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## Brain Cross-Protection against SARS-CoV-2 Variants by a Lentiviral Vaccine in New Transgenic Mice

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1 **Brain Cross-Protection against SARS-CoV-2 Variants by a Lentiviral Vaccine in New Transgenic**  
2 **Mice**

3  
4 Running title: A COVID-19 Intranasal Lentiviral Vaccine

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26 **Abstract**

27 COVID-19 vaccines already in use or in clinical development may have reduced efficacy against  
28 emerging SARS-CoV-2 variants. In addition, although the neurotropism of SARS-CoV-2 is well  
29 established, the vaccine strategies currently developed have not taken into account protection of the  
30 central nervous system. Here, we generated a transgenic mouse strain expressing the human Angiotensin  
31 Converting Enzyme 2, and displaying unprecedented brain permissiveness to SARS-CoV-2 replication, in  
32 addition to high permissiveness levels in the lung. Using this stringent transgenic model, we  
33 demonstrated that a non-integrative lentiviral vector, encoding for the spike glycoprotein of the ancestral  
34 SARS-CoV-2, used in intramuscular prime and intranasal boost elicits sterilizing protection of lung and  
35 brain against both the ancestral virus, and the Gamma (P.1) variant of concern, which carries multiple  
36 vaccine escape mutations. Beyond induction of strong neutralizing antibodies, the mechanism underlying  
37 this broad protection spectrum involves a robust protective T-cell immunity, unaffected by the recent  
38 mutations accumulated in the emerging SARS-CoV-2 variants.

39

## 40 Introduction

41 Prolongation of the worldwide pandemic coronavirus disease 2019 (COVID-19) requires the  
42 development of effective and safe prophylactic second generation vaccines against the Severe Acute  
43 Respiratory Syndrome beta-coronavirus 2 (SARS-CoV-2). Although SARS-CoV-2 primarily targets the  
44 respiratory tract, its neurotropism, similar to that of SARS-CoV and Middle East Respiratory Syndrome  
45 (MERS)-CoV (Glass *et al*, 2004; Li *et al*, 2016; Netland *et al*, 2008) has been regularly reported  
46 (Aghagoli *et al*, 2020; Fotuhi *et al*, 2020; Hu *et al*, 2020; Politi *et al*, 2020; Roman *et al*, 2020; von  
47 Weyhern *et al*, 2020; Whittaker *et al*, 2020). Moreover, expression of the SARS-CoV-2 receptor,  
48 Angiotensin Converting Enzyme 2 (ACE2), by neuronal and glial cells makes the brain susceptible to  
49 neuro-invasion (Chen *et al*, 2020; Xu & Lazartigues, 2020) and COVID-19 human patients frequently  
50 present neurological symptoms (Bourgonje *et al*, 2020; Hu *et al*, 2020; Mao *et al*, 2020).

51 A large multicenter prospective study found neurological manifestations in 80% of hospitalized  
52 COVID-19 patients. The most frequent self-reported symptoms were headache, anosmia, ageusia and  
53 syncope, while among the clinically verified neurological disorders, present in 53% of the patients, the  
54 most frequent symptoms were acute encephalopathy and coma. Other neurological symptoms included  
55 strokes, seizures, meningitis and abnormal brainstem reflexes. In addition to the possibly devastating  
56 consequences of these acute manifestations, the long-term and debilitating effects of post-COVID-19  
57 neurological sequelae and prolonged symptoms, such as fatigue, headaches, dizziness, anosmia or “brain  
58 fog”, represent an increasingly recognized matter of concern (Ali Awan *et al*, 2021; Wijeratne &  
59 Crewther, 2020). An autopsic study of COVID-19 deceased patients demonstrated the presence of the  
60 envelop spike glycoprotein of SARS-CoV-2 ( $S_{CoV-2}$ ) in epithelial and neural/neuronal cells of the  
61 olfactory mucosa, while viral RNA was detected in neuroanatomical areas receiving olfactory tract  
62 projections, suggesting that the olfactory mucosa could serve as a portal for neuro-invasion followed by  
63 retrograde axonal dissemination (Meinhardt *et al*, 2021). Hematogenous spread can also be involved as  
64 suggested by visualization of viral antigen in brain endothelial cells in the same study (Meinhardt *et al*,  
65 2021). Based on autopsy and animal studies, it has been suggested that human coronaviruses can establish  
66 persistent infection in the brain (Desforges *et al*, 2014). Therefore, it is critical to focus on the protective  
67 properties of COVID-19 vaccine candidates, not only in the respiratory tract, but also in the brain.

68 New SARS-CoV-2 variants of concern resulting from mutations accumulating in  $S_{CoV-2}$  have been  
69 identified by genome sequencing in diverse geographical locations throughout the world.  $S_{CoV-2}$  is  
70 composed of S1 and S2 subunits. The former harbors a Receptor Binding Domain (RBD) that  
71 encompasses the Receptor Binding Motif (RBM), which is the main functional motif interacting with  
72 human ACE2 (hACE2) (Hoffmann *et al*, 2020; Shang *et al*, 2020). RBD and RBM are prone to mutations  
73 that can further improve the fitness of  $S_{CoV-2}$  for binding to hACE2. A crucial consideration for such

74 mutations is the alteration in RBD/RBM B-cell epitopes, which can lead to the escape from the action of  
75 neutralizing antibodies (NAbs) raised in individuals previously infected with ancestral SARS-CoV-2 or  
76 immunized with S<sub>CoV-2</sub>-based vaccines. Among the many SARS-CoV-2 variants that have emerged since  
77 the start of the pandemic, four have been classified as variants of concern by the WHO because they pose  
78 an increased risk to global public health ([https://www.who.int/en/activities/tracking-SARS-CoV-2-](https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/)  
79 [variants/](https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/)). These variants harbor multiple S<sub>CoV-2</sub> mutations conferring increased resistance to therapeutic  
80 antibodies and to sera from convalescents or vaccinees (Hoffmann *et al*, 2021; Lazarevic *et al*, 2021).  
81 Among them the Gamma (P.1) variant contains the highest number of S<sub>CoV-2</sub> mutations, including a  
82 critical triplet (K417T, E484K, N501Y) within the RBD that is present almost identical (K417N, E484K,  
83 N501Y) in the Beta (B.1.351) variant (Hoffmann *et al.*, 2021; Lazarevic *et al.*, 2021).

84 We have recently established the high performance of a non-integrative lentiviral vector (LV)  
85 encoding the full-length sequence of S<sub>CoV-2</sub> of the ancestral strain (LV::S), when used in systemic prime  
86 followed by intranasal (i.n.) boost (Ku *et al*, 2021). LVs allow transgene insertion up to 5 kb in length and  
87 offer outstanding potential for gene transfer to the nuclei of host cells (Di Nunzio *et al*, 2012; Hu *et al*,  
88 2011; Ku *et al*, 2020; Zennou *et al*, 2000). LVs display *in vivo* tropism for immune cells, notably  
89 dendritic cells (Arce *et al*, 2009). They are non-replicative, non-cytopathic and scarcely inflammatory  
90 (Lopez *et al*, Submitted). These vectors induce long-lasting B- and T-cell immunity (Di Nunzio *et al.*,  
91 2012; Hu *et al.*, 2011; Ku *et al*, 2020; Zennou *et al.*, 2000). LVs are pseudo-typed with the surface  
92 glycoprotein of Vesicular Stomatitis Virus, to which the human population has limited exposure. This  
93 prevents these vectors from being targeted by preexisting immunity in humans, unlike adenoviral vectors  
94 of human serotypes (Rosenberg *et al*, 1998; Schirmbeck *et al*, 2008). The safety of LV has been  
95 established in humans in a phase I/II Human Immunodeficiency Virus (HIV)-1 vaccine trial (2011-  
96 006260-52 EN).

97 Here, we generated new hACE2 transgenic mice with unprecedented brain permissiveness to SARS-  
98 CoV-2 replication resulting in marked brain inflammation and lethality following infection. They are also  
99 prone to SARS-CoV-2 infection in the lung, yet with milder inflammation than in the brain. Using this  
100 stringent preclinical animal model, we demonstrated the capability of i.m.-i.n. prime-boost immunization  
101 with our LV-based vaccine candidate to reach full protection of both lungs and Central Nervous System  
102 (CNS) against SARS-CoV-2 infection. Importantly, the LV encoding for S<sub>CoV-2</sub> of the ancestral SARS-  
103 CoV-2 induced sterilizing prophylaxis of lung and brain against both the ancestral and the Gamma (P.1)  
104 variant of concern. Beside the induction of strong NAbs, the mechanism underlying this protection is  
105 linked to a strong poly-specific T-cell immunity, not affected by the mutations accumulated in S<sub>CoV-2</sub> of  
106 the emerging SARS-CoV-2 variants.

107

## Results

### Generation of new hACE2 transgenic mice with high brain permissiveness to SARS-CoV-2 replication

To set up a mouse model permissive to SARS-CoV-2 replication allowing assessment of our vaccine candidates, based on the previously produced B6.K18-ACE2<sup>2PrImn/JAX</sup> mice (McCray *et al.*, 2007), we generated C57BL/6 transgenic mice with an LV (Nakagawa & Hoogenraad, 2011) carrying the *hACE2* gene under the human cytokeratin 18 promoter, namely “B6.K18-hACE2<sup>IP-THV</sup>” (Appendix Figure S1). The permissiveness of these mice to SARS-CoV-2 replication was evaluated after one generation backcross to WT C57BL/6 (N1). N1 mice with varying number of *hACE2* transgene copies per genome (Figure 1A) were sampled and inoculated i.n. with the ancestral SARS-CoV-2. At day 3 post-inoculation (3 dpi), the mean  $\pm$  SD of lung viral RNA content was as high as  $(3.3 \pm 1.6) \times 10^{10}$  copies of SARS-CoV-2 RNA/lung in permissive mice (Figure 1B). SARS-CoV-2 RNA copies per lung  $< 1 \times 10^7$  correspond to the genetic material derived from the input in the absence of viral replication (Ku *et al.*, 2021). We also noted that the lung viral RNA content (Figure 1B) was not proportional to the *hACE2* transgene copy number per genome (Figure 1A) or to the amount of hACE2 protein expression in the lungs (Figure 1C, D). Remarkably, viral RNA content, as high as  $(5.7 \pm 7.1) \times 10^{10}$  copies of SARS-CoV-2 RNA, were also detected in the brain of the permissive mice (Figure 1B). Virus replication/dissemination was also observed, although to a lesser extent, in the heart and kidneys. In another set of experiment with mice from our B6.K18-hACE2<sup>IP-THV</sup> colony, we also established the replication kinetics of ancestral SARS-CoV-2 in the lungs and brain by measuring viral RNA contents (Figure 1E) and viral loads determined by PFU counting (Figure 1F) over time. Viral RNA contents reached a plateau at 1 dpi in the lungs, while they increased between 1 and 3 dpi in the brain. We observed a 5-10% weight loss at 2-3dpi (Figure 1G). At this time point, mice started to be lethargic, with hunched posture and ruffled hair coat, reaching the humane endpoint. Comparatively, B6.K18-hACE2<sup>JAX</sup> transgenic mice reportedly experienced significant weight loss from 4 dpi on, with an average of 20% weight loss recorded between 5 and 7 dpi (Winkler *et al.*, 2020).

At 3 dpi, lung histological sections of SARS-CoV-2-inoculated B6.K18-hACE2<sup>IP-THV</sup> mice displayed significant interstitial inflammation (Figure 2A-F) and alveolar exudates (Figure 2D and F), accompanied by peribronchiolar and perivascular infiltration (Figure 2C) and minimal to moderate alterations of the bronchiolar epithelium (Figure 2E). Thus, SARS-CoV-2 infection essentially induced an alveolo-interstitial syndrome in B6.K18-hACE2<sup>IP-THV</sup> mice, similarly to what was reported in the B6.K18-ACE2<sup>2PrImn/JAX</sup> transgenic mouse model (Winkler *et al.*, 2020). At the same time-point, immunohistochemistry (IHC) analysis of the brain of infected B6.K18-hACE2<sup>IP-THV</sup> mice, by use of a

141 SARS-CoV-2 nucleocapsid protein (N<sub>CoV-2</sub>)-specific polyclonal antibody, revealed multiple clusters of  
142 N<sub>CoV-2</sub><sup>+</sup> cells (Figure 2G, H).

143 We compared the replication of SARS-CoV-2 in lungs and brain and the viral dissemination to various  
144 organs in B6.K18-hACE2<sup>IP-THV</sup> and B6.K18-ACE2<sup>2PrImn/JAX</sup> mice (McCray *et al.*, 2007) (Figure 3A). The  
145 lung viral RNA contents were slightly lower in B6.K18-hACE2<sup>IP-THV</sup> compared with B6.K18-  
146 ACE2<sup>2PrImn/JAX</sup> mice. However, viral RNA contents in the brain of B6.K18-hACE2<sup>IP-THV</sup> mice were ~ 4  
147 log higher than in their B6.K18-ACE2<sup>2PrImn/JAX</sup> counterparts (Figure 3A). Viral RNA contents were also  
148 assessed by a sub-genomic E<sub>CoV-2</sub> RNA (Esg) qRT-PCR, which is an indicator of active viral replication  
149 (Chandrashekar *et al.*, 2020; Tostanoski *et al.*, 2020; Wolfel *et al.*, 2020). Measurement of brain RNA  
150 contents by Esg qRT-PCR detected  $(7.55 \pm 7.74) \times 10^9$  copies of SARS-CoV-2 RNA in B6.K18-  
151 hACE2<sup>IP-THV</sup> mice and no copies of this replication-related RNA in 4 out of 5 B6.K18-ACE2<sup>2PrImn/JAX</sup>  
152 mice. This dramatic difference of SARS-CoV-2 replication in the brain of the two transgenic strains was  
153 associated with significantly higher *hACE2* mRNA expression in the brain of B6.K18-hACE2<sup>IP-THV</sup> mice  
154 (Figure 3B). However, *hACE2* mRNA expression in the lungs of B6.K18-hACE2<sup>IP-THV</sup> mice was also  
155 higher than in B6.K18-ACE2<sup>2PrImn/JAX</sup> mice, despite of the lower viral replication rate in the lungs of the  
156 former. A trend towards higher viral RNA contents was also observed in the kidneys and heart of  
157 B6.K18-hACE2<sup>IP-THV</sup> compared with B6.K18-ACE2<sup>2PrImn/JAX</sup> mice (Figure 3A).

158 In accordance with the lower lung viral RNA contents, B6.K18-hACE2<sup>IP-THV</sup> mice displayed less  
159 pulmonary inflammation than B6.K18-ACE2<sup>2PrImn/JAX</sup> mice, as evaluated by qRT-PCR study of 20  
160 inflammatory analytes, applied to RNA extracted from total lung homogenates (Figure 3C). This same  
161 assay applied to RNA extracted from total brain homogenates detected robust inflammation in B6.K18-  
162 hACE2<sup>IP-THV</sup> — but not B6.K18-ACE2<sup>2PrImn/JAX</sup> — mice (Figure 3C).

163 Also, as mentioned above, B6.K18-hACE2<sup>IP-THV</sup> mice generally reached the humane endpoint between  
164 3 and 4 dpi and therefore displayed a more rapidly lethal SARS-CoV-2-mediated disease than their  
165 B6.K18-ACE2<sup>2PrImn/JAX</sup> counterparts (Winkler *et al.*, 2020) (Appendix Figure S1). Therefore, large  
166 permissiveness to SARS-CoV-2 replication in both lung and CNS, marked brain inflammation and rapid  
167 development of a lethal disease are major distinctive features offered by this new B6.K18-hACE2<sup>IP-THV</sup>  
168 transgenic model. Difference in pathology between B6.K18-hACE2<sup>IP-THV</sup> and B6.K18-ACE2<sup>2PrImn/JAX</sup>  
169 mice suggests that some future results can be model dependent.

### 170 **Protection of lungs and brain in LV::S-immunized B6.K18-hACE2<sup>IP-THV</sup> mice**

171 We generated an LV encoding for the prefusion form of S<sub>CoV-2</sub> derived from the ancestral strain. This  
172 prefusion S<sub>CoV-2</sub> antigen has the  $\Delta 675-685$  deletion which encompasses the RRAR furin cleavage site in  
173 order to limit its conformational dynamics and to maintain better exposure of the S1 B-cell epitopes

174 (McCallum *et al.*, 2020). For an improved half-life, the sequence also harbors the K<sup>986P</sup> and V<sup>987P</sup>  
175 consecutive proline substitutions in S2 (Appendix Figure S2A) (Walls *et al.*, 2020). C57BL/6 mice primed  
176 (i.m.) and boosted (i.n.) with LV encoding the wild type or prefusion S<sub>CoV-2</sub> possessed high serum titers of  
177 anti-S<sub>CoV-2</sub> IgG (Appendix Figure S2B), high titers of anti-S<sub>CoV-2</sub> IgG and IgA in the lung extracts  
178 (Appendix Figure S2C), and comparable sero-neutralizing activity (Appendix Figure S2D). These results  
179 indicate that the modifications in the prefusion form does not impact positively or negatively its capacity  
180 to induce Ab responses against native S<sub>CoV-2</sub>.

181 We then evaluated the vaccine efficacy of LV::S in B6.K18-hACE2<sup>IP-THV</sup> mice. In a first set of  
182 experiments with these mice, we used an integrative version of the vector. Individuals ( $n = 6/\text{group}$ )  
183 were primed i.m. with  $1 \times 10^7$  TU/mouse of LV::S or an empty LV (sham) at wk 0 and then boosted i.n.  
184 at wk 3 with the same dose of the same vectors (Figure 4A). Mice were then challenged with the  
185 ancestral SARS-CoV-2 at wk 5. A high serum neutralizing activity was detected in LV::S-vaccinated  
186 mice (Figure 4B). Many studies use PFU counting to determine viral loads in vaccine efficacy studies.  
187 We noticed that large amounts of NAbs in the lungs of intranasally vaccinated individuals, although not  
188 necessarily spatially in contact with circulating viral particles in live animals, can come to contact with  
189 and neutralize viral particles in the lung homogenates in vitro, causing the PFU assay to underestimate  
190 the amounts of cultivable viral particles. Therefore, in the following studies, we evaluated the viral  
191 contents/replication by use of E or Esg qRT-PCR. In the lungs, but also in the brain, vaccination  
192 conferred complete protection against SARS-CoV-2 replication, maintaining the viral RNA content  
193 close to the input level (Figure 4C top). Lung viral RNA content assessed by Esg qRT-PCR, did not  
194 detect any viral replication in vaccinated mice (Figure 4C bottom). Remarkably, Esg qRT-PCR  
195 quantitation of viral RNA contents in brain detected no copies of this replication-related SARS-CoV-2  
196 RNA in LV::S-vaccinated mice *versus*  $(7.55 \pm 7.84) \times 10^9$  copies in the brain of the sham-vaccinated  
197 controls (Figure 4C bottom).

198 At 3 dpi, cytometric investigation of the lung innate immune cell subsets (Figure EV1A) detected  
199 significantly lower proportions of NK cells (CD11b<sup>int</sup> NKp46<sup>+</sup>) and neutrophils (CD11b<sup>+</sup> CD24<sup>+</sup> SiglecF<sup>-</sup>  
200 Ly6G<sup>+</sup>) among the lung CD45<sup>+</sup> cells in the LV::S-vaccinated and protected B6.K18-hACE2<sup>IP-THV</sup> mice,  
201 than in the sham-vaccinated controls (Figure 4D). Both cell populations have been associated with  
202 enhanced lung inflammation and poor outcome in the context of COVID-19 (Cavalcante-Silva *et al.*,  
203 2021; Masselli *et al.*, 2020). Frequencies of the other lung innate immune cell subsets were not  
204 significantly distinct in the protected and unprotected groups (Figure EV1B). This protective anti-  
205 inflammatory effect of the vaccine was also recorded in the brain, as expression levels of the  
206 inflammatory mediators IFN- $\alpha$ , TNF- $\alpha$ , IL-5, IL-6, IL-10, IL-12p40, CCL2, CCL3, CXCL9 and  
207 CXCL10 were significantly lower in LV::S-immunized animals than in the sham group (Figure 4E). In

208 the lungs, where SARS-CoV-2 infection in non- or sham-vaccinated animals does not induce strong  
209 cytokine and chemokine expression (Figures 3C and Figure EV1C), qRT-PCR analysis rather detected a  
210 modest increase in the level of factors classically produced during T-cell responses, such as TNF- $\alpha$  and  
211 IL-2 (Figure EV1C), which probably results from the vaccine immunogenicity. Sham-vaccinated and  
212 challenged B6.K18-hACE2<sup>IP-THV</sup> mice reached the humane endpoint, being hunched and lethargic with  
213 ruffled hair coat, at 3 dpi while the LV::S-vaccinated counterparts had no detectable symptoms.  
214 Therefore, an i.m.-i.n. prime-boost with LV::S prevents SARS-CoV-2 replication in both lung and CNS  
215 and inhibits virus-mediated lung infiltration, as well as neuro-inflammation.

### 216 **Immune response and protection in LV::S-vaccinated B6.K18-hACE2<sup>IP-THV</sup> mice**

217 For further characterization of the protective properties of LV::S in B6.K18-hACE2<sup>IP-THV</sup> mice, we  
218 used the safe and non-integrative version of LV (Ku *et al.*, 2021). Thus, “LV::S” hereafter refers to this  
219 non-integrative version. B6.K18-hACE2<sup>IP-THV</sup> mice were primed i.m. at wk 0 and boosted i.n. at wk 5  
220 with LV::S. Sham-vaccinated controls received an empty LV following the same regimen. At wk 7,  
221 IFN- $\gamma$ -producing CD8<sup>+</sup> T cells, specific to several S<sub>CoV-2</sub> epitopes, were detected in the lungs (Figure  
222 5A) and spleen (Appendix Figure S3A left) of LV::S-vaccinated mice. Small numbers of IFN- $\gamma$ -  
223 producing CD4<sup>+</sup> T cells were also detected in the spleen (Appendix Figure S3A right) and lungs  
224 (Appendix Figure S3B) of these mice. The proportion of effector memory (Tem) and resident memory  
225 (Trm) cells among CD8<sup>+</sup> T cells of the lung was higher in LV::S i.m.-i.n.-vaccinated mice than in their  
226 sham counterparts (Figure 5B, C). By use of a H-2D<sup>b</sup>-S<sub>CoV-2:538-546</sub> dextramer, we further focused on a  
227 fraction of S<sub>CoV-2</sub>-specific CD8<sup>+</sup> T cells in the lungs of LV::S- or sham-vaccinated mice (Figure 5D, E).  
228 In contrast to LV::S-vaccinated mice, no dextramer<sup>+</sup> cells were detected in lung CD8<sup>+</sup> T cells of the  
229 sham group. Inside this specific CD8<sup>+</sup> T-cell subset, the proportions of central memory (Tcm) and Tem  
230 were comparable and a Trm subset was identifiable. High titers of serum and lung anti-S<sub>CoV-2</sub> IgG and  
231 IgA (Figure 5F), and notable serum and lung SARS-CoV-2 neutralizing activity (Figure 5G) were  
232 detected in LV::S-vaccinated mice.

233 To assess the impact of LV::S vaccination route on brain or lung protection in this murine model,  
234 B6.K18-hACE2<sup>IP-THV</sup> mice were vaccinated by the i.m. or i.n. route at wk 0 and then left untreated or  
235 boosted by the i.m. or i.n. route at wk 5. Mice were challenged with SARS-CoV-2 at wk 7. At 3 dpi, the  
236 highest brain protection was observed in mice that were primed i.m. or i.n. and boosted i.n. (Figure 6A).  
237 An i.m.-i.m. prime-boost or a single i.m. or i.n. immunization with LV::S was not sufficient to  
238 significantly reduce the viral RNA content in the brain. In the lungs, a single i.m. or i.n. administration  
239 of LV::S failed to confer protection in the lungs of these highly susceptible B6.K18-hACE2<sup>IP-THV</sup> model  
240 (Figure 6A). The prime-boost vaccination regimen led to the highest levels of lung protection,  
241 regardless of the immunization route tested. In nasal washes from the LV::S i.m.-i.n. immunized group,

242 viral RNA contents were lower than in the sham group, although the difference did not reach statistical  
243 significance (Figure 6A). This result is consistent with the observation that systemic or mucosal immune  
244 responses significantly reduces viral loads and tissue damage in the lungs of hamsters intranasally  
245 challenged with SARS-CoV-2, but not in their nasal turbinate (Zhou *et al*, 2021). Administration of a  
246 single i.n. dose of the chimpanzee adenovirus-vectorized SARS-CoV-2 (ChAd-SARS-CoV-2-S)  
247 vaccine to wild-type C57BL/6 mice, pretreated with a hACE2-encoding serotype 5 adenoviral vector  
248 (Ad5::hACE2) prior to SARS-CoV-2 challenge, resulted in complete elimination of viral RNA from  
249 nasal washes, measured at 4-8 dpi (Hassan *et al*, 2020). The discrepancy between these results in  
250 Ad5::hACE2-pretreated mice and those observed here in B6.K18-hACE2<sup>IP-THV</sup> mice, may be explained  
251 by the differences in the characteristics of the murine models used and the time points studied. I.n.  
252 immunization of hamsters with ChAd-SARS-CoV-2-S also resulted in minimal or no viral RNA content  
253 in nasal swabs and nasal olfactory neuroepithelium (Bricker *et al*, 2021). However, in rhesus monkeys,  
254 ChAd-SARS-CoV-2-S i.n. vaccination did not result in significant reduction of the viral RNA contents  
255 in nasal swabs at 3 and 5 dpi, although statistical significance was reached at 7 dpi (Hassan *et al*, 2021).  
256 Thus, the differences in pre-clinical models and the kinetics studied appear to well impact the reduction  
257 of viral loads in the upper respiratory tract.

258 At 3 dpi, H&E analysis of the lung sections in the sham group showed the same kind of lesions  
259 detailed in Figure 2B-E. Compared to the sham group, inflammation seriousness and interstitial syndrome  
260 were reduced in the LV::S-vaccinated mice, even if some degree of inflammation was present (Figure 6B,  
261 C). The inflamed zones from LV::S- and sham-vaccinated controls contained N<sub>CoV-2</sub> antigen detected by  
262 IHC study of contiguous lung sections (Figure 6B), indicating that, even if the virus replication has been  
263 largely reduced in the i.m.-i.n. vaccinated mice (Figure 6A), the infiltration and virus remnants have not  
264 yet been completely resorbed at the early time point of 3 dpi.

265 We also detected higher density of CD3<sup>+</sup> T cells in the olfactory bulbs of LV::S vaccinated and  
266 protected mice than in the sham individuals (Figure 7A). As expected with this LV vaccine, the T-cell  
267 response was polarized towards the CD8<sup>+</sup> compartment, as evidenced by the higher proportion of CD8<sup>+</sup> T  
268 cells in the olfactory bulbs of protected animals (Figure 7B) and by the presence of high amounts of anti-  
269 S<sub>CoV-2</sub> CD8<sup>+</sup> T-cell responses in the spleen, while specific CD4<sup>+</sup> T cells were few (Appendix Figure S3A).  
270 A very few specifically reacting CD4<sup>+</sup> T cells was found in the lungs (Appendix Figure S3B), and in the  
271 olfactory bulb, CD4<sup>+</sup> T cells had no distinctive activated or migratory phenotype, as assessed by their  
272 surface expression of CD69 or CCR7 (Appendix Figure S3C). In line with the absence of CCR7  
273 expression on these T cells, and unlike Murine Hepatitis Virus (MHV) infection (Cupovic *et al*, 2016),  
274 we saw no up-regulation of CCL19 and CCL21 (CCR7 ligands) in the brain, regardless of the protected  
275 status of the mice (Appendix Figure S3D). At 3 dpi, qRT-PCR analysis of olfactory bulbs detected very

276 low levels of inflammation, ranging from -2 to +2 log<sub>2</sub> fold change compared with untreated negative  
277 controls, with no significant difference between the LV::S and sham groups (Appendix Figure S3E).  
278 Compared to the LV::S-vaccinated and protected group, there were more neutrophils (CD11b<sup>+</sup> Ly6C<sup>+</sup>  
279 Ly6G<sup>+</sup>) in the olfactory bulbs (Figure 7C) and inflammatory monocytes (CD11b<sup>+</sup> Ly6C<sup>+</sup> Ly6G<sup>-</sup>) in the  
280 brain (Figure 7D) of unprotected sham mice, reflecting a higher level of neuroinflammation in these mice.  
281 Histological examination of brains did not reveal gross alterations of the organ. However, in each of the  
282 three infected sham-vaccinated mice studied, periventricular alterations were visible. In two out of three  
283 mice studied, there were infiltrates of predominately mononuclear leukocytes (Figure 7E), and in the third  
284 mouse, a small periventricular hemorrhage was observed (not shown). Such alterations were not detected  
285 in any of the four LV::S-vaccinated and SARS-CoV-2-challenged mice studied.

### 286 **Complete cross-protection induced by LV::S against the genetically distant SARS-CoV-2** 287 **Gamma variant**

288 A critical issue regarding the COVID-19 vaccines currently in use is the protective potency against  
289 emerging variants. To assess this question with the vaccine candidate developed here, B6.K18-hACE2<sup>IP-</sup>  
290 <sup>THV</sup> mice were primed i.m. (wk 0) and boosted i.n. (wk 5) with LV::S or sham (Figure 8A). Mice were  
291 then challenged at wk 7 with the SARS-CoV-2 Gamma strain which is among the most genetically  
292 distant SARS-CoV-2 variants so far described (Buss *et al*, 2021). Determination of the brain and lung  
293 viral loads at 3 dpi demonstrated that prime-boost vaccination with LV encoding the S<sub>CoV-2</sub> from the  
294 ancestral sequence induced full protection of the brain and lungs against SARS-CoV-2 Gamma (Figure  
295 8B). Studies involving H&E and IHC staining of serial lung sections were performed to visualize the  
296 N<sub>CoV-2</sub> antigen in the tissue and to localize it with respect to the inflammatory foci (Figure EV2A, B).  
297 H&E images did not reveal significant differences in the extent and severity of pulmonary inflammatory  
298 lesions between LV::S- and sham-vaccinated mice (Figure EV2, rows 1 and 3). However, within the  
299 inflammatory areas, as inferred from the contiguous H&E-stained sections, N<sub>CoV-2</sub><sup>+</sup> patches were readily  
300 discernable in lungs of sham mice, even at low magnification, while they were less frequent in LV::S-  
301 vaccinated mice (Figure EV2A, rows 2 and 4). Moreover, the brains of infected sham controls contained  
302 multiple areas positive for N<sub>CoV-2</sub> staining and enumeration at the single cell level revealed significantly  
303 less N<sub>CoV-2</sub><sup>+</sup> cells in the brains of LV::S-vaccinated mice (Figure EV2C, D).

304 The markedly decreased ability of the sera of LV::S-vaccinated mice to neutralize S<sub>Beta</sub> or S<sub>Gamma</sub>  
305 pseudo-viruses, compared to S<sub>Ancestral</sub>, S<sub>D614G</sub> or S<sub>Alpha</sub> pseudo-viruses, (Figure 8C), raised the possibility  
306 of T-cell involvement in this total protection. To evaluate this possibility, we vaccinated C57BL/6 WT or  
307  $\mu$ MT KO mice following the same i.m.-i.n. protocol as above (Figure S4A).  $\mu$ MT KO are deficient in  
308 mature B-cell compartment and therefore lack Ig/antibody response (Kitamura *et al*, 1991). To make  
309 these non-transgenic mice permissive to SARS-CoV-2 replication, they were pre-treated 4 days before the

310 SARS-CoV-2 challenge with  $3 \times 10^8$  IGU of Ad5::hACE2 (Ku *et al.*, 2021). Determination of lung viral  
311 loads at 3 dpi showed complete protection of the lungs in vaccinated WT mice as well as a highly  
312 significant protection in vaccinated  $\mu$ MT KO mice (Figure 8E). This observation determined that B-cell  
313 independent and antigen-specific cellular immunity, specifically the T-cell response, plays a major role in  
314 LV-mediated protection. This is consistent with the strong T-cell responses induced by LV::S at the  
315 systemic level (Figure 8F) and in the lungs (Figure 5A-E), and the recruitment of CD8<sup>+</sup> T cells in the  
316 olfactory bulbs, detectable in vaccinated and challenged mice (Figure 7A, B, Figure 8D). Importantly, all  
317 murine and human CD8<sup>+</sup> T-cell epitopes identified on the ancestral S<sub>CoV-2</sub> sequence are preserved in the  
318 mutated S<sub>CoV-2</sub> Gamma (Appendix Table S1). These observations indicate the strong potential of LV at  
319 inducing full protection of lungs and brain against ancestral and emerging SARS-CoV-2 variants by  
320 eliciting strong B and T cell-responses. In contrast to the B-cell epitopes which are targets of NAbs  
321 (Hoffmann *et al.*, 2021), the so far identified T-cell epitopes have not been impacted by mutations  
322 accumulated in the S<sub>CoV-2</sub> of the emerging variants.

## Discussion

LV-based platforms emerged recently as a powerful vaccination approach against COVID-19, notably when used as a systemic prime followed by mucosal i.n. boost, inducing sterilizing immunity against lung SARS-CoV-2 infection in preclinical animal models (Ku *et al.*, 2021). In the present study, to investigate the efficacy of our vaccine candidates, we generated a new transgenic mouse model, using the LV-based transgenesis approach (Nakagawa & Hoogenraad, 2011). The ILV used in this strategy encodes for hACE2 under the control of the cytokeratin K18 promoter, i.e., the same promoter as previously used by Perlman's team to generate B6.K18-ACE2<sup>2PrImn/JAX</sup> mice (McCray *et al.*, 2007), with a few adaptations to the lentiviral FLAP transfer plasmid. However, the new B6.K18-hACE2<sup>IP-THV</sup> mice have certain distinctive features, as they express much higher levels of hACE2 mRNA in the brain and display markedly increased brain permissiveness to SARS-CoV-2 replication, in parallel with a substantial brain inflammation and development of a lethal disease in <4 days post infection. These distinctive characteristics can arise from differences in the hACE2 expression profile due to: (i) alternative insertion sites of ILV into the chromosome compared to naked DNA, and/or (ii) different effect of the Woodchuck Posttranscriptional Regulatory Element (WPRE) *versus* the alfalfa virus translational enhancer (McCray *et al.*, 2007), in B6.K18-hACE2<sup>IP-THV</sup> and B6.K18-ACE2<sup>2PrImn/JAX</sup> animals, respectively (Appendix Figure S1). Other reported *hACE2* humanized mice express the transgene under: (i) murine ACE2 promoter, without reported hACE2 mRNA expression in the brain (Yang *et al.*, 2007), (ii) "hepatocyte nuclear factor-3/forkhead homologue 4" (HFH4) promoter, i.e., "HFH4-hACE2" C3B6 mice, in which lung is the principal site of infection and pathology (Jiang *et al.*, 2020; Menachery *et al.*, 2016), and (iii) "CAG" mixed promoter, i.e. "AC70" C3H × C57BL/6 mice, in which hACE2 mRNA is expressed in various organs including lungs and brain (Tseng *et al.*, 2007). Comparison of AC70 and B6.K18-hACE2<sup>IP-THV</sup> mice could yield information to assess the similarities and distinctions of these two models. The B6.K18-hACE2<sup>IP-THV</sup> murine model not only has broad applications in COVID-19 vaccine studies, but also provides a unique rodent model for exploration of COVID-19-derived neuropathology. Based on the substantial permissiveness of the brain to SARS-CoV-2 replication and development of a lethal disease, this pre-clinical model can be considered as even more stringent than the golden hamster model. The report of a new transgenic mouse to provide a model to assess the efficiency of vaccine candidates against the highly critical neurological component of the disease is an important breakthrough. This new and unique model will also be beneficial for the research community to have an accelerated understanding on the immune protection against neural COVID-19 disease, so far a neglected niche due to the lack of a model.

The source of neurological manifestations associated with COVID-19 in patients with comorbid conditions can be: (i) direct impact of SARS-CoV-2 on CNS, (ii) infection of brain vascular endothelium

357 and, (iii) uncontrolled anti-viral immune reaction inside CNS. ACE2 is expressed in human neurons,  
358 astrocytes and oligodendrocytes, located in middle temporal gyrus and posterior cingulate cortex, which  
359 may explain the brain permissiveness to SARS-CoV-2 in patients (Song *et al.*, 2020). Previous reports  
360 have demonstrated that respiratory viruses can invade the brain through neural dissemination or  
361 hematogenous route (Desforges *et al.*, 2014). Besides that, the direct connection of olfactory system to  
362 the CNS via the frontal cortex also represents a plausible route for brain invasion (Mori *et al.*, 2005).  
363 Neural transmission of viruses to the CNS can occur as a result of direct neuron invasion through axonal  
364 transport in the olfactory mucosa. Subsequent to intraneuronal replication, the virus spreads to synapses  
365 and disseminate to anatomical CNS zones receiving olfactory tract projections (Berth *et al.*, 2009;  
366 Koyuncu *et al.*, 2013; Roman *et al.*, 2020; Zubair *et al.*, 2020). However, the detection of viral RNA in  
367 CNS regions without connection with olfactory mucosa suggests the existence of another viral entry into  
368 the CNS, including migration of SARS-CoV-2-infected immune cells crossing the hemato-encephalic  
369 barrier or direct viral entry pathway via CNS vascular endothelium (Meinhardt *et al.*, 2021). Although at  
370 steady state, viruses cannot penetrate into the brain through an intact blood-brain barrier (Berth *et al.*,  
371 2009), inflammation mediators which are massively produced during cytokine/chemokine storm, notably  
372 TNF- $\alpha$  and CCL2, can disrupt the integrity of blood-brain barrier or increase its permeability, allowing  
373 paracellular blood-to-brain transport of the virus or virus-infected leukocytes (Aghagoli *et al.*, 2020; Hu  
374 *et al.*, 2011). The use of the highly stringent B6.K18-hACE2<sup>IP-THV</sup> mice demonstrated the importance of  
375 i.n. booster immunization for inducing sterilizing protection of CNS by our LV-based vaccine candidate  
376 developed against SARS-CoV-2. Olfactory bulb may control viral CNS infection through the action of  
377 local innate and adaptive immunity (Durrant *et al.*, 2016). In line with these observations, we detected  
378 increased frequencies of CD8<sup>+</sup> T cells at this anatomically strategic area in i.m.-i.n. vaccinated and  
379 protected mice. In addition, substantial reduction in the inflammatory mediators was also found in the  
380 brain of the i.m.-i.n. vaccinated and protected mice, as well as decreased proportions of neutrophils and  
381 inflammatory monocytes respectively in the olfactory bulbs and brain. Regardless of the mechanism of  
382 the SARS-CoV-2 entry into the brain, we provide evidence of the full protection of the CNS against  
383 SARS-CoV-2 by i.n. booster immunization with LV::S.

384 Importantly, while multiple SARS-CoV-2 variants are emerging around the world, serious questions  
385 are being raised about the protection potential of the vaccines currently in use against these variants  
386 (Hoffmann *et al.*, 2021). The LV::S vaccine candidate provides full cross-protection against one of the  
387 most genetically distant variants, Gamma (P.1), without antigen sequence adaptation. However, existing  
388 RNA- or adenoviral-based vaccines showed several folds reduction in neutralizing efficacy of NABs and  
389 protection potential in humans against the new SARS-CoV-2 variants (Moore & Offit, 2021).  
390 Technically, the sequence of the spike can be replaced or adapted easily in all kinds of vaccines.  
391 However, switching the spike sequence of new variants for a second or third booster shot can pose the  
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392 problem of “original sin”. According to this well-documented fact, individuals already vaccinated with  
393 the first ancestral S<sub>CoV-2</sub> sequence, might not be able to mount a new antibody response against the new  
394 S<sub>CoV-2</sub>, but can rather develop a reinforced antibody response against the firstly encountered ancestral  
395 S<sub>CoV-2</sub> (Brown & Essigmann, 2021). It is our belief that the LV::S vaccine candidate remains fully  
396 protective against the distant variants contributed by: (i) the high antibody titers with strong neutralizing  
397 activity, induced following prime immunization and, (ii) the remarkable capacity of LV to induce strong  
398 and long-lasting CD8<sup>+</sup> T-cell immunity against multiple MHC-I epitopes which are not modified by the  
399 mutations so far accumulated in S<sub>CoV-2</sub> of emerging variants. These two arms of adaptive immunity,  
400 strengthened and targeted, by the LV::S i.n. boost, to the principal entry point of the virus efficiently  
401 avoid the infection of main anatomical sites, i.e., lungs and brain by generating a mucosal immunity,  
402 which is poorly addressed by vaccinal strategies currently deployed.

403 It has been shown that the sequences of 97% of the MHC-I and -II T-cell epitopes are conserved in  
404 Alpha, Beta and Gamma variants (Tarke *et al*, 2021). Thus, it is implausible that mutations in SARS-  
405 CoV-2 variants would allow the virus to efficiently evade T-cell immunity. The high conservation of  
406 CD8<sup>+</sup> T-cell epitopes is also linked to their short sequences (9-10 amino acids), as opposed to epitopes  
407 recognized by NABs that are longer and/or conformational. It is also worth mentioning that the high HLA  
408 polymorphism in human populations covers distinct epitope repertoires between individuals, making it  
409 highly unlikely that SARS-CoV-2 would completely escape T-cell surveillance at the populational level.

410 This lack of local immunity can result in a transient presence of SARS-CoV-2 in the respiratory tract,  
411 leading to some contagiousness. The partial resistance of the variants to the NABs generated by the first-  
412 generation vaccines may exacerbate this issue in the future, avoiding a complete containment of the  
413 outbreak by mass vaccination. The sterilizing protection of the brain and lungs against the ancestral and  
414 the most distant variants of SARS-CoV-2 conferred by LV::S immunization provides a promising  
415 COVID-19 vaccine candidate of second generation. A phase I/IIa clinical trial is currently in preparation  
416 for the use of i.n. boost by LV::S in previously vaccinated persons or in COVID-19 convalescents. This  
417 LV::S i.n. boost can be used to induce long-term protection or to broaden the specificity of the protective  
418 response. Protection of the brain, so far not directly addressed by other vaccine strategies, has also to be  
419 taken into account, considering the multiple and sometimes severe neuropathological manifestations  
420 associated with COVID-19.

421 Considering: (i) the prolongation of the pandemic, (ii) a real need for new vaccines including in  
422 developing countries, and (iii) the relatively short-lived and limited specificity of mRNA vaccines which  
423 mainly promote antibody responses, the LV::S vaccine candidate has a true potential for prophylactic use  
424 against COVID-19. LV::S has a strong capacity to induce protective T-cell responses, unaffected by the  
425 current escape mutations of the variants of concern. In addition, because of its non-inflammatory

426 properties (Cousin *et al.*, 2019; Lopez *et al.*), LV is a well suited vaccinal vector for i.n. immunization,  
427 with its widely recognized benefits in the establishment of IgA and resident immune cells in the  
428 respiratory tract (Lund & Randall, 2021), and for its effectiveness in reducing virus transmission (van  
429 Doremalen *et al.*, 2021).

## 430 **Methods**

### 431 **Construction and production of LV**

432 A codon-optimized prefusion S sequence (1-1262) (Appendix Table S2) was amplified from pMK-  
433 RQ\_S-2019-nCoV and inserted into pFlap by restriction/ligation between BamHI and XhoI sites, between  
434 the native human ieCMV promoter and a mutated Woodchuck Posttranscriptional Regulatory Element  
435 (WPRE) sequence. The *atg* starting codon of WPRE was mutated (mWPRE) to avoid transcription of the  
436 downstream truncated “X” protein of Woodchuck Hepatitis Virus for safety concerns (Appendix Figure  
437 S4). Plasmids were amplified and used to produce LV as previously described (Ku *et al.*, 2021).

### 438 **Mice**

439 Female C57BL/6JRj mice (Janvier, Le Genest Saint Isle, France) were used between the age of 7 and  
440 12 wks.  $\mu$ MT KO mice were bred at Institut Pasteur animal facilities and were a kind gift of Dr P. Vieira  
441 (Institut Pasteur). Transgenic B6.K18-ACE2<sup>2PrImn/JAX</sup> mice (JAX stock #034860) were from Jackson  
442 Laboratories and were a kind gift of Dr J. Jaubert (Institut Pasteur). Transgenic B6.K18-hACE2<sup>IP-THV</sup>  
443 mice were generated and bred, as detailed below, at the Centre for Mouse Genetic Engineering, CIGM of  
444 Institut Pasteur. During the immunization period transgenic mice were housed in individually-ventilated  
445 cages under specific pathogen-free conditions. Mice were transferred into individually filtered cages in  
446 isolator for SARS-CoV-2 inoculation at the Institut Pasteur animal facilities. Prior to i.n. injections, mice  
447 were anesthetized by i.p. injection of Ketamine (Imalgene, 80 mg/kg) and Xylazine (Rompun, 5 mg/kg).

### 448 **Mouse Transgenesis**

449 The human K18 promoter (GenBank: AF179904.1 nucleotide 90 to 2579) was amplified by nested  
450 PCR from A549 cell lysate, as previously described (Chow *et al.*, 1997; Koehler *et al.*, 2000). The “i6x7”  
451 intron (GenBank: AF179904.1 nucleotide 2988 to 3740) was synthesized by Genscript. The K18<sup>JAX</sup>  
452 (originally named K18i6x7PA) promoter includes the K18 promoter, the i6x7 intron at 5' and an  
453 enhancer/polyadenylation sequence (PA) at 3' of the *hACE2* gene. The<sup>K18 IP-ThV</sup> promoter, instead of PA,  
454 contains the stronger wild-type WPRE element at 3' of the *hACE2* gene. Unlike the K18<sup>JAX</sup> construct  
455 which harbors the 3' regulatory region containing a polyA sequence, the K18<sup>IP-ThV</sup> construct uses the  
456 polyA sequence already present within the 3' Long Terminal Repeats (LTR) of the lentiviral plasmid. The  
457 i6x7 intronic part was modified to introduce a consensus 5' splicing donor and a 3' donor site sequence.  
458 The AAGGGG donor site was further modified for the AAGTGG consensus site. Based on a consensus  
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459 sequence logo (Dogan *et al.*, 2007), the poly-pyrimidine tract preceding splicing acceptor site  
460 (TACAATCCCTC in original sequence GenBank: AF179904.1 and TTTTTTTTTTTT in K18<sup>JAX</sup>) was  
461 replaced by CTTTTTCCTTCC to limit incompatibility with the reverse transcription step during  
462 transduction. Moreover, original splicing acceptor site CAGAT was modified to correspond to the  
463 consensus sequence CAGGT. As a construction facilitator, a ClaI restriction site was introduced between  
464 the promoter and the intron. The construct was inserted into a pFLAP plasmid between the MluI and  
465 BamHI sites. *hACE2* gene cDNA was introduced between the BamHI and XhoI sites by  
466 restriction/ligation. Integrative LV::K18-hACE2 was produced as described in (Ku *et al.*, 2021) and  
467 concentrated by two cycles of ultracentrifugation at 22,000 rpm 1h 4°C.

468 ILV of high titer ( $4.16 \times 10^9$  TU/ml) carrying K18-hACE2<sup>IP-THV</sup> was used in transgenesis by subzonal  
469 micro-injection under the pellucida of fertilized eggs, and transplantation into the pseudo-pregnant  
470 B6CBAF1 females. LV allows particularly efficient transfer of the transgene into the nuclei of the  
471 fertilized eggs (Nakagawa & Hoogenraad, 2011). At N0 generation,  $\approx 11\%$  of the mice, i.e., 15 out of  
472 139, had at least one copy of the transgene per genome. Eight N0 *hACE2*<sup>+</sup> males were crossed with  
473 female WT C57BL/6 mice. At N1 generation,  $\approx 62\%$  of the mice, i.e., 91 out of 147, had at least one copy  
474 of the transgene per genome.

#### 475 **Genotyping and quantitation of *hACE2* gene copy number/genome in transgenic mice**

476 Genomic DNA (gDNA) from transgenic mice was prepared from the tail biopsies by phenol-  
477 chloroform extraction. Sixty ng of gDNA were used as a template of qPCR with SYBR Green using  
478 specific primers listed in Appendix Table S3. Using the same template and in the same reaction plate,  
479 mouse *pkdl* (Polycystic Kidney Disease 1) and *gapdh* were also quantified. All samples were run in  
480 quadruplicate in 10  $\mu$ l reaction as follows: 10 min at 95°C, 40 cycles of 15 s at 95°C and 30 sec at 60°C.  
481 To calculate the transgene copy number, the  $2^{-\Delta\Delta C_t}$  method was applied using the *pkdl* as a calibrator and  
482 *gapdh* as an endogenous control. The  $2^{-\Delta\Delta C_t}$  provides the fold change in copy number of the *hACE2* gene  
483 relative to *pkdl* gene.

#### 484 **Western blot**

485 Levels of hACE2 in the lungs of transgenic mice were assessed by western blotting. Lung cell  
486 suspensions were resolved on 4%–12% NuPAGE Bis-Tris protein gels (Thermo Fisher Scientific), then  
487 transferred onto a nitrocellulose membrane (Biorad, France). The nitrocellulose membrane was blocked  
488 in 5% non-fat milk in PBS-T for 2 h at room temperature and probed overnight with goat anti-hACE2  
489 primary Ab at 1 mg/mL (AF933, R&D systems). Following three wash intervals of 10 min with PBS-T,  
490 the membrane was incubated for 1 h at room temperature with HRP-conjugated anti-goat secondary Ab  
491 and HRP-conjugated anti- $\beta$ -actin (ab197277, Abcam). The membrane was washed with PBS-T thrice

492 before visualization with enhanced chemiluminescence via the super signal west femto maximum  
493 sensitivity substrate (Thermo Fisher Scientific) on ChemiDoc XRS+ (Biorad, France). PageRuler Plus  
494 prestained protein ladder was used as size reference. Relative quantification of western blots was  
495 performed using ImageJ program. Images from the same blot were taken with the same exposure time  
496 and were inverted before measuring the protein band intensity. The ratio of hACE2 to  $\beta$ -actin was  
497 calculated to indicate the relative expression of hACE2 in each sample.

#### 498 **Ethical Approval of Animal Studies**

499 Experimentation on mice was realized in accordance with the European and French guidelines  
500 (Directive 86/609/CEE and Decree 87-848 of 19 October 1987) subsequent to approval by the Institut  
501 Pasteur Safety, Animal Care and Use Committee, protocol agreement delivered by local ethical  
502 committee (CETEA #DAP20007, CETEA #DAP200058) and Ministry of High Education and Research  
503 APAFIS#24627-2020031117362508 v1, APAFIS#28755-2020122110238379 v1.

#### 504 **Humoral and T-cell immunity, Inflammation**

505 As recently detailed elsewhere (Ku *et al.*, 2021), T-splenocyte responses were quantitated by IFN- $\gamma$   
506 ELISPOT and anti-S IgG or IgA Abs were detected by ELISA by use of recombinant stabilized S<sub>CoV-2</sub>.  
507 NAb quantitation was performed by use of LV particles pseudo-typed with S<sub>CoV-2</sub> from the diverse  
508 variants, as previously described (Anna *et al.*, 2020; Sterlin *et al.*, 2020). The qRT-PCR quantification of  
509 inflammatory mediators in the lungs, brain and olfactory bulbs was performed as recently detailed (Ku *et*  
510 *al.*, 2021) on total RNA extracted by TRIzol reagent (Invitrogen) and immediately stored at  $-80^{\circ}\text{C}$ . The  
511 RNA quality was assessed using a Bioanalyzer 2100 (Agilent Technologies). RNA samples were  
512 quantitated using a NanoDrop Spectrophotometer (Thermo Scientific NanoDrop). The RNA Integrity  
513 Number (RIN) was 7.5-10.0. CCL19 and CCL21 expression were verified using the following primer  
514 pairs: forward primers were 5'-CTG CCT CAG ATT ATC TGC CAT-3' for CCL19 and 5'- AAG GCA  
515 GTG ATG GAG GGG-3' for CCL21; reverse primers were 5'- AGG TAG CGG AAG GCT TTC AC -3'  
516 for CCL19 and 5'- CGG GGT AAG AAC AGG ATT G -3' for CCL21.

#### 517 **SARS-CoV-2 inoculation**

518 Transgenic B6.K18-hACE2<sup>IP-THV</sup> or B6.K18-ACE2<sup>2PrImn/JAX</sup> were anesthetized by i.p. injection of  
519 Ketamine and Xylazine mixture, transferred into a level 3 biosafety cabinet and inoculated i.n. with  $0.3 \times$   
520  $10^5$  TCID<sub>50</sub> of the BetaCoV/France/IDF0372/2020 or Gamma (P.1) SARS-CoV-2 clinical isolate  
521 (Lescure *et al.*, 2020). Mice were inoculated i.n. with 20  $\mu\text{l}$  of viral inoculum and were housed in an  
522 isolator in BioSafety Level 3 animal facilities of Institut Pasteur. The organs recovered from the infected  
523 mice were manipulated according to the approved standard procedures of these facilities. Ad5::hACE2  
524 pretreatment of WT of  $\mu\text{MT}$  KO mice before SARS-CoV-2 inoculation was performed as previously

525 described (Ku *et al.*, 2021). All experiments with SARS-CoV-2 were performed in a biosafety level 3  
526 laboratory and with approval from the department of hygiene and security of Institut Pasteur, under the  
527 protocol agreement # 20.070 A-B.

### 528 **Determination of viral RNA content in the organs**

529 Organs from mice were removed aseptically and immediately frozen at -80°C. RNA from circulating  
530 SARS-CoV-2 was prepared from lungs as recently described (Ku *et al.*, 2021). Briefly, lung homogenates  
531 were prepared by thawing and homogenizing of the organs in lysing matrix M (MP Biomedical) with 500  
532 µl of ice-cold PBS using a MP Biomedical Fastprep 24 Tissue Homogenizer. RNA was extracted from  
533 the supernatants of lung homogenates centrifuged during 10 min at 2000g, using the Qiagen Rneasy kit  
534 according to the manufacturer instructions, except that the neutralization step with AVL buffer/carrier  
535 RNA was omitted. These RNA preparations were used to determine viral RNA content by E-specific  
536 qRT-PCR. Alternatively, total RNA was prepared from lungs or other organs using lysing matrix D (MP  
537 Biomedical) containing 1 mL of TRIzol reagent and homogenization at 30 s at 6.0 m/s twice using MP  
538 Biomedical Fastprep 24 Tissue Homogenizer. Total RNA was extracted using TRIzol reagent  
539 (ThermoFisher). These RNA preparations were used to determine viral RNA content by Esg-specific  
540 qRT-PCR, hACE2 expression level or inflammatory mediators. RNA was isolated from nasal washes  
541 using QIAamp Viral RNA Mini Kit (Qiagen).

542 SARS-CoV-2 E gene (Corman *et al.*, 2020) or E sub-genomic mRNA (Esg RNA) (Wolfel *et al.*, 2020),  
543 was quantitated following reverse transcription and real-time quantitative TaqMan® PCR, using  
544 SuperScript™ III Platinum One-Step qRT-PCR System (Invitrogen) and specific primers and probe  
545 (Eurofins) (Appendix Table S4). The standard curve of Esg mRNA assay was performed using in vitro  
546 transcribed RNA derived from PCR fragment of “T7 SARS-CoV-2 Esg mRNA”. The in vitro transcribed  
547 RNA was synthesized using T7 RiboMAX Express Large Scale RNA production system (Promega) and  
548 purified by phenol/chloroform extraction and two successive precipitations with isopropanol and ethanol.  
549 Concentration of RNA was determined by optical density measurement, diluted to 10<sup>9</sup> genome  
550 equivalents/µL in RNase-free water containing 100µg/mL tRNA carrier, and stored at -80°C. Serial  
551 dilutions of this in vitro transcribed RNA were prepared in RNase-free water containing 10µg/ml tRNA  
552 carrier to build a standard curve for each assay. PCR conditions were: (i) reverse transcription at 55°C for  
553 10 min, (ii) enzyme inactivation at 95°C for 3 min, and (iii) 45 cycles of denaturation/amplification at  
554 95°C for 15 s, 58°C for 30 s. PCR products were analyzed on an ABI 7500 Fast real-time PCR system  
555 (Applied Biosystems). PFU assay was performed as previously described (Ku *et al.*, 2021).

### 556 **Cytometric analysis of immune lung and brain cells**

557 Isolation and staining of lung innate immune cells were largely detailed recently (Ku *et al.*, 2021).  
558 Cervical lymph nodes, olfactory bulb and brain from each group of mice were pooled and treated with  
559 400 U/ml type IV collagenase and DNase I (Roche) for a 30-minute incubation at 37°C. Cervical lymph  
560 nodes and olfactory bulbs were then homogenized with glass homogenizer while brains were  
561 homogenized by use of GentleMacs (Miltenyi Biotech). Cell suspensions were then filtered through 100  
562 µm-pore filters, washed and centrifuged at 1200 rpm during 8 minutes. Cell suspensions from brain were  
563 enriched in immune cells on Percoll gradient after 25 min centrifugation at 1360 g at RT, without brakes.  
564 The recovered cells from lungs were stained as recently described elsewhere (Ku *et al.*, 2021). The  
565 recovered cells from brain were stained by appropriate mAb mixture as follows. (i) To detect innate  
566 immune cells, Near IR Live/Dead (Invitrogen), FcγII/III receptor blocking anti-CD16/CD32 (BD  
567 Biosciences), BV605-anti-CD45 (BD Biosciences), PE-anti-CD11b (eBioscience), and PE-Cy7-  
568 antiCD11c (eBioscience) were used. (ii) To detect NK, neutrophils, Ly-6C<sup>±</sup> monocytes and  
569 macrophages, Near IR DL (Invitrogen), FcγII/III receptor blocking anti-CD16/CD32 (BD Biosciences),  
570 BV605-anti-CD45 (BD Biosciences), PE-anti-CD11b (eBioscience), PE-Cy7-antiCD11c (eBioscience),  
571 APC-anti-Ly6G (Miltenyi), BV711-anti-Siglec-F (BD), AF700-anti-NKp46 (BD Biosciences), and FITC-  
572 anti-Ly6C (ab25025, Abcam), were used. (iii) To detect adaptive immune cells, Near IR Live/Dead  
573 (Invitrogen), FcγII/III receptor blocking anti-CD16/CD32 (BD Biosciences), APC-anti-CD45 (BD),  
574 PerCP-Cy5.5-anti-CD3 (eBioscience), FITC-anti-CD4 (BD Pharmingen), BV711-anti-CD8 (BD  
575 Horizon), BV605-anti-CD69 (Biolegend), PE-anti-CCR7 (eBioscience) and VioBlue-Anti-B220  
576 (Miltenyi), were used. (iv) To identify lung memory CD8<sup>+</sup> T-cell subsets, PerCP-Vio700-anti-CD3,  
577 BV510-anti-CD8, PE-anti-CD62L, APC-anti-CD69, APC-Cy7-anti-CD44 and FITC-anti-CD103 were  
578 used. (v) To identify S<sub>CoV-2</sub>-specific CD8<sup>+</sup> T-cells, a dextramer of H2-D<sup>b</sup> combined to S<sub>CoV-2</sub>:538-546  
579 (CVNFNFNGL) epitope (Zhuang *et al.*, 2021) was used (Immudex, Danmark). Lung cells were first  
580 stained with the PE-conjugated dextramer for 30 min in dark at room temperature prior at the addition of  
581 a cocktail of Yellow Live/Dead (Invitrogen) and PerCP-Vio700-anti-CD3, BV510-anti-CD8, BV421-  
582 anti-CD62L, APC-anti-CD69, APC-Cy7-anti-CD44 and FITC-anti-CD103 mAbs. Cells were incubated  
583 with appropriate mixtures for 25 minutes at 4°C, washed in PBS containing 3% FCS and fixed with  
584 Paraformaldehyde 4% by an overnight incubation at 4°C. Samples were acquired in an Attune NxT  
585 cytometer (Invitrogen) and data analyzed by FlowJo software (Treestar, OR, USA).

## 586 **Histopathology**

587 Samples from the lungs or brain of transgenic mice were fixed in formalin for 7 days and embedded in  
588 paraffin. Paraffin sections (5-µm thick) were stained with Hematoxylin and Eosin (H&E). In some cases,  
589 serial sections were prepared for IHC analyses. Slides were scanned using the AxioScan Z1 (Zeiss)  
590 system and images were analyzed with the Zen 2.6 software. Histopathological lesions were qualitatively

591 described and when possible scored, using: (i) distribution qualifiers (i.e., focal, multifocal, locally  
592 extensive or diffuse), and (ii) a five-scale severity grade, i.e., 1: minimal, 2: mild, 3: moderate, 4: marked  
593 and 5: severe. For the histological heatmaps, the scores were determined as follows: the percentage of  
594 abnormal zone was estimated from low magnification images of scanned slides. All other scores were  
595 established at higher magnification (20 to 40× in the Zen program); the interstitial and alveolar syndrome  
596 scores reflected the extent of the syndrome, while the inflammation seriousness represented an evaluation  
597 of the intensity of the inflammatory reaction, i.e., abundance of inflammatory cells and exudate,  
598 conservation or disruption of the lung architecture; the bronchiolar epithelium alteration score was  
599 derived from both the extent and the severity of the lesions. IHC was performed as described elsewhere.  
600 Rabbit anti-N<sub>Cov-2</sub> antibody (NB100-56576, Novus Biologicals, France) and biotinylated goat anti-rabbit  
601 Ig secondary antibody (E0432, Dako, Agilent, France) were used in IHC.

## 602 **Statistical analyses**

603 Experiments were performed with numbers of animals previously determined as sufficient for a  
604 correct statistical assessment, based on biostatistical prediction. The Mann-Whitney statistical test was  
605 applied to the results, using Graph Pad Prism8 software.

## 606 **Data Availability**

607 This study includes no data deposited in external repositories. Information/data required will be  
608 available by the corresponding author upon request.

609

610 **References**

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772

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789 **Author Contributions**

790 Study concept and design: MWK, MB, FA, FLV, LM, PC, acquisition of data: MWK, PA, MB, FA,  
791 AN, BV, FN, JL, PS, CB, KN, LM, construction and production of LV and technical support: PA, AN,  
792 FM, PS, CB, IF, analysis and interpretation of data: MWK, PA, MB, FA, FG, FLV, LM, PC, mouse  
793 transgenesis: SC, IL, DC, FLV, histology: DH, FG, drafting of the manuscript: MWK, PA, FG, LM.

794 **Conflict of Interests**

795 PC is the founder and CSO of TheraVectys. MWK, FA, PA, AN, FM, BV, FN, JL, IF and KN are  
796 employees of TheraVectys. Other authors declare no competing interests. MWK, FA, AN, FLV, LM and  
797 PC are inventors of a pending patent directed to the B6.K18-hACE2<sup>IP-THV</sup> transgenic mice and the  
798 potential of i.n. LV::S vaccination at protecting brain against SARS-CoV-2.

799 **For more information**

800 Author's website: <https://theravectys.site/>

801

802 **The Paper Explained**

803 **PROBLEM:** Prolongation of the pandemic COVID-19 requires the development of effective second-  
804 generation vaccines. Although lung is the main site of SARS-CoV-2 infection, the virus can infect the  
805 central nervous system leading to headache, myalgia, smell loss and taste impairment and reduced  
806 consciousness, with possible long-term consequences. However, for want of a relevant model, it has been  
807 difficult to evaluate the protective effects of current COVID-19 vaccines on the brain. In addition, these  
808 first-generation vaccines seem to induce only partial protection against several SARS-CoV-2 emerging  
809 variants.

810  
811 **RESULTS:** We have recently showed the high vaccine efficacy of a lentiviral vector targeting the Spike  
812 antigen from SARS-CoV-2 (LV::S), when used for intramuscular prime followed by intranasal boost.  
813 LV::S not only induces strong antibody responses but is particularly effective at inducing T-cell  
814 responses. Here, we generated a murine transgenic preclinical model with high permissiveness of both  
815 lung and brain to SARS-CoV-2 infection. In this model, we demonstrated that LV::S induces sterilizing  
816 protection of lung and brain, not only against the ancestral SARS-CoV-2, but also against one of the most  
817 genetically distant SARS-CoV-2 variants of concern known to date. The strong T-cell response induced  
818 by the LV::S vaccine candidate probably plays an important role in this cross-protection, as antigenic  
819 motifs recognized by the LV::S-induced T cells are not target of mutations accumulating in the emerging  
820 SARS-CoV-2 variants of concern.

821  
822 **IMPACT:** The LV-based vaccination strategy is recent, effective and promising. LV vector has an  
823 excellent safety record as demonstrated in a previous clinical trial. This vector is safe and non-  
824 inflammatory and is appropriate for mucosal immunization which protects, in addition to the lungs, the  
825 brain of vaccinated animals. Therefore LV::S emerges as a vaccine candidate of choice to boost waning  
826 immunity in patients convalescing from the first epidemic waves or individuals vaccinated with first  
827 generation vaccines. This vaccine candidate should avoid neurological complications related to COVID-  
828 19 and extend the protective potential against new emerging variants. A Phase I/II clinical trial is  
829 currently in preparation.

830 **Figure Legend**

831 **Figure 1. Large permissiveness of the lungs and brain of B6.K18-hACE2<sup>IP-THV</sup> transgenic**  
832 **mice to SARS-CoV-2 replication.**

833 (A) Representative genotyping results from 15 N1 B6.K18-hACE2<sup>IP-THV</sup> mice as performed by  
834 qPCR to determine their *hACE2* gene copy number per genome. Dots represent individual mice.

835 (B) Phenotyping of the same mice, presented in the same order, inoculated i.n. with  $0.3 \times 10^5$   
836 TCID<sub>50</sub> of SARS-CoV-2 at the age of 5-7 wks and viral RNA content was determination in the  
837 indicated organs at 3 dpi by conventional E-specific qRT-PCR. Red lines indicate the qRT-PCR  
838 limits of detection.

839 (C, D) Quantification (C) and images (D) of hACE2 protein expression level by Western blot  
840 from the lungs of the same mice as in panels A and B, presented in the same order.

841 (E, F) Kinetics of SARS-CoV-2 replication in the lungs and brain of B6.K18-hACE2<sup>IP-THV</sup> mice as  
842 followed by measuring viral RNA contents by conventional E-specific qRT-PCR (E) or by PFU  
843 counting (F).

844 (G) Percentage of initial weight measured from the mice of panels E and F.

845

846 **Figure 2. Histology of the lungs and brain of B6.K18-hACE2<sup>IP-THV</sup> transgenic mice after**  
847 **SARS-CoV-2 inoculation.**

848 (A) Representative H&E whole-lung section at 3 dpi in B6.K18-hACE2<sup>IP-THV</sup> transgenic mice  
849 inoculated i.n. with  $0.3 \times 10^5$  TCID<sub>50</sub> of SARS-CoV-2, compared to non-infected controls (NI). In  
850 the infected lung, less transparent, purple-red areas resulted from inflammatory lesions of the lung  
851 parenchyma. Scale bar: 500  $\mu$ m.

852 (B-E) Examples of lesions observed in the lungs of infected mice at higher magnification. The two  
853 top panels depict mild (B) (scale bar: 100  $\mu$ m) and mild to moderate (C) interstitium thickening  
854 accompanied by dense inflammatory infiltrates predominantly localized in the vicinity of bronchioles  
855 (yellow stars) and also present around blood vessels (green stars) (scale bar: 200  $\mu$ m). (D) Alveoli  
856 filled with a proteinaceous exudate containing a few cells (green arrows) (scale bar: 50  $\mu$ m). (E)  
857 Discrete degenerative lesions of the bronchiolar epithelium, such as perinuclear clear spaces (blue  
858 arrows), hyper-eosinophilic cells with condensed nuclei (orange arrow) and some intraluminal  
859 fibrinous and cell debris (black arrow) in an overall well-preserved epithelium (scale bar: 20  $\mu$ m).

860 (F) Heatmap representing the histological scores for various parameters in infected mice at 3 dpi,  
861 compared to NI controls ( $n = 3/\text{group}$ ). Statistical significance was evaluated by Mann-Whitney test  
862 ( $*= p < 0.05$ ,  $**= p < 0.01$ ,  $***= p < 0.0001$ , ns = not significant).

863 (G) Representative  $N_{\text{CoV-2}}$ -specific IHC image of the brain in NI control or SARS-CoV-2-infected  
864 mice at 3 dpi. Scale bar: 500  $\mu\text{m}$ .

865 (H) Closer view of a  $N_{\text{CoV-2}}$  positive area from an infected mouse. Scale bar: 500  $\mu\text{m}$ .

866 **Figure 3. Comparison of SARS-CoV-2 replication and infection-derived inflammation in**  
867 **B6.K18-hACE2<sup>IP-THV</sup> and B6.K18-hACE2<sup>prlmm/Jax</sup> transgenic mice.**

868 (A) Comparative permissiveness of various organs from B6.K18-hACE2<sup>IP-THV</sup> and B6.K18-  
869 ACE2<sup>prlmm/Jax</sup> transgenic mice ( $n = 5\text{-}6/\text{group}$ ) to SARS-CoV-2 replication, as determined at 3 dpi by  
870 conventional E-specific or sub-genomic Esg-specific qRT-PCR. Red lines indicate the qRT-PCR  
871 limits of detection.

872 (B) Comparative quantitation of *hACE-2* mRNA in the lungs and brain of B6.K18-hACE2<sup>IP-THV</sup>  
873 and B6.K18-ACE2<sup>prlmm/Jax</sup> transgenic mice ( $n = 5\text{-}6/\text{group}$ ).

874 (C) Heatmaps represent  $\log_2$  fold change in cytokine and chemokine mRNA expression in the  
875 lungs or brain of B6.K18-hACE2<sup>IP-THV</sup> and B6.K18-ACE2<sup>prlmm/Jax</sup> mice at 3 dpi ( $n = 5\text{-}6/\text{group}$ ). Data  
876 were normalized versus untreated controls.

877 Data information: Statistical significance was evaluated by Mann-Whitney test ( $*= p < 0.05$ ,  $**= p$   
878  $< 0.01$ , ns = not significant).

879 **Figure 4. Vaccination with LV::S protects both lungs and central nervous system from**  
880 **SARS-CoV-2 infection in B6.K18-hACE2<sup>IP-THV</sup> transgenic mice.**

881 (A) Timeline of prime-boost LV::S vaccination and SARS-CoV-2 challenge in B6.K18-hACE2<sup>IP-</sup>  
882 <sup>THV</sup> mice ( $n = 6/\text{group}$ ). The LVs used in this experiment were integrative.

883 (B) Serum neutralization capacity of anti- $S_{\text{CoV-2}}$  Abs in LV::S-vaccinated mice compared to sham  
884 mice ( $n = 6/\text{group}$ ).

885 (C) Viral RNA content as determined in various organs at 3 dpi ( $n = 6/\text{group}$ ) by use of  
886 conventional E-specific or sub-genomic Esg-specific qRT-PCR. Red lines indicate the qRT-PCR  
887 detection limits.

888 (D) Percentages of NK cells or neutrophils in the lungs of LV::S- or sham-vaccinated and SARS-  
889 CoV-2-challenged B6.K18-hACE2<sup>IP-THV</sup> transgenic mice at 3 dpi ( $n = 6/\text{group}$ ). Percentages were  
890 calculated versus total lung live CD45<sup>+</sup> cells.

891 (E) Relative log<sub>2</sub> fold change in cytokine and chemokine mRNA expression in the brain of LV::S-  
892 or sham-immunized and SARS-CoV-2-challenged B6.K18-hACE2<sup>IP-THV</sup> transgenic mice at 3 dpi (*n*  
893 = 6/group). Means ± SD are shown. Data were normalized versus untreated controls.

894 Data information: Statistical significance was evaluated by Mann-Whitney test (\* = *p*<0.05, \*\* =  
895 *p*<0.01).

896 **Figure 5. Cellular and humoral immunity in LV::S-vaccinated B6.K18-hACE2<sup>IP-THV</sup> mice.**

897 B6.K18-hACE2<sup>IP-THV</sup> mice were primed (i.m.) at wk 0 and boosted (i.n.) at wk 5 (*n* = 5) with non-  
898 integrative LV::S. Control mice were injected with an empty LV (sham).

899 (A) Representative IFN-γ response by lung CD8<sup>+</sup> T cells of as studied at wk 7 after in vitro  
900 stimulation with the indicated S<sub>CoV-2</sub>-derived peptides.

901 (B, C) Cytometric strategy to detect lung CD8<sup>+</sup> T central memory (T<sub>cm</sub>, CD44<sup>+</sup>CD62L<sup>+</sup>CD69<sup>-</sup>), T  
902 effector memory (T<sub>em</sub>, CD44<sup>+</sup>CD62L<sup>-</sup>CD69<sup>-</sup>) and T resident memory (T<sub>rm</sub>, CD44<sup>+</sup>CD62L<sup>-</sup>  
903 CD69<sup>+</sup>CD103<sup>+</sup>) and (C) percentages of these subsets among CD8<sup>+</sup> T-cells in LV::S-vaccinated (*n* =  
904 9) or sham (*n* = 5) mice.

905 (D) Cytometric strategy to detect S<sub>CoV-2</sub>-specific CD8<sup>+</sup> T cells by use of the H-2D<sup>b</sup>-S<sub>CoV-2</sub>:538-546  
906 dextramer in the lungs of LV::S or sham-vaccinated mice. Inside CD8<sup>+</sup> dextramer<sup>+</sup> T-cell subset,  
907 T<sub>cm</sub>, T<sub>em</sub> and T<sub>rm</sub> have been distinguished.

908 (E) Percentages of dextramer<sup>+</sup> cells were calculated versus CD8<sup>+</sup> T cells in both mouse groups and  
909 those of T<sub>cm</sub>, T<sub>em</sub> and T<sub>rm</sub> were calculated versus dextramer<sup>+</sup> CD44<sup>+</sup> cells in LV::S-vaccinated  
910 mice (*n* = 4/group). N/A= not applicable.

911 (F, G) Anti-S<sub>CoV-2</sub> IgG or IgA titers (F) and neutralizing activity (EC50) (G) in the sera or lung  
912 homogenates at 3 dpi. Samples from individual mice (*n* = 4/group) were studied.

913 Data information: Statistical significance of the difference between the two groups was evaluated by  
914 Mann-Whitney test (\* = *p* < 0.05, \*\* = *p* < 0.01).

915 **Figure 6. Comparison of vaccination routes in the protective efficacy of LV::S.**  
916 **Comparative histopathology of lungs from unprotected and LV::S-vaccinated and protected**  
917 **mice.**

918 (A) B6.K18-hACE2<sup>IP-THV</sup> mice were immunized with LV::S via i.m. or i.n. at wk 0 and boosted  
919 via i.m. or i.n. at wk 5 (*n* = 5/group) with non-integrative LV::S. Control mice were injected i.m. i.n.  
920 with an empty LV (sham). Viral RNA contents were determined by conventional E-specific RT-PCR  
921 at 3 dpi, in the brain, lung and nasal washes.

922 (B) Lung histology in B6.K18-hACE2<sup>IP-THV</sup> mice, vaccinated with LV::S after SARS-CoV-2  
923 inoculation. H&E (rows 1 and 3) (scale bar: 500  $\mu$ m) and N<sub>CoV-2</sub>-specific IHC (rows 2 and 4) (scale  
924 bar: 50  $\mu$ m) staining of whole lung sections (scale bar: 50  $\mu$ m) from the primed (i.m.), boosted (i.n.)  
925 and challenged B6.K18-hACE2<sup>IP-THV</sup> mice compared with their sham controls. H&E and N<sub>CoV-2</sub>-  
926 specific IHC were performed on contiguous sections. The IHC fields correspond to the rectangles in  
927 the corresponding H&E images above them. A representative N<sub>CoV-2</sub>-specific IHC on a lung section  
928 from a non-infected (NI) mouse is also shown.

929 (C) Heatmap representing the histological scores for various parameters in LV::S-vaccinated or  
930 sham mice at 3 dpi ( $n = 6$ /group).

931 Data information: Statistical significance was evaluated by Mann-Whitney test (\*=  $p < 0.05$ , \*\*=  $p$   
932  $< 0.01$ , \*\*\*\*=  $p < 0.0001$ , ns = not significant).

933 **Figure 7. Features of olfactory bulbs or brains in the protected LV::S- or unprotected sham-**  
934 **vaccinated B6.K18-hACE2<sup>IP-THV</sup> mice.** Mice are those detailed in the Figure 6.

935 (A) Example of CD3-positive cells in an olfactory bulb from an LV::S i.m.-i.n. vaccinated and  
936 protected mice and representative results from this group versus sham-vaccinated and unprotected  
937 mice at 3 dpi ( $n = 7-9$ /group). Scale bar: 50  $\mu$ m. Statistical significance was evaluated by Mann-  
938 Whitney test (\*\*=  $p < 0.01$ ).

939 (B-D) Cytometric analysis of cells extracted from pooled olfactory bulbs from the same groups.  
940 (C, D) Innate immune cells in the olfactory bulbs (C) or brain (D). The CD11b<sup>+</sup> Ly6C<sup>+</sup> Ly6G<sup>+</sup>  
941 population in the olfactory bulbs are neutrophils and the CD11b<sup>+</sup> Ly6C<sup>+</sup> Ly6G<sup>-</sup> cells of the brain are  
942 inflammatory monocytes.

943 (E) Brain H&E histology at 3 dpi. The top right and both bottom panels show examples, in two  
944 different mice, of leukocyte clusters (arrows) alongside the ventricular wall. No such clusters were  
945 detected in the LV::S i.m.-i.n. vaccinated mice (top left panel). Scale bar: 200  $\mu$ m. The close up view  
946 (bottom right panel) highlights the thickened, disorganized ependymal lining, compared to the  
947 normal ependymal cells and cilia of an LV::S i.m.-i.n. vaccinated mouse (top left panel). Scale bar:  
948 50  $\mu$ m.

949 **Figure 8. Full protective capacity of LV::S against the SARS-CoV-2 Gamma variant.**

950 (A) Timeline of LV::S i.m.-i.n. immunization and challenge with SARS-CoV-2 Gamma in  
951 B6.K18-hACE2<sup>IP-THV</sup> mice ( $n = 5$ /group). The LVs used in this experiment were non-integrative.  
952 Olfactory bulbs, brains and lungs were collected at 3 dpi.

953 (B) Brain or lung viral RNA contents, determined by conventional E-specific or sub-genomic Esg-  
954 specific qRT-PCR at 3 dpi. Two mice out of the 5 sham-vaccinated mice did not have detectable viral  
955 RNA in the lungs despite high viral RNA content in the brain and hACE2 mRNA expression levels  
956 comparable to that of the other mice in the same group.

957 (C) Neutralizing activity (EC50) of sera from individual LV::S-vaccinated mice against pseudo-  
958 viruses harboring S<sub>CoV-2</sub> from the ancestral strain or D614G, Alpha, Beta or Gamma variants. Red  
959 asterisk indicates significance versus ancestral, blue asterisk indicates significance versus D614G  
960 variant, while orange asterisk indicates significance versus Alpha variant. Statistical comparisons  
961 were made at the respective boosting timepoint. In homologous settings, sera from mice immunized  
962 with LV::S<sub>Beta</sub> or LV::S<sub>Gamma</sub>, fully inhibited pseudoviruses bearing S from Beta or Gamma,  
963 validating the assay for all pseudo-viruses used.

964 (D) Cytometric analysis of CD8<sup>+</sup> T cells in pooled olfactory bulbs of LV::S i.m.-i.n. vaccinated  
965 and protected mice versus sham-vaccinated and unprotected mice.

966 (E) Wild type or  $\mu$ MT KO mice ( $n = 5-9$ /group) were injected by LV::S or sham following the  
967 time line shown in (A), then pretreated with Ad5::hACE2 4 days before challenge with the ancestral  
968 SARS-CoV-2 strain. Lung viral RNA contents were determined at 3 dpi.

969 (F) T-splenocyte responses in LV::S-primed and -boosted C57BL/6 WT mice or sham controls ( $n$   
970 = 3-5/group), evaluated by IFN- $\gamma$  ELISPOT using 15-mer peptides encompassing S<sub>CoV-2</sub> MHC-I-  
971 restricted epitopes.

972 Data information: Statistical significance was evaluated by Mann-Whitney test (\*=  $p < 0.05$ , \*\*=  $p <$   
973  $0.01$ , \*\*\*\*=  $p < 0.0001$ ).

974

975 **Expanded View Figure Legends**

976 **Figure EV1. Inflammation status of the lungs.**

977 (A) Cytometric analysis of innate cell population and qRT-PCR analysis of cytokines and  
978 chemokines in the lungs of LV::S- or sham-vaccinated and SARS-CoV-2-challenged B6.K18-  
979 hACE2<sup>IP-THV</sup> transgenic mice. Cytometric gating strategy to quantify various lung innate immune  
980 cells at 3 dpi. Cells were first gated on hematopoietic CD45<sup>+</sup> cells and then by sequential gates,  
981 through three distinct paths.

982 (B) Percentages of selected innate immune subsets versus total lung CD45<sup>+</sup> cells were  
983 determined in individual mice ( $n = 6/\text{group}$ ).

984 (C) Heatmap representing log<sub>2</sub> fold change in cytokine and chemokine mRNA expression in the  
985 lungs of LV::S- or sham-vaccinated mice at 3 dpi ( $n = 6/\text{group}$ ). Data were normalized versus  
986 untreated controls. Statistical significance was evaluated by Mann-Whitney test ( $* = p < 0.05$ ,  $** = p$   
987  $< 0.01$ , ns = not significant).

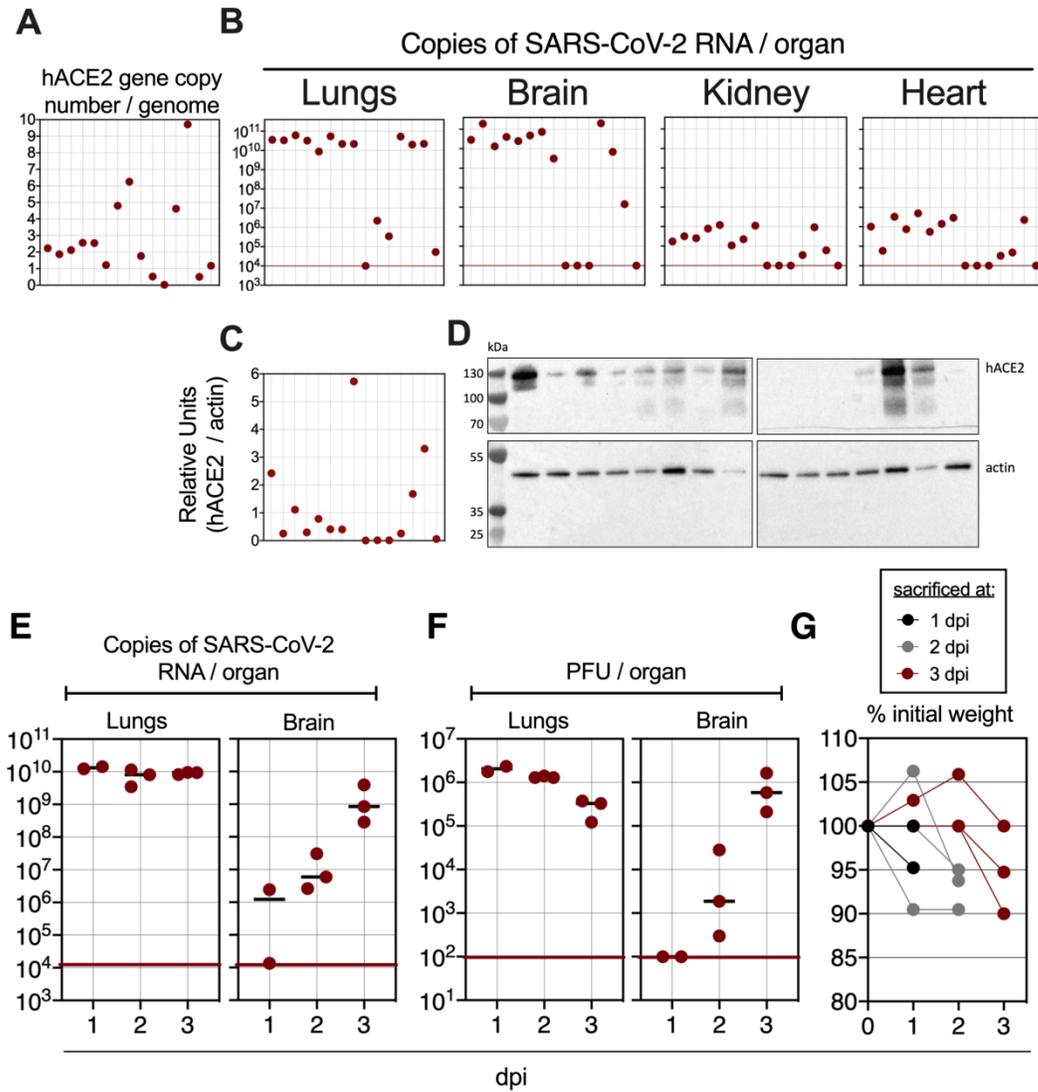
988 **Figure EV2. Lung and brain histology in B6.K18-hACE2<sup>IP-THV</sup> mice, vaccinated with LV::S**  
989 **and challenged with SARS-CoV-2 Gamma.**

990 (A, B) H&E (rows 1 and 3) and N<sub>CoV-2</sub>-specific IHC (rows 2 and 4) staining of 3 dpi whole-lung  
991 sections from B6.K18-hACE2<sup>IP-THV</sup> mice, LV::S- or sham-vaccinated and challenged, following the  
992 time line in Figure 7A. H&E and IHC were performed on contiguous sections. Scale bar: 500  $\mu\text{m}$ .  
993 The boxed area in the IHC images harbor N<sub>CoV-2</sub>-specific labeling, as exemplified at higher  
994 magnification in (B) Scale bar: 100  $\mu\text{m}$ . They correspond to inflammatory infiltrates seen in the  
995 corresponding H&E-stained sections.

996 (C) Representative images of N<sub>CoV-2</sub>-specific IHC staining of whole-brain section in sham or  
997 LV::S-vaccinated mice. Boxes highlight clusters of N<sup>CoV-2</sup> positive cells. Scale bar: 500  $\mu\text{m}$ .

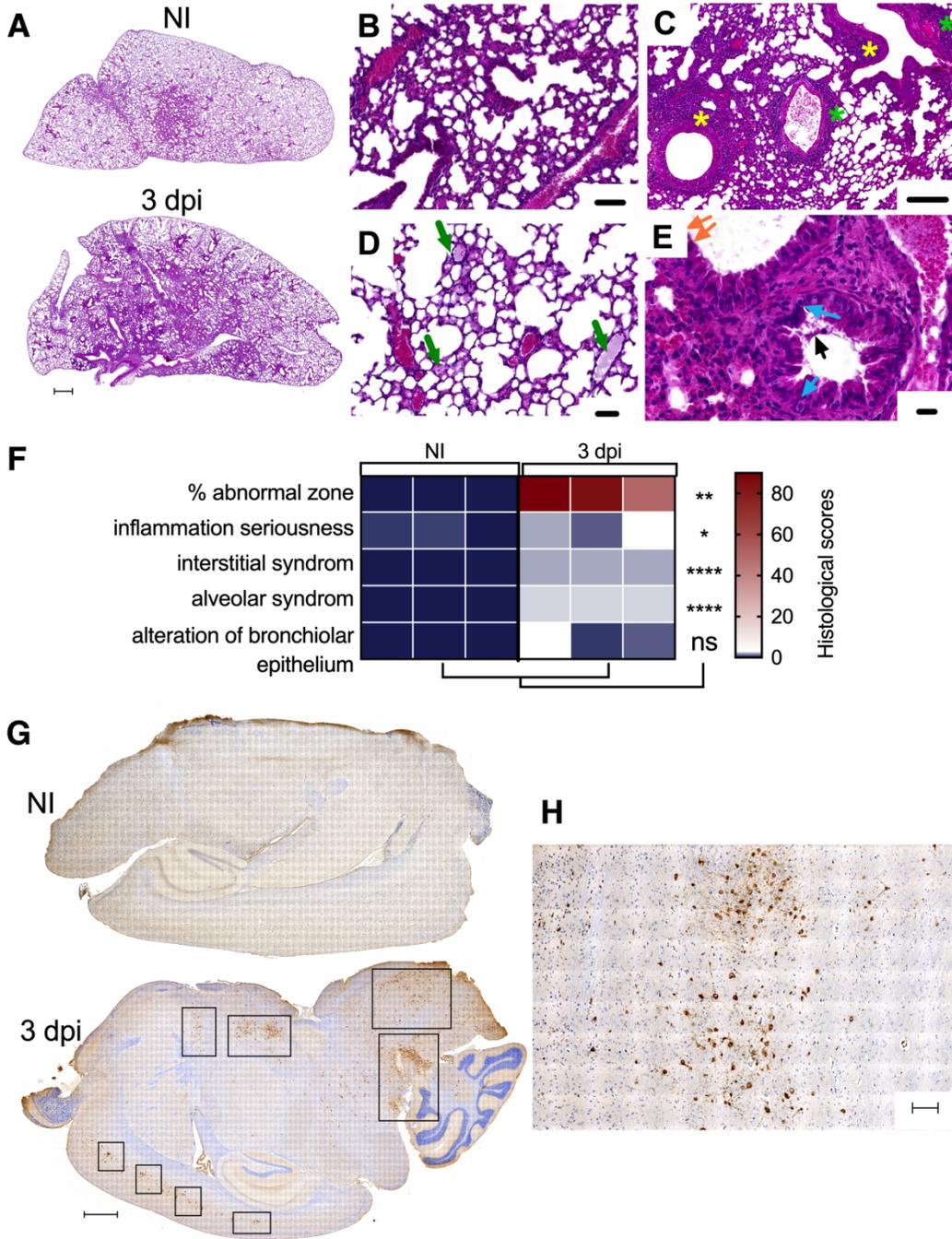
998 (D) An example of brain IHC signal at higher magnification and frequency of N<sub>CoV2</sub><sup>+</sup> cells in  
999 brains from vaccinated and sham animals. Scale bar: 100  $\mu\text{m}$ . Numbers of N<sub>CoV2</sub><sup>+</sup> cells per mm<sup>2</sup> of  
1000 brain were determined in individual mice ( $n = 5/\text{group}$ ). Statistical significance was evaluated by  
1001 Mann-Whitney test ( $** = p < 0.01$ ).

Figure 1



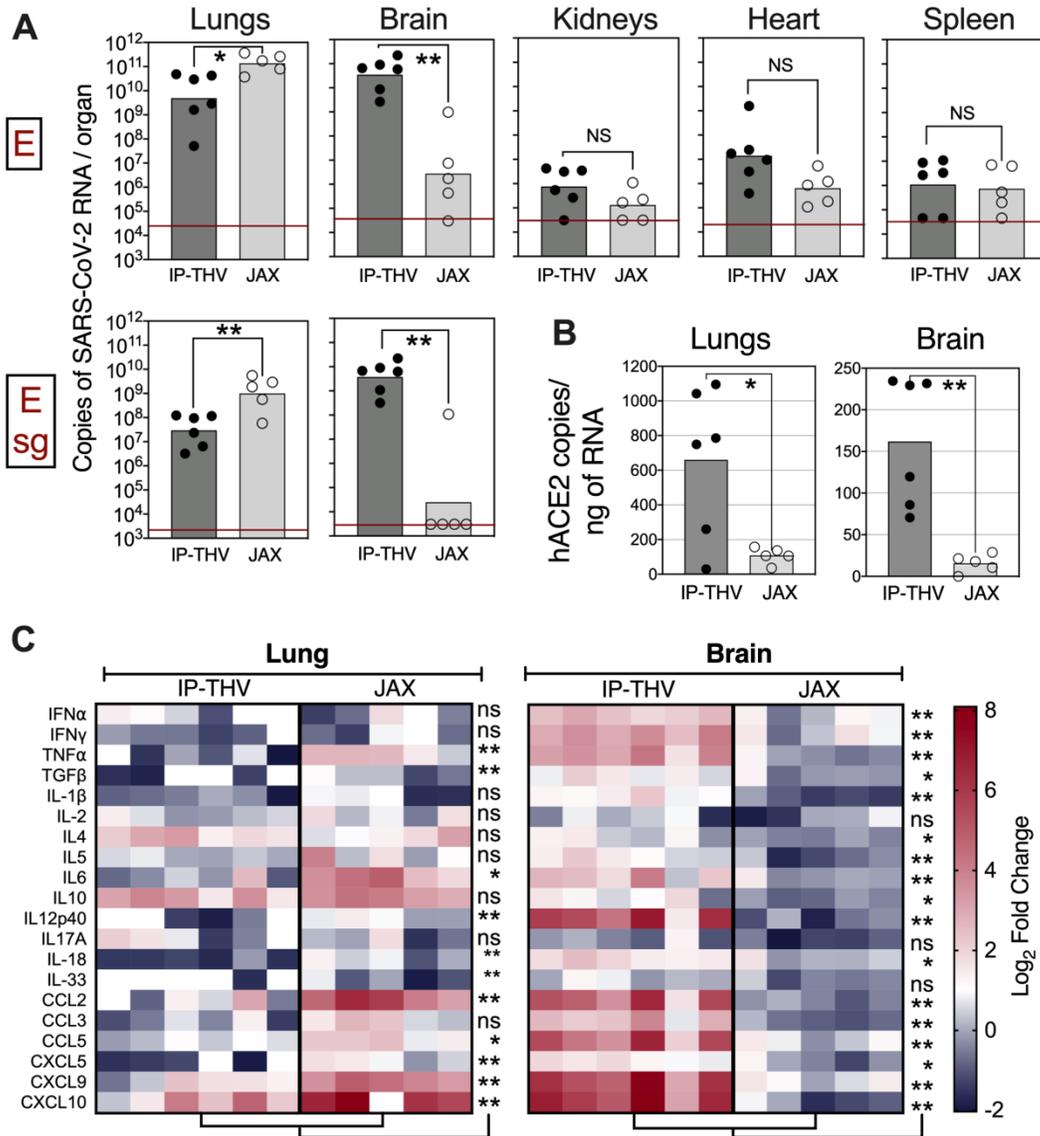
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Figure 2

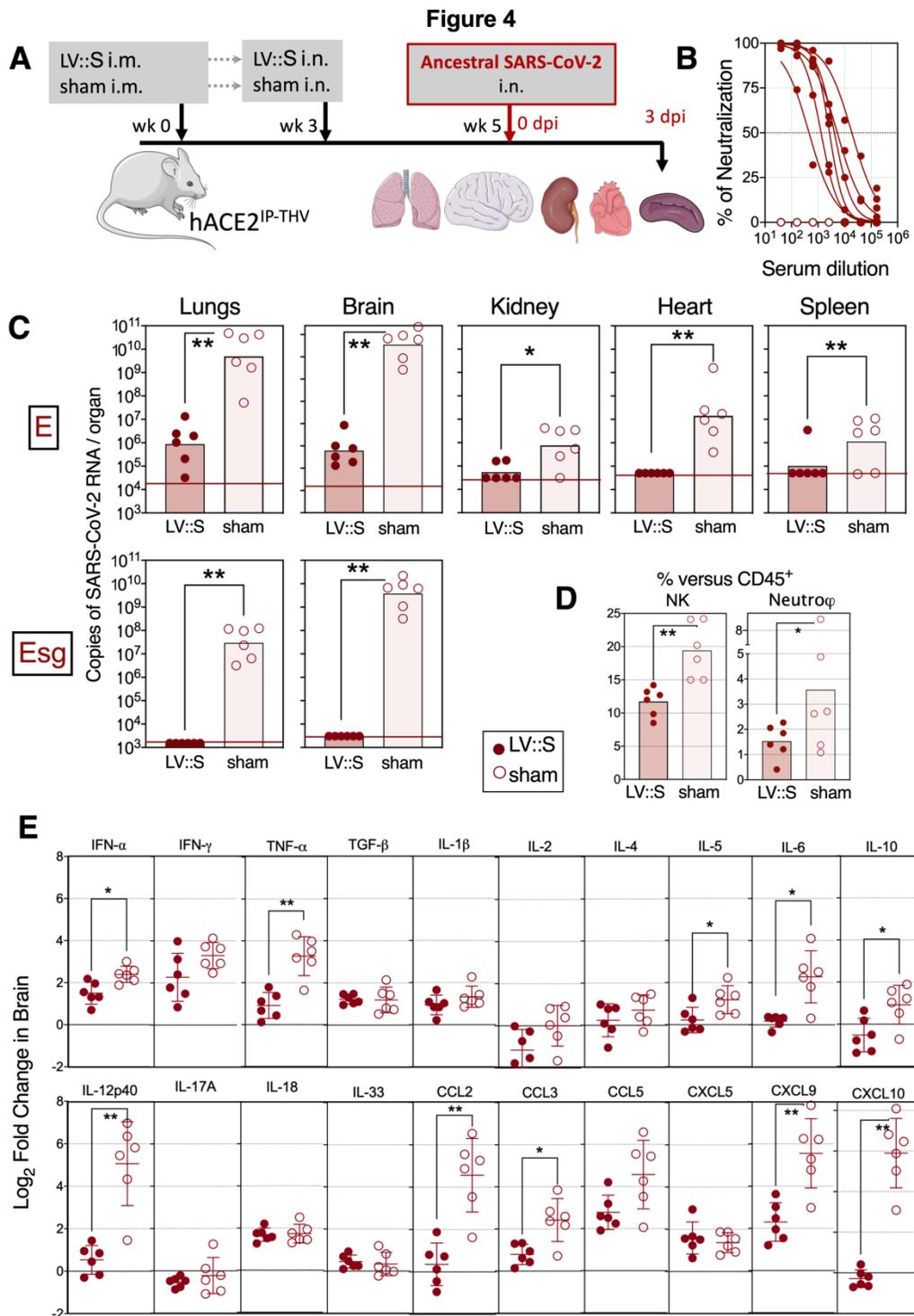


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Figure 3

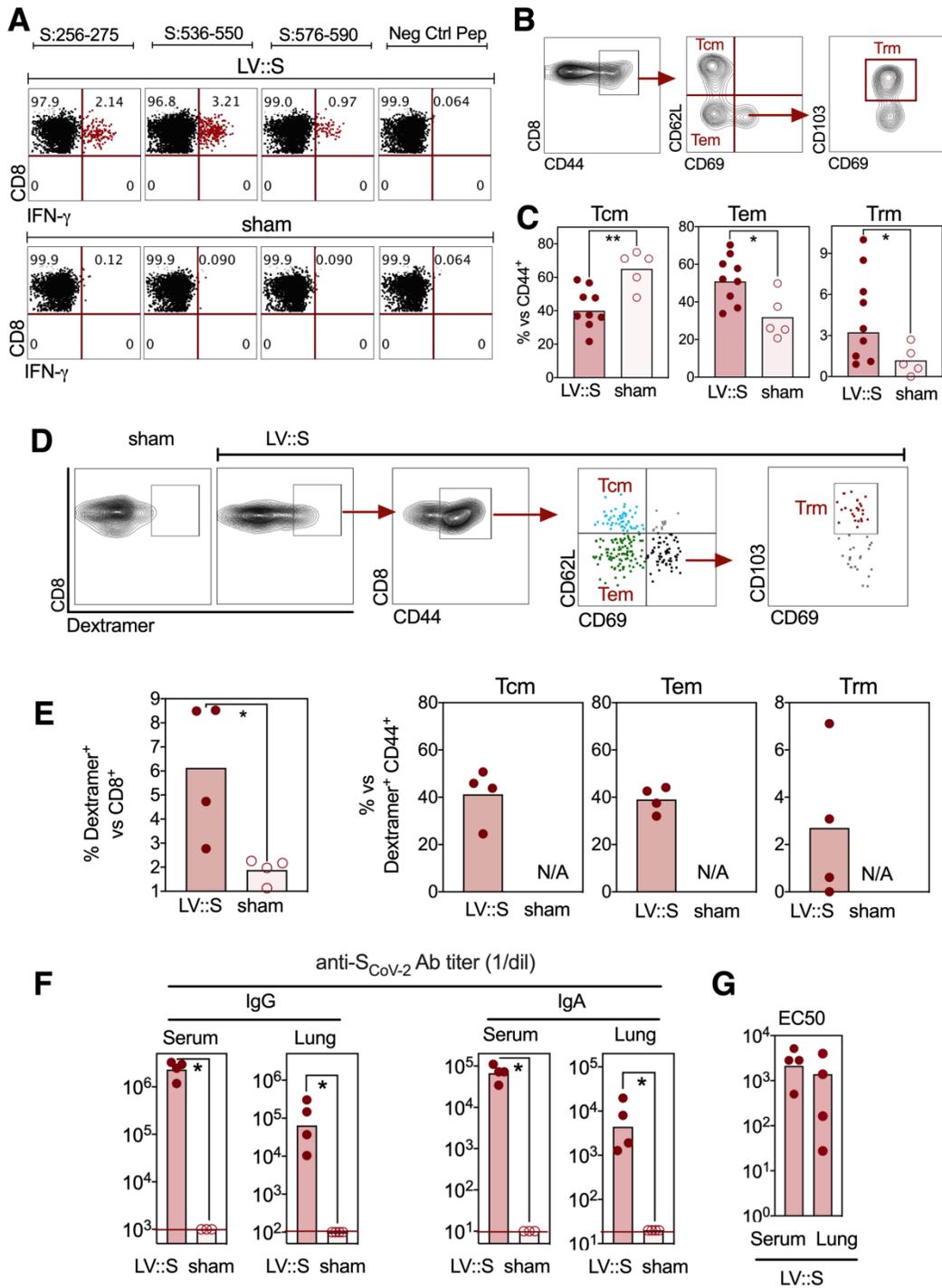


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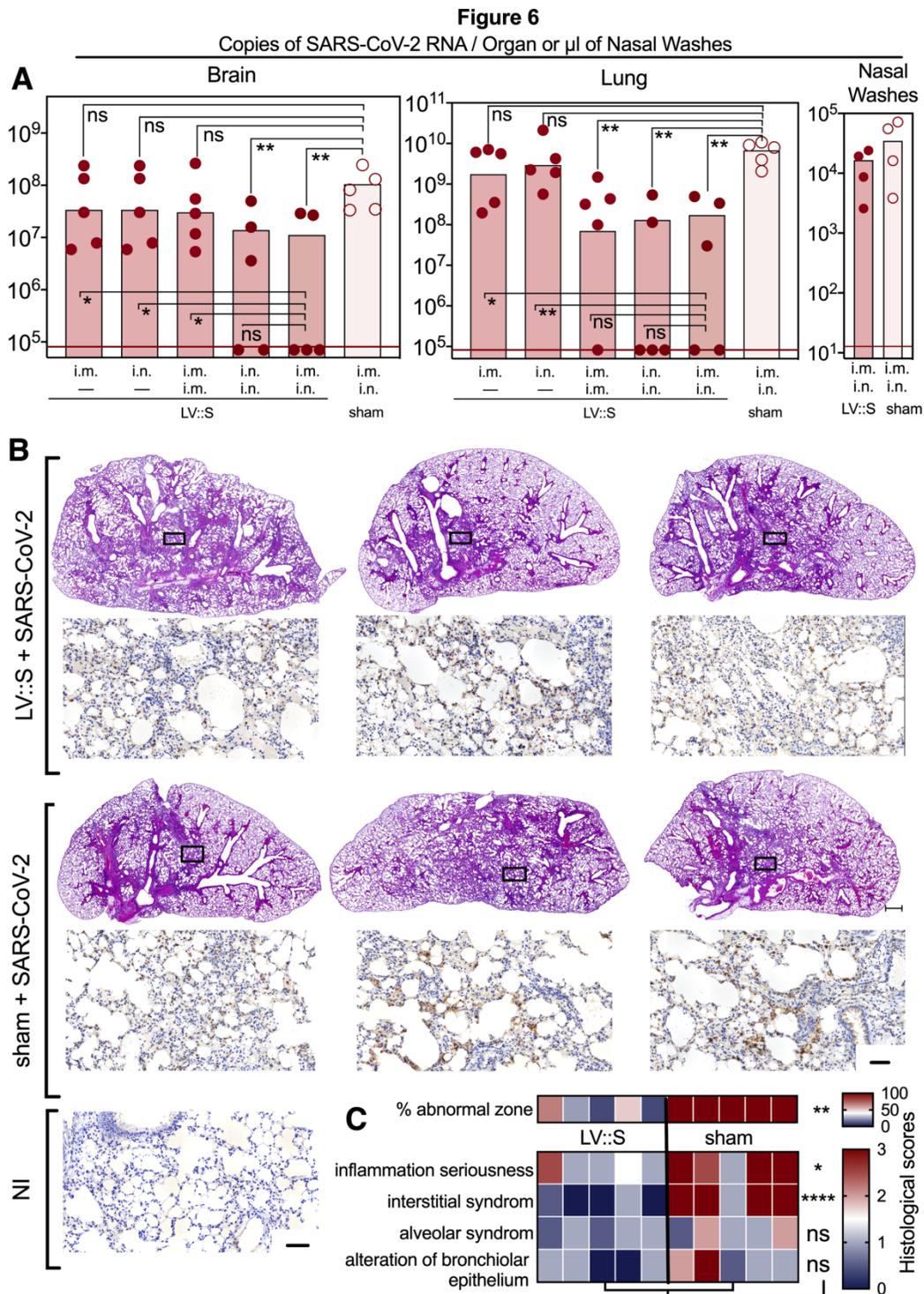


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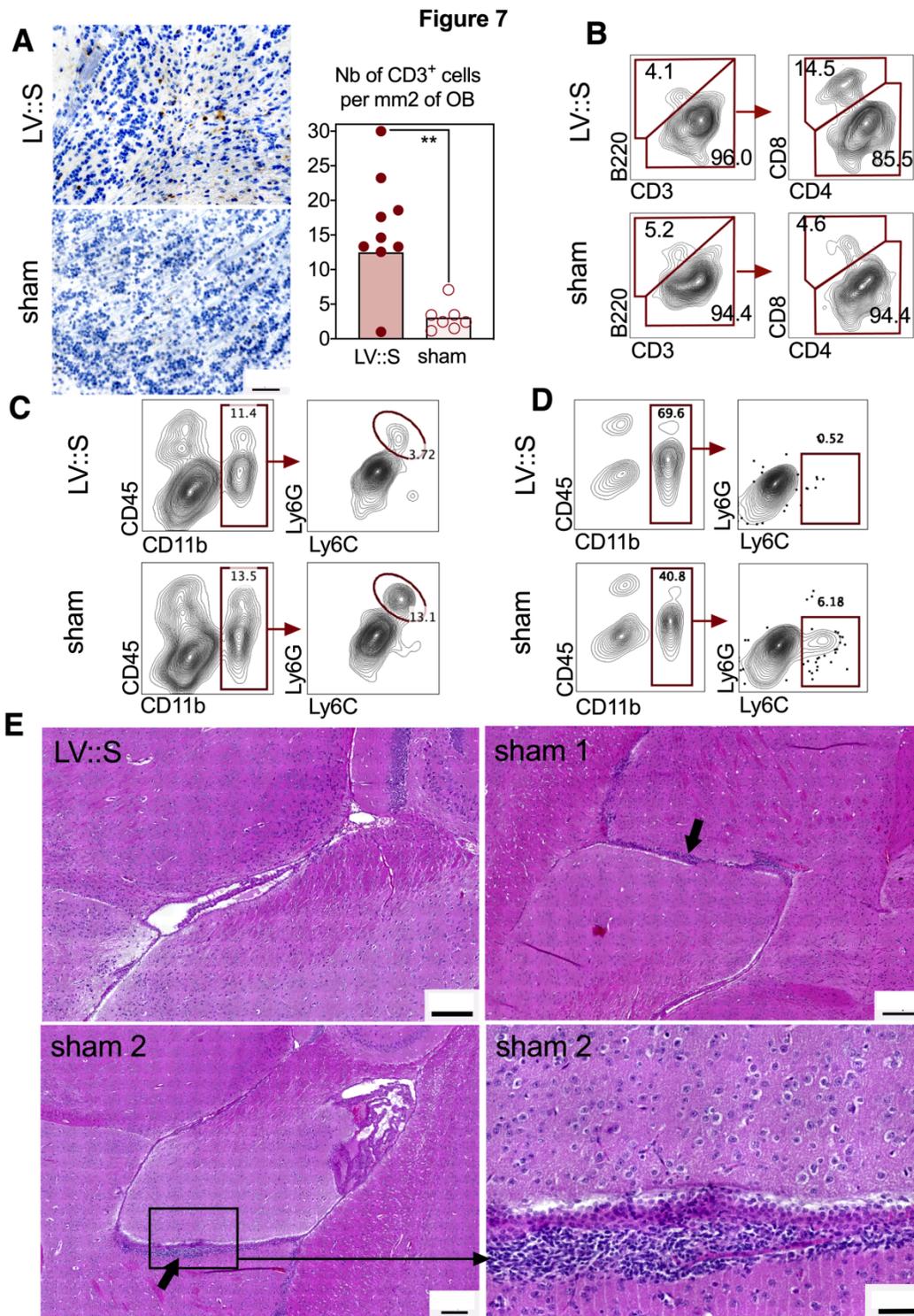
**Figure 5**



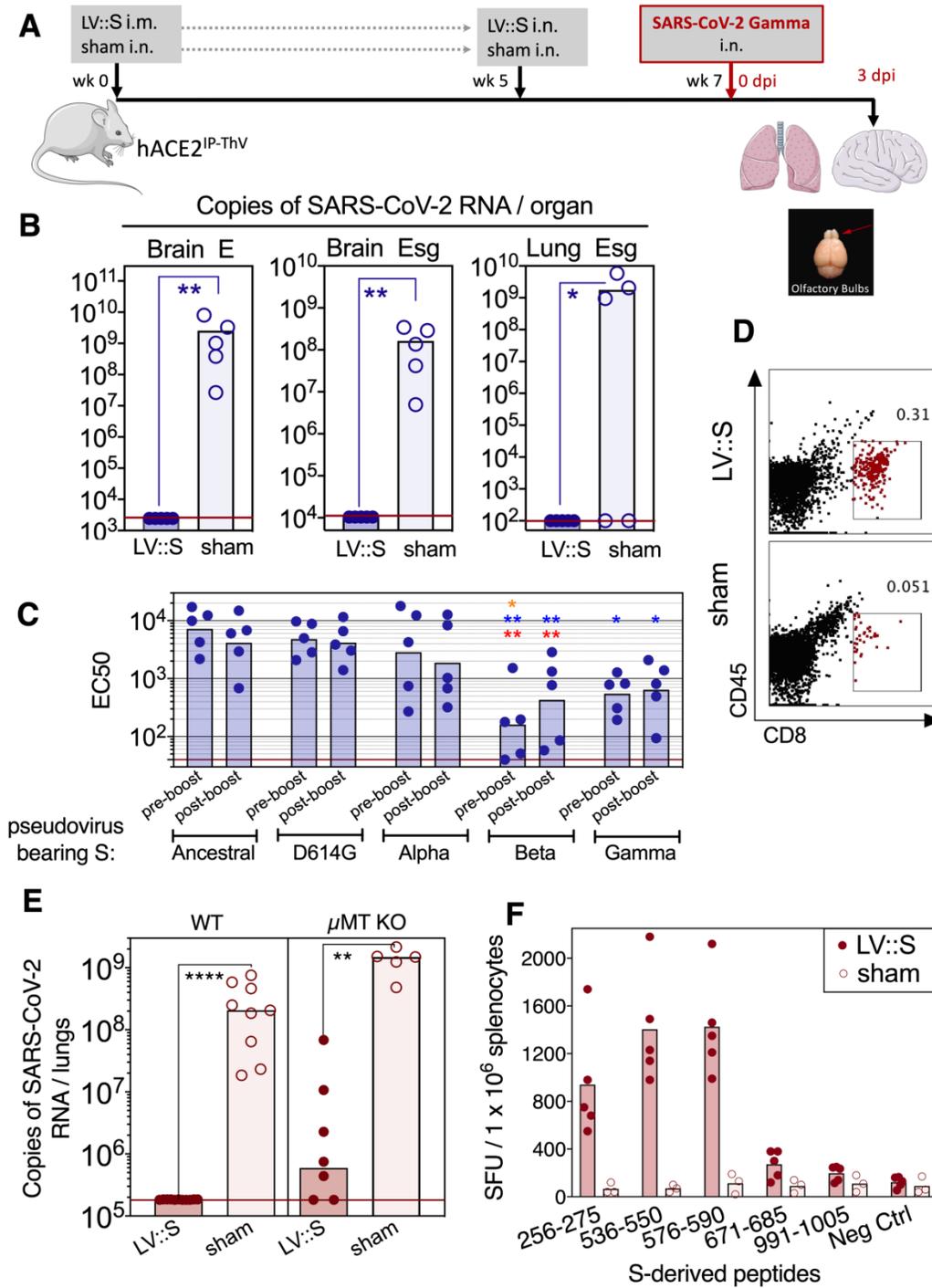
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**Figure 8**



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