15	85	20
Ritonavir	721.0/268.0	120	5	30	40

585 *Used for quantification

586 Supplemental Table 1: MRM signals were identified and optimized for ritonavir and PF-
587 07321332 (nirmatrelvir) from standards. Key: MRM: multiple reaction monitoring; DP:
588 declustering potential; EP: entrance potential; CE: collision cell entrance potential; CXP:
589 collision cell exit potential

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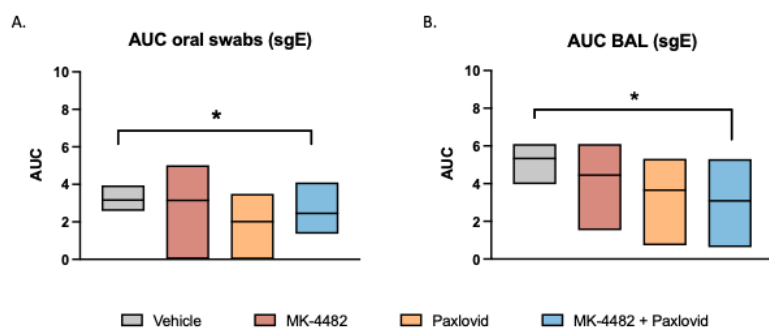
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Figure S1



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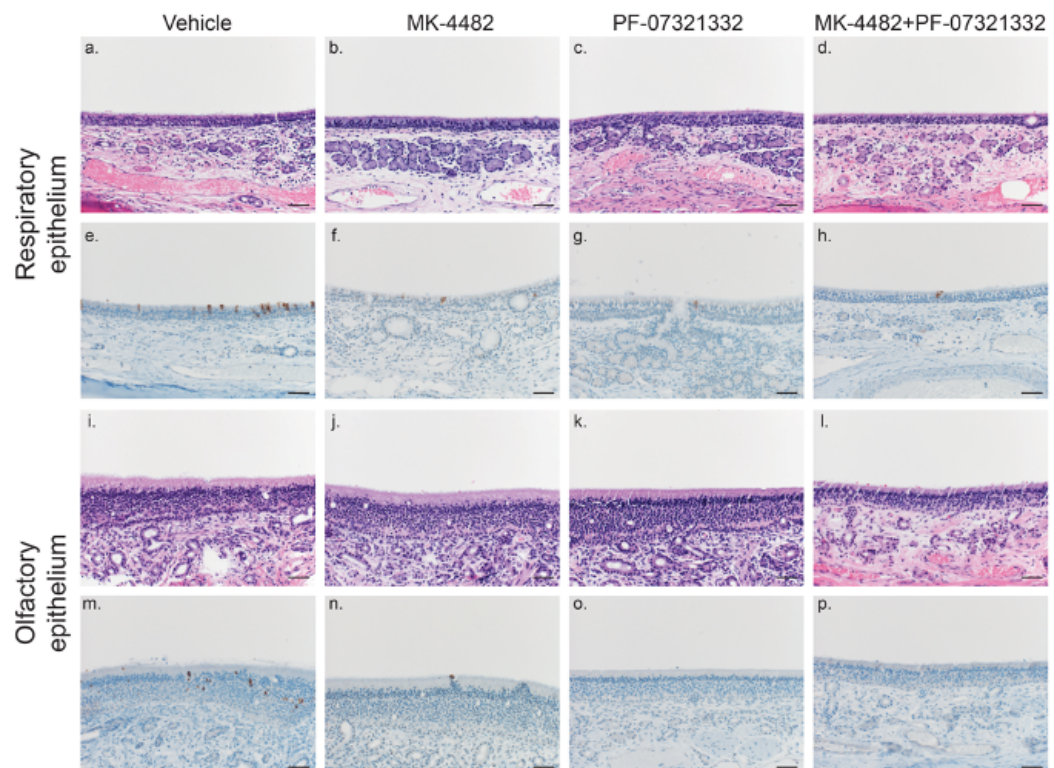
597 **Supplementary figure 1. AUC analysis of oral swabs and BAL fluid.** Viral RNA loads from
598 oral swabs (**A**) and BAL (**B**) samples were determined by quantitative RT-PCR targeting sgE
599 RNA as a surrogate for replication and shedding. Copy numbers of viral genomes were
600 calculated for each animal per day, AUC was then calculated over the course of the study and
601 displayed in a boxplot with the mean displayed. Ordinary one-way ANOVA with multiple
602 comparisons were used to evaluate significance (*P-value = 0.01 to 0.05).

603

604

605

Figure S2



606

607 **Supplementary figure 2. Combination therapy reduced antigen load in olfactory and**
608 **respiratory epithelium from nasal turbinates in SARS-CoV-2 infected rhesus macaques.**
609 Tissues were collected on 4dpi and stained with H&E or IHC for analysis. **H&E staining of**
610 **representative tissues sections of the respiratory epithelium (A-D).** No pathology was found
611 in H&E stains of the nasal turbinates. **IHC staining of SARS-CoV-2 antigen in respiratory**
612 **epithelium (E-H).** Reduced or no IHC stain was found in the respiratory epithelium of MK-
613 4482, PF-07321332 and MK-4482 + PF-07321332 treated animals compared to vehicle controls.
614 **H&E staining of representative tissues sections of the olfactory epithelium from nasal**
615 **turbinate (I-L).** No pathology was found in the olfactory epithelium. **IHC staining of SARS-**
616 **CoV-2 antigen in olfactory epithelium (M-P).** IHC analysis did show scattered
617 immunoreactivity in the vehicle treated animals and little to no observable viral antigen in the
618 MK-4482, PF-07321332 and MK-4482 + PF-07321332 treated animals 200X, Bar=50µm

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