

1 **Prophylactic intranasal administration of a TLR2 agonist reduces**
2 **upper respiratory tract viral shedding in a SARS-CoV-2 challenge**
3 **ferret model**

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52 **Abstract:**

53

54 Respiratory viruses such as coronaviruses represent major ongoing global threats,
55 causing epidemics and pandemics with huge economic burden. Rapid spread of virus
56 through populations poses an enormous challenge for outbreak control. Like all
57 respiratory viruses, the most recent novel human coronavirus SARS-CoV-2, initiates
58 infection in the upper respiratory tract (URT). Infected individuals are often
59 asymptomatic, yet highly infectious and readily transmit virus. A therapy that restricts
60 initial replication in the URT has the potential to prevent progression of severe lower
61 respiratory tract disease as well as limiting person-to-person transmission.

62 We show that prophylactic intra-nasal administration of the TLR2/6 agonist INNA-051 in
63 a SARS-CoV-2 ferret infection model effectively reduces levels of viral RNA in the nose
64 and throat. The results of our study support clinical development of a therapy based on
65 prophylactic TLR2/6 innate immune activation in the URT to reduce SARS-CoV-2
66 transmission and provide protection against COVID-19.

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68

69 **Keywords: Ferret; COVID-19; SARS-CoV-2; animal model; coronavirus; viral**
70 **shedding; TLR-2; transmission; INNA-051.**

71 Coronaviruses (CoV) are pleomorphic, positive-sense, single-stranded RNA-enveloped
72 viruses, members of the *Coronaviridae* family, that mainly infect wild animals and cause
73 mild disease¹. In addition, seven human CoVs that belong to either the
74 Alphacoronavirus- or Betacoronavirus-genus have now been identified. Four of these
75 human CoVs usually cause a mild, upper respiratory tract illness (common cold). Another
76 three novel human CoVs have emerged in the past two decades through transmission
77 to humans via an intermediate animal host², and caused outbreaks of significant
78 respiratory morbidity and mortality: in 2003, Severe Acute Respiratory Syndrome
79 (SARS) CoV in China³, in 2012 Middle Eastern Respiratory Syndrome (MERS) CoV in
80 Saudi Arabia⁴ and more recently in December 2019, SARS-CoV-2, a novel CoV type
81 identified in lower respiratory tract of patients presenting viral pneumonia in Wuhan,
82 China⁵. Unlike the highly pathogenic SARS or MERS CoVs, SARS-CoV-2 infections
83 have spread rapidly around the globe, causing broad spectrum respiratory symptoms,
84 from very mild to severe, life-threatening disease (COVID-19) mostly in at risk
85 populations such as the elderly and those with comorbidities.

86 As with other respiratory CoVs, SARS-CoV-2 primarily spreads via the airborne route,
87 with respiratory droplets expelled by infected individuals⁶. Virus can be transmitted
88 from symptomatic, as well as pre- or asymptomatic individuals^{7,8}, with asymptomatic
89 individuals being able to shed virus, and therefore being capable to transmit the disease,
90 for longer than those with symptoms⁹. As with other respiratory viruses such as influenza,
91 recent evidence suggests that, the epithelium of the upper respiratory tract (URT) is the
92 initial site of SARS-CoV-2 infection^{10,11}. This is consistent with the abundant nasal
93 epithelial cell expression of the SARS-CoV-2 receptor, angiotensin-converting enzyme 2
94 (ACE2) and its decreasing expression throughout the lower respiratory tract¹¹.

95 A topical treatment of the URT that boosts anti-viral immunity and restricts viral replication
96 is a promising method to promote viral clearance, reduce viral shedding and
97 transmission. The TLRs are key microbe-recognition receptors with a crucial role in
98 activation of host defence and protection from infections and therefore attractive drug
99 targets against infectious diseases¹²⁻¹⁴. Synthetic agonists of the intracellular viral
100 DNA/RNA-recognising TLR molecules, TLR3, TLR7/8 and TLR9, are capable of
101 boosting protective innate immune responses against respiratory viruses. However,
102 their success in the clinic has been limited, due to short-duration of benefit or induction
103 of adverse effects, related to the release of pro-inflammatory cytokines and activation
104 of the type-1 Interferon pathway¹⁵⁻¹⁸. TLRs expressed on the cell surface such as TLR2

105 offer an alternative approach. TLR2 dimerizes with TLR1 or TLR6 to recognize a broad
106 variety of commensal and pathogenic microbial molecules and its activation is tightly
107 regulated to maintain immune homeostasis¹⁹. A series of novel synthetic molecules,
108 named the INNA compounds, have been developed with TLR2/6 agonist properties.
109 Importantly, TLR2/6 agonists of the INNA compound series do not directly activate
110 Type-1 interferons (unpublished data).

111 Airways administration of INNA compounds has been shown to protect from lethal
112 influenza virus infection, prevent viral transmission and secondary bacterial
113 superinfections in mouse disease models²⁰⁻²². Intranasal (i.n.) treatment with INNA
114 compounds also reduces viral load and lung inflammation in mouse models of
115 rhinovirus infection (unpublished data). The demonstrated prophylactic benefit is
116 associated with fast TLR2/6-mediated up-regulation of a series of innate immune
117 response elements in airway epithelial cells, defined by early, rapid expression of NF-
118 κ B-regulated anti-microbial genes, including IFN- λ and chemokines, that precede
119 immune cell recruitment and support prolonged antiviral defence, suppresses viral
120 load and virus-induced pulmonary inflammation (unpublished data).

121 To determine whether TLR2/6 agonists are also active against SARS-CoV-2, we used
122 prophylactic i.n. administration of the novel compound INNA-051, in a SARS-CoV-2
123 challenge ferret model²³.

124 Ferret challenge models are commonly used to understand human respiratory virus-
125 induced diseases and to evaluate the efficacy of related vaccines and drugs^{24,25}. Use of
126 ferrets is appropriate in the case of SARS-CoV-2 infection, as they express the virus
127 entry ACE2 receptor in their airways²⁶⁻²⁸ and SARS-CoV-2 i.n. inoculation in ferrets
128 results in virus replication in the URT and dose-dependent viral shedding^{23,28}.

129 In the present study, ferrets received two i.n. administrations of INNA-051 or vehicle-
130 control (PBS), at 4 days before and 1 day prior to i.n. challenge with 5.0×10^6 plaque-
131 forming units (PFU) of SARS-CoV-2 in 1ml volume (day 0). Four groups (6
132 animals/group) were used: Group 1 received two 1 ml doses (100 μ g/mL) INNA-051
133 (high dose); Group 2 received two 1 ml doses (20 μ g/mL) INNA-051 doses (low dose);
134 Group 3 received one 1ml dose (20 μ g/mL) INNA-051 at -4 days and one 1 ml dose
135 INNA-051 (100 μ g/mL) at -1 day (mixed dose); Group 4 received two doses of vehicle
136 (PBS) alone. After inoculation with SARS-CoV-2, ferrets were monitored for 12 days.
137 In life samples were taken at days 1, 3, 5, 7, 10 and 12, with scheduled culls at days
138 3 (n=6) and end of study days 12-14 (n=18) (Fig 1A).

139 Previous *in vivo* studies in mice have shown that respiratory application of INNA
140 compounds have a good safety profile, without significant pro-inflammatory side
141 effects or systemic cytokine release syndrome (unpublished data). Intranasal
142 administration to ferrets of two doses of INNA-051, prior to SARS-CoV-2 challenge,
143 did not induce observable or, measurable clinical signs of inflammation or changes in
144 the animal's activity.

145 Assessment of body temperature revealed some variation between treatment groups
146 (Fig 1B), with 2 of 6 ferrets in the INNA-051 high dose group 1 showing a transient
147 increase of temperature $>40.0^{\circ}\text{C}$, only after the first, but not the second dose. No
148 significant changes from baseline were noticed in body weight, with the variation
149 among groups being less than 2% (Fig 1C).

150 It has been previously described that SARS-CoV-2 infection in ferrets is not associated
151 with the development of severe symptomatology, but it represents a robust model of
152 mild disease that allows the study of respiratory viral replication^{23,29}. In this context, no
153 obvious differences in clinical disease signs were observed among treatment groups
154 in this experimental study. Assessment of body temperature (Fig 1B) and weight loss
155 (Fig 1C) did not reveal significant variation between the INNA-051 and PBS-treated
156 groups.

157 To follow the dynamics of SARS-CoV-2 viral replication and assess the impact of
158 INNA-051 prophylactic treatment, nasal wash and throat swab samples were taken 4
159 days before viral challenge and at 1, 3, 5, 7, 10 and 12-days post challenge (dpc).
160 Analysis of viral RNA in nasal wash samples at 1 dpc confirmed infection in all
161 treatment groups, with lower viral RNA levels detected in INNA-051 treatment Group
162 3 (Fig. 2A). Reduction of viral RNA in treatment Group 3 was also evident at 3 dpc
163 ($p=0.0155$) (Fig. 2A). By 5 dpc, all INNA-051 treated groups had significantly (>10 -
164 fold) reduced viral RNA compared to the vehicle-control group (2-way ANOVA
165 Dunnett's multiple comparison test: Group 1 $p=0.0244$; Group 2 $p=0.0107$; Group 3
166 $p=0.0071$ compared to vehicle-control Group 4) (Fig 2A). On 5 dpc, the viral RNA
167 levels in the nasal washes of the majority of INNA-051 treated animals remained low
168 or below quantifiable limits throughout the course of infection. Viral RNA levels were
169 found to be below the level of quantification in nasal washes of PBS-treated animals
170 from 10 dpc onwards (Fig 2A).

171 Analysis of viral RNA in throat swabs provided further evidence of the capacity of
172 INNA-051 treatment to reduce SARS-CoV-2 in the URT (Fig 2B). On 3 dpc lower viral

173 RNA levels were found in throat swabs of INNA-051 treated animals, with significantly
174 greater reduction again observed ($p=0.0345$) in INNA-051 treatment Group 3. By 5
175 dpc, all groups treated with INNA-051 had significantly reduced (>10 fold) viral RNA
176 levels, compared to the vehicle control group (2-way ANOVA Dunnett's multiple
177 comparison: Group 1 $p=0.0002$, Group 2 $p=<0.0001$ and Group 3 $p=0.0039$ compared
178 to vehicle control Group 4). Highly significantly reduction in viral RNA in the throat of
179 INNA-051 treated animals was also apparent on 7 dpc (2-way ANOVA Dunnett's
180 multiple comparison: Group 1 $p=0.0014$, Group 2 $p=<0.0001$ and Group 3 $p=0.0002$
181 compared to vehicle control Group 4), while by 10 dpc to the end of the study, the
182 levels of viral RNA were below the limit of quantitation in all treatment groups (Fig 2B).
183 Because all INNA-051 treatment groups exhibited reduced viral RNA in the nose and
184 throat, we combined these groups into a single data set (supplementary figures) and
185 compared to the group treated with vehicle. Using 2-way ANOVA Sidak's multiple
186 comparison test, significant (>10 fold) reduction in nasal viral RNA was observed at 5
187 dpc ($p=0.0057$) and highly significant ($p<0.0001$), greater than 10-fold reduction in
188 throat viral RNA was apparent from 5 to 7 dpc following INNA-051 i.n. treatment
189 (Figure S1). Group 2 (20ug/mL) appears to be the most optimal dosing in this study
190 After 5 days post-exposure to SARS-CoV-2, INNA-051 had statistically significant
191 reduction of virus in throat swabs ($p=<0.0001$) with 24 fold (96% reduction) and nasal
192 wash ($p=0.0107$) 15 fold (93% reduction) in this group compared to untreated animals.
193 These results indicate a similar profile with the protective effects of natural acquired
194 immunity in the ferret challenge model, as observed following re-challenge with SARS-
195 CoV-2²³.

196 To assess SARS-CoV-2 detected beyond the URT, lung tissue samples were
197 collected, on scheduled cull day 3 (6/24 animals) and days 12-14 (18/24 animals) dpc
198 and analysed for viral RNA levels. On day 3 dpc, two culled ferrets from the control
199 vehicle group had detectable viral RNA levels (7.42×10^4 and 2.86×10^4 copies/ml) (Fig
200 2C). There was one ferret in Group 1 showing detectable, but below the quantifiable
201 limit, viral RNA, and no other INNA-051 treated ferrets showing detectable viral RNA
202 in lung tissue on day 3 and days 12-14 dpc

203 This study provides evidence supporting a novel approach to prevent SARS-CoV-2
204 transmission, based on reduced viral shedding, following prophylactic i.n
205 administration of INNA-051. Global efforts for prevention of SARS-CoV-2 infection
206 have so far been mostly focused on social distancing and hygiene measures as well

207 as on R&D efforts for the development of vaccines. Our data demonstrate, for the first
208 time, in an *in vivo* SARS-CoV-2 infection model, that INNA-051 is highly effective at
209 reducing URT viral shedding, providing the potential to control virus transmission and
210 COVID-19 disease.

211 TLR2 stimulation at mucosal surfaces triggers rapid up-regulation of protective, innate
212 immune defence responses, and also activates counter-regulatory signalling that
213 suppresses development of excessive inflammation and tissue damage and promotes
214 the integrity of local epithelial barrier function^{30,31}. In addition, the INNA compounds
215 have been specifically designed to exert TLR2-mediated pharmacological activity on
216 mucosal epithelium, without being systemically absorbed (Ena Respiratory
217 unpublished data), a property that is expected to facilitate their development as safe,
218 antiviral drug candidates. The lack of obvious clinical signs of inflammation following
219 the administration of two doses of INNA-051 administered i.n supports this view.
220 Histopathology from the study indicates that i.n. INNA-051 administration does not
221 exacerbate SARS-CoV-2 pathology in the ferret lung in this setting (Figure S2).

222 It has been previously shown that i.n administration of an INNA compound in a mouse
223 model of influenza triggers a cascade of innate immune signals that results in
224 reduction of viral load, prevention of lower-respiratory infection and viral transmission
225 between animals²⁰⁻²². In a mouse rhinovirus infection model, we have shown that
226 prophylactic treatment with an INNA compounds primes airway mucosal immunity
227 providing prolong protection (unpublished data). Effective and accelerated anti-viral
228 responses against rhinovirus have also been demonstrated following INNA compound
229 treatment in an *in vitro* model of primary bronchial epithelial cells from healthy
230 individuals and donors with asthma (unpublished data).

231 Although the ferret SARS-CoV-2 model has limitations and may not represent the severe
232 spectrum of COVID-19 disease, our findings are highly encouraging and indicative of the
233 potential impact i.n. administration of INNA-051 prophylactically may have against
234 SARS-CoV-2 in humans. The fact that a significant reduction of URT viral RNA levels
235 was observed in INNA-051-treated outbred ferrets during the peak of viral replication
236 (5-7 days dpc) in this model²³ implies airway immunity priming and enhancement of
237 antiviral host defence. The predictive value of antiviral effectiveness data from
238 respiratory viral infection ferret models and translation into human infectious disease has
239 been established^{25,32}. For this reason, the SARS-CoV-2 ferret model has been used,
240 during the current pandemic, to evaluate the therapeutic effect of a number of FDA-

241 approved/repurposed drugs including, lopinavir-ritonavir, hydroxychloroquine sulfate,
242 or emtricitabine-tenofovir³³. These drugs were found to have no or only modest (~4
243 fold for emtricitabine-tenofovir) effect against SARS-CoV2 viral replication, as
244 measured by viral titres in nasal wash from the ferrets³³.

245 Substantial reduction of SARS-CoV viral shedding in the URT and therefore control of
246 respiratory virus transmission may not be easily achievable without potentiation of
247 airways antiviral immune defences³⁴. Systemic antiviral drugs, as well as vaccines
248 may not be effective in halting respiratory viral transmission even if they achieve
249 suppression of clinical disease and in fact preliminary results from an experimental
250 study with one of the leading SARS-CoV-2 vaccine candidates (an adenovirus-
251 construct expressing SARS-CoV-2 spike protein) in non-human primates have shown
252 little effect on the virus load in nasal washes³⁵. To address these potential limitations,
253 particularly during the urgent circumstances of an epidemic, parallel use of an i.n.
254 administered innate immune modulator with the characteristics of INNA-051 may be
255 highly appropriate to rapidly boost innate immunity at the primary site of respiratory
256 infection which is protective within days of treatment. The use of i.n. INNA-051 for
257 antiviral respiratory prophylaxis therefore offers several additional advantages,
258 including fast-acting protection, and is in contrast to vaccines that take 2-4 weeks to
259 mount a protective response. The limited risk for development of antiviral resistance,
260 the option of self-administration and the non-prohibitive cost for large-scale
261 manufacturing are also especially attractive factors.

262 In conclusion, this study provides evidence that prophylactic i.n. administration of the
263 TLR2/6 agonist INNA-051 offers a promising approach for prevention and
264 management of SARS-CoV-2 infection that can be used as a stand-alone method of
265 antiviral prophylaxis and is complimentary to potential vaccination programs. This
266 approach is particularly appealing to individuals at elevated risk of community
267 transmission or development of severe disease, including front-line health care
268 workers, vulnerable communities, the elderly, the immunocompromised and those
269 with existing comorbidities.

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271

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276

277 **Contributions**

278 N.W.B, B.Y.C, C.D, D.C.J, F.M, P.P, D.T and M.W.C. contributed to the concept and
279 design of the study.

280 P.P and D.T. wrote the manuscript.

281 N.W.B, B.Y.C, C.D, S.D, D.C.J, F.M. and M.W.C. provided critical review.

282 P.P co-ordinated the study and performed analysis on data generated.

283 J.D. and M.G.C. provided virus strain.

284 K.R.B. grew viral stock, provided challenge material and assisted with statistical
285 analysis.

286 S.A.F and P.Y. managed all animal procedures.

287 B.E.C, R.C, C.M.K.H, V.L, D.N, K.A.R. and S.T. contributed to the processing of all
288 animal samples at containment level 3.

289 E.R directed histology experiments and performed critical assessment of pathology.

290 M.J.A, N.I.W and R.J.W. performed the extraction and PCR of samples.

291 G.S performed quality control and analytical assistance on PCR data.

292

293 **Conflicts of Interest**

294 No conflicts of interest declared.

295

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312 **Methods**

313 **Animals.**

314 Twenty-four healthy, female outbred ferrets (*Mustela putorius furo*) aged 6-8 months
315 were obtained from a UK Home Office accredited supplier. The mean weight at the
316 time of first INNA-051 treatment was 845g/ferret (range 740-1040g). Animals were
317 housed in social groups of six prior to and post INNA-051 treatment at Advisory
318 Committee on Dangerous Pathogens (ACDP) containment level 2. Animals were
319 transferred to ACDP containment level 3 and housed in pairs post SARS-CoV-2
320 challenge. Cages met with the UK Home Office *Code of Practice for the Housing and*
321 *Care of Animals Bred, Supplied or Used for Scientific Procedures* (December 2014).
322 Access to food and water was *ad libitum* and environmental enrichment was provided.
323 All experimental work was conducted in accordance with and under the authority of a
324 UK Home Office approved project licence that had been subject to local ethical review
325 at PHE Porton Down by the Animal Welfare and Ethical Review Body (AWERB) as
326 required by the *Home Office Animals (Scientific Procedures) Act 1986*. Animals were
327 sedated by intramuscular injection of ketamine/xylazine (17.9 mg/kg and 3.6 mg/kg
328 bodyweight) for administering of treatments, in-life sampling and viral challenge.

329

330 **INNA-051.**

331 INNA-051 belongs to a series of closely-related, pegylated synthetic analogues of the
332 diacylated lipopeptide, S-[2,3-bis(palmitoyl oxy)propyl] cysteine (Pam₂Cys) (INNA
333 compound series), with selective TLR2/TLR6 agonist activity. Pam₂Cys is inherently
334 insoluble and has been rendered soluble by others through addition of the amino acid
335 motif SK4³⁶. Oligo lysine sequences have, however, been shown to be toxic, albeit at
336 high concentration³⁷ and to modulate viral infection processes independent of TLR
337 activation³⁸. Any off-target effects were mitigated by incorporating polyethylene glycol
338 as a solubilising agent, in the INNA compound series³⁸. The EC₅₀s for INNA-051 for
339 the human TLR2/6 receptor is calculated at 40.1 pg/mL or ~19pM.

340 Freeze dried INNA-051 provided by Ena Respiratory, Melbourne, Australia was
341 resuspended in phosphate buffered saline (PBS) (1mg/ml) and stored 2-8°C.
342 Immediately prior to treatment, INNA-051 (1mg/ml) was further diluted in PBS to the
343 required treatment doses; high dose (100µg/ml), low dose (20µg/ml) and mixed dose
344 (20µg/ml first dose and 100µg/ml second dose).

345

346 **Inoculum.**

347 SARS-CoV-2 Victoria/01/2020³⁹ was generously provided by Peter Doherty Institute
348 for Infection and Immunity, Melbourne, Australia at P1 and passaged twice in
349 Vero/hSLAM cells [ECACC 04091501], obtained from the European Collection of
350 Authenticated Cell Cultures (ECACC) PHE, Porton Down, UK. Whole genome
351 sequencing was performed, on the challenge isolate, SISPA protocol and then
352 sequenced using Nanopore as described previously⁴⁰. Virus titre was determined by
353 plaque assay on Vero/E6 cells [ECACC 85020206]. Challenge substance dilutions
354 were conducted in Phosphate Buffer Saline (PBS). Challenge virus was delivered by
355 intranasal instillation (1.0 ml total, 0.5 ml per nostril) at 5.0×10^6 pfu/ml.

356

357 **Experimental Design.**

358 Experimental design and viral challenge dose were informed by a previous dose-
359 dependent ferret study²³. Prior to commencing the experiment, animals were randomly
360 assigned to the four treatment groups, to minimise bias. A temperature/ID chip (Bio-
361 Thermo Identichip, Animalcare Ltd, UK) was inserted subcutaneously into the dorsal
362 cervical region of each animal.

363

364 INNA-051 was delivered by intranasal instillation (1.0 ml total, 0.5 ml per nostril) to
365 three groups (n=6) of ferrets 4 days and 1 day prior to challenge. On each day, group
366 1 received a high dose [100µg/ml], group 2 a low dose [20µg/ml] and group 3 received
367 a 20µg/ml dose 4 days prior to challenge and a 100µg/ml] dose 1 day before challenge.
368 PBS was delivered to control group ferrets (n=6) 4 days and 1 day prior to challenge.
369 Two ferrets each from the high dose, low dose and control groups were scheduled for
370 euthanasia on day 3 (n=6). Remaining ferrets (n=18) were scheduled for euthanasia
371 on days 12-14; high and low dose [day 12 n=1, day 13 n=2, day 14 n=1], mixed dose
372 [n=2 days 12-14] and control [n=2 days 12 and 14].

373

374 Nasal washes and throat swabs for all ferrets were taken prior to first treatment, at
375 days 1 and 3 pc (n=24) and at days 5, 7, 10 and 12 pc for surviving ferrets (n=18). At
376 necropsy, tissue samples were taken for histopathology and analysed by PCR. Nasal
377 washes were obtained by flushing the nasal cavity with 2 ml PBS. Cotton throat swabs
378 (Koehler Technische Produkten, VWR) were gently stroked across the back of the

379 pharynx in the tonsillar area and retained in viral transport media (VTM). Throat swabs
380 were processed, and aliquots were stored in AVL at -80°C until assay.

381

382 **Clinical and euthanasia observations.**

383 Animals were monitored for clinical signs of disease twice daily (approximately 8 hours
384 apart) for the entirety of the experiment. Clinical signs of disease were assigned a
385 score based upon the following criteria. Activity was scored as follows; 0 = alert and
386 playful, 1 = alert, playful when stimulated, 2 = alert, not playful when stimulated, 3 =
387 not alert or playful. No clinical signs were noted throughout the experiment. To meet
388 the requirement of the project license, immobility, neurological signs or a sudden drop
389 in temperature were predetermined automatic euthanasia criteria. Animals were also
390 deemed to have reached a humane endpoint if their body weight was at or below 30%
391 baseline. If any ferret reached any of these three criteria, they were to be immediately
392 euthanised using a UK Home Office approved Schedule 1 procedure. No animals
393 reached these end-points during this study.

394

395 Temperature was taken using a microchip reader and implanted temperature/ID chip.
396 Temperature was recorded at each clinical scoring point using the chip to ensure any
397 peak of fever was recorded. Animals were weighed at the same time each day
398 throughout the experiment.

399

400 **Necropsy Procedures.**

401 Ferrets were anaesthetised with ketamine/xylazine (17.9 mg/kg and 3.6 mg/kg
402 bodyweight) and exsanguination was effected via cardiac puncture, followed by
403 injection of an anaesthetic overdose (sodium pentobarbitone Dolelethal, Vetquinol UK
404 Ltd, 140 mg/kg). A necropsy was performed immediately after confirmation of death.
405 The left lung was dissected and used for subsequent virology procedures.

406

407 **RNA Extraction.**

408 RNA was isolated from nasal wash, throat swabs, EDTA treated whole blood, BAL
409 and lung tissue. Weighed lung tissue was homogenised and inactivated in RLT
410 (Qiagen) supplemented with 1%(v/v) Beta-mercaptoethanol. Tissue homogenate was
411 then centrifuged through a QIAshredder homogenizer (Qiagen) and supplemented
412 with ethanol as per manufacturer's instructions. Non-tissue samples were inactivated

413 in AVL (Qiagen) and ethanol. Downstream extraction on all inactivated samples was
414 then performed using the BioSprint™96 One-For-All vet kit (Indical) and Kingfisher
415 Flex platform as per manufacturer's instructions.

416

417 **Quantification of Viral Loads by RT-qPCR.**

418 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) targeting a
419 region of the SARS-CoV-2 nucleocapsid (N) gene was used to determine viral loads
420 and was performed using TaqPath™ 1-Step RT-qPCR Master Mix, CG (Applied
421 Biosystems™), 2019-nCoV CDC RUO Kit (Integrated DNA Technologies) and
422 QuantStudio™ 7 Flex Real-Time PCR System. Sequences of the N1 primers and
423 probe were: 2019-nCoV_N1-forward, 5' GACCCCAAATCAGCGAAAT 3'; 2019-
424 nCoV_N1-reverse, 5' TCTGGTACTGCCAGTTGAATCTG 3'; 2019-nCoV_N1-probe,
425 5' FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1 3'. The cycling conditions were:
426 25°C for 2 minutes, 50°C for 15 minutes, 95°C for 2 minutes, followed by 45 cycles of
427 95°C for 3 seconds, 55°C for 30 seconds. The quantification standard was *in vitro*
428 transcribed RNA of the SARS-CoV-2 N ORF (accession number NC_045512.2) with
429 quantification between 1×10^1 and 1×10^6 copies/ μ l. Positive samples detected below
430 the lower limit of quantification (LLOQ) were assigned the value of 5 copies/ μ l, whilst
431 undetected samples were assigned the value of < 2.3 copies/ μ l, equivalent to the
432 assays lower limit of detection (LLOD).

433

434 **Histopathological Analysis.**

435 Samples from the left cranial and left caudal lung lobe together with nasal cavity, were
436 fixed by immersion in 10% neutral-buffered formalin and processed routinely into
437 paraffin wax. Nasal cavity samples were decalcified using an EDTA-based solution
438 prior to embedding. 4 μ m sections were cut and stained with haematoxylin and eosin
439 (H&E) and examined microscopically. In addition, samples were stained using the
440 RNAscope technique to identify the SARS-CoV-2 virus RNA. Briefly, tissues were pre-
441 treated with hydrogen peroxide for 10 minutes (room temperature), target retrieval for
442 15 mins (98-101°C) and protease plus for 30 mins (40°C) (Advanced Cell Diagnostics).
443 A V-nCoV2019-S probe (Cat No. 848561, Advanced Cell Diagnostics) was incubated
444 on the tissues for 2 hours at 40°C. Amplification of the signal was carried out following
445 the RNAscope protocol using the RNAscope 2.5 HD Detection kit – Red (Advanced
446 Cell Diagnostics).

447

448 **Statistical analysis.**

449 Virology data were compared by two-way ANOVA with Dunnett's multiple comparisons
450 test and two-way ANOVA with Sidak's multiple comparisons test. Transformed values
451 fitted to a straight line in a QQ plot confirming normal distributions for comparison. A
452 p-value <0.05 was considered statistically significant. Statistical analyses were
453 performed using GraphPad Prism, version 8.4.2.

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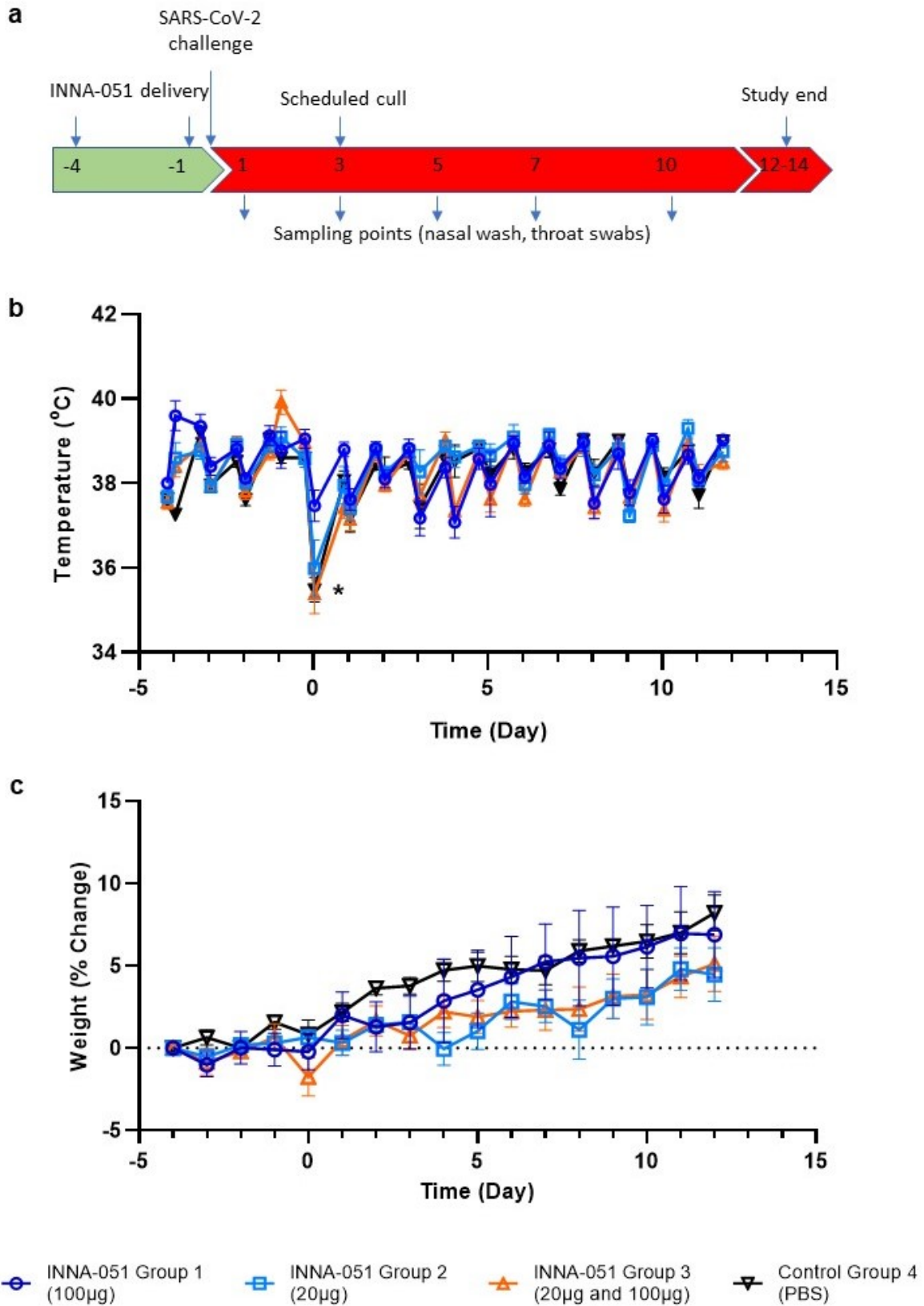
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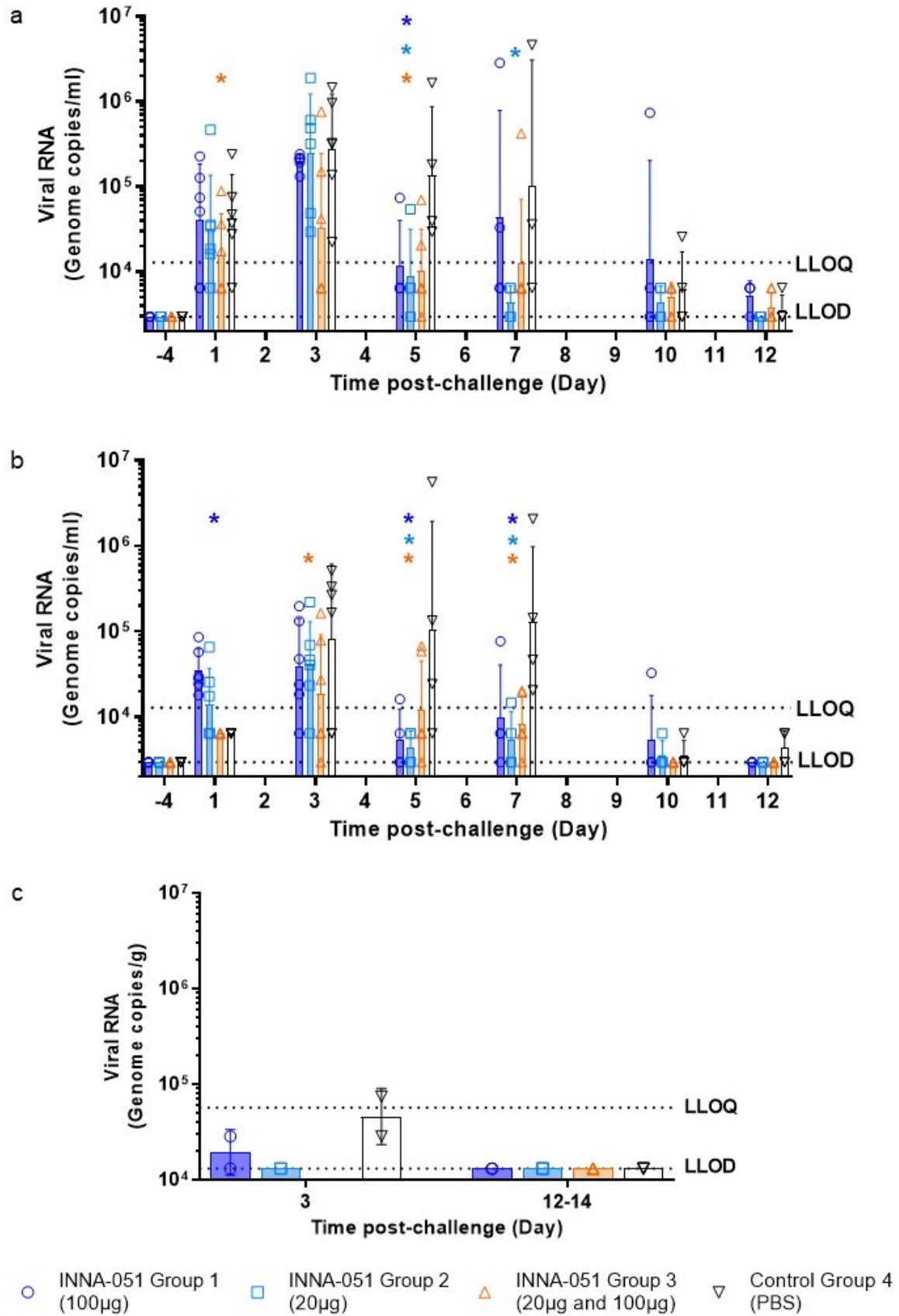
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584 **Figure 1. Clinical observations.** (a) Schematic of experimental design. Ferrets
585 received INNA-051 and PBS treatments 4 days and 1 day prior to challenge with 5.0
586 $\times 10^6$ pfu/ml SARS-CoV-2. Nasal wash and throat swabs were collected at days 1, 3,
587 5, 7, 10 & 12 post challenge (p.c.) for all treatment groups and control group.
588 Scheduled culls were performed for 6/24 ferrets on day 3 p.c. and 18/24 ferrets on
589 days 12-14 p.c. (b) Temperatures were measured twice daily (approximately 8 hours
590 apart), using implanted temperature/ID chips. Mean temperatures \pm standard error
591 of the mean (SEM) are displayed. Temperature dip post SARS-CoV-2 challenge (*)
592 was attributed to sedation. (b) Weight was recorded daily and percentage change from
593 the ferret weight prior to treatment plotted. Mean percentage weight change \pm SEM
594 are displayed.
595



596

597 **Figure 2. Viral RNA shedding following SARS-CoV-2 challenge.** Nasal wash and
598 throat swabs were collected at days 1, 3, 5, 7, 10 & 12 p.c. for all treatment groups
599 and vehicle control group. Lung tissue was collected at necropsy on scheduled cull
600 day 3 (n=6) and end cull days 12-14 (n=18). Viral RNA was quantified by RT-qPCR.
601 (a) Nasal wash (b) Throat swab (c) Lung tissue. Geometric mean +/- standard
602 deviation are displayed on the graphs. Dashed horizontal lines denote the lower limit
603 of quantification (LLOQ) and lower limit of detection (LLOD). Statistical significance
604 (95% CI of differences) in comparison to the control group using two-way ANOVA
605 Dunnett's multiple comparisons test are displayed above the error bars (*). Day 7 nasal
606 wash for group 4 had viral RNA quantified for 3/4 ferrets; no sample was available for
607 processing.
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