

1 **Full Brain and Lung Prophylaxis against SARS-CoV-2 by Intranasal Lentiviral Vaccination in a**  
2 **New hACE2 Transgenic Mouse Model or Golden Hamsters**

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## 0 **Summary**

1 Non-integrative, non-cytopathic and non-inflammatory lentiviral vectors are particularly suitable for  
2 mucosal vaccination and recently emerge as a promising strategy to elicit sterilizing prophylaxis against  
3 SARS-CoV-2 in preclinical animal models. Here, we demonstrate that a single intranasal administration of  
4 a lentiviral vector encoding a prefusion form of SARS-CoV-2 spike glycoprotein induces full protection of  
5 respiratory tracts and totally avoids pulmonary inflammation in the susceptible hamster model. More  
6 importantly, we generated a new transgenic mouse strain, expressing the human Angiotensin Converting  
7 Enzyme 2, with unprecedented brain permissibility to SARS-CoV-2 replication and developing a lethal  
8 disease in <4 days post infection. Even though the neurotropism of SARS-CoV-2 is now well established,  
9 so far other vaccine strategies under development have not taken into the account the protection of central  
0 nervous system. Using our highly stringent transgenic model, we demonstrated that an intranasal booster  
1 immunization with the developed lentiviral vaccine candidate achieves full protection of both respiratory  
2 tracts and brain against SARS-CoV-2.

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## 5 **Keywords**

6 Lentiviral vectors; beta-coronavirus; pre-fusion spike glycoprotein; intranasal vaccination; mucosal  
7 immunity; lung inflammation; brain inflammation; central nervous system; olfactory bulb; golden  
8 hamsters; hACE2 transgenic mice.

## 9 Introduction

0 Severe Acute Respiratory Syndrome beta-coronavirus 2 (SARS-CoV-2) is the causative agent of  
1 pandemic coronavirus disease 2019 (COVID-19), hence the imminent necessity to develop effective and  
2 safe prophylactic vaccines. We have recently established the high performance of lentiviral vector (LV)-  
3 based vaccination against SARS-CoV-2 in pre-clinical animal models (Ku et al., 2021). Our reported  
4 COVID-19 LV-based vaccine candidate is currently in progress to proceed to clinical trial. Both integrative  
5 (ILV) and non-integrative (NILV) forms of LV allow transgene insertion up to 8kb in length and offer  
6 outstanding potential of gene transfer to the nuclei of host cells (Di Nunzio et al., 2012; Hu et al., 2011; Ku  
7 et al., 2020; Zennou et al., 2000). LVs display *in vivo* tropism for immune cells, notably dendritic cells  
8 (Arce et al., 2009, our unpublished data). They are non-replicative, non-cytopathic and scarcely  
9 inflammatory (unpublished data). These vectors induce long-lasting B- and T-cell immunity (Di Nunzio et  
0 al., 2012; Hu et al., 2011; Ku et al., 2020; Zennou et al., 2000). LV are pseudo-typed with the surface  
1 glycoprotein of Vesicular Stomatitis Virus, to which the human population has limited exposure, avoiding  
2 these vectors to be targeted by preexisting immunity in humans, which is in net contrast to adenoviral  
3 vectors (Rosenberg et al., 1998; Schirmbeck et al., 2008). The safety of LV has been established in human  
4 in a phase I/II Human Immunodeficiency Virus (HIV)-1 vaccine trial (2011-006260-52 EN).

5 “LV::S<sub>FL</sub>” encodes the full-length sequence of the surface class I fusion spike (S) glycoprotein of SARS-  
6 CoV-2 (S<sub>CoV-2</sub>) that is able to elicit strong neutralizing antibodies (NAbs) and CD8<sup>+</sup> T-cell responses against  
7 a large spectrum of S<sub>CoV-2</sub> MHC-I-restricted epitopes (Ku et al., 2021). Notably, a systemic prime, followed  
8 by intranasal (i.n.) boost with LV::S<sub>FL</sub> is associated with a robust prophylactic effect against lung SARS-  
9 CoV-2 replication, accompanied by strong reduction of infection-derived lung inflammation and tissue  
0 injury. These observations have been made in mice in which the expression of human Angiotensin-  
1 Converting Enzyme 2 (hACE2) was induced in the respiratory tracts by instillation of an adenoviral vector  
2 serotype 5 (Ad5::hACE2), or in the highly susceptible *Mesocricetus auratus*, i.e., outbred golden hamsters.  
3 These results indicated the requirement for i.n. boost and induction of mucosal adaptive immunity to reach  
4 almost sterilizing lung protection against SARS-CoV-2 (Ku et al., 2021).

5 Although lung is the organ of predilection for SARS-CoV-2, its neurotropism, similar to that of SARS-  
6 CoV and Middle East Respiratory Syndrome (MERS)-CoV, (Glass et al., 2004; Li et al., 2016; Netland et  
7 al., 2008) has been reported (Aghagoli et al., 2020; Fotuhi et al., 2020; Hu et al., 2020; Liu et al., 2020;  
8 Politi et al., 2020; Roman et al., 2020; von Weyhern et al., 2020; Whittaker et al., 2020). Moreover,  
9 expression of ACE2 in neuronal and glial cells has been described (Chen et al., 2020; Xu and Lazartigues,  
0 2020). Accordingly, COVID-19 human patients can present symptoms like headache, myalgia, anosmia,  
1 dysgeusia, impaired consciousness and acute cerebrovascular disease (Bourgonje et al., 2020; Hu et al.,  
2 2020; Mao et al., 2020). Analysis of autopsies of COVID-19 deceased patients demonstrated presence of

3 SARS-CoV-2 in nasopharynx and brain, and virus entry into central nervous system (CNS) via neural-  
4 mucosal interface of olfactory mucosa (Meinhardt et al., 2020). These observations align with the previous  
5 report of viruses capable of gaining access to the brain through neural dissemination or hematogenous route  
6 (Desforges et al., 2014). Therefore, it is critical to focus hereinafter on the protective properties of COVID-  
7 19 vaccine candidates, not only in the respiratory tracts, but also in the brain.

8 Contribution of S<sub>CoV-2</sub> is instrumental in host cell invasion through cellular attachment and membrane  
9 fusion subsequent to engagement with hACE2 (Walls et al., 2020). Following this interaction, the  
0 extracellular domain of the metastable prefusion S<sub>CoV-2</sub> is cleaved at the 682<sup>RRAR</sup>685 site of subtilisin-like  
1 protease furin (Guo et al., 2020; Walls et al., 2020). This gives rise to: (i) S1 subunit, harboring the ACE2  
2 Receptor Binding Domain (RBD) that harbors main B-cell epitopes targeted by NAbS (Walls et al., 2020),  
3 and (ii) S2 subunit, containing the membrane-fusion machinery. This cleavage leads to substantial  
4 conformational rearrangements resulting in a highly stable post-fusion form of S<sub>CoV-2</sub> that initiates the  
5 fusion reaction with the host cell membrane (Sternberg and Naujokat, 2020). Similar to envelop  
6 glycoproteins of several other viruses that harbor furin cleavage site, including respiratory syncytial virus,  
7 HIV, Ebola virus, human metapneumovirus, and Lassa virus (Bos et al., 2020). It is possible to engineer  
8 S<sub>CoV-2</sub> to limit its conformational dynamics and avoid the stabilization under its prefusion conformation to  
9 maintain better exposure of the S1 B-cell epitopes and improve immunogen availability (McCallum et al.,  
0 2020).

1 Here, we first demonstrated that a single i.n. administration of an LV encoding a prefusion form of S<sub>CoV-2</sub>  
2 was as protective as a systemic prime followed by an i.n. boost by conferring sterilizing pulmonary  
3 protection against SARS-CoV-2 in the pre-clinical hamster model. A single i.n. injection of this LV was as  
4 protective as a systemic prime followed by an i.n. boost in these animals. More importantly, we generated  
5 a new hACE2 transgenic mouse strain with unprecedented permissibility of the brain to SARS-CoV-2  
6 replication. Using this unique preclinical animal model, we demonstrated the importance of i.n. booster  
7 immunization with the developed LV-based vaccine candidate to reach full protection of not only lungs but  
8 also CNS against SARS-CoV-2. Our results indicated that i.n. vaccination with non-cytopathic and non-  
9 inflammatory LV is a performant and safe strategy to elicit sterilizing immunity in the main anatomical  
0 sites affected by COVID-19.

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## 2 Results

### 3 Adaptive Immunity Elicited by LV::S<sub>ΔF2P</sub> in Comparison to LV::S<sub>FL</sub>

4 We generated LV encoding a prefusion form of S<sub>CoV-2</sub> under transcriptional control of the  
5 cytomegalovirus promoter. This prefusion S<sub>CoV-2</sub> variant (S<sub>ΔF2P</sub>) has a deletion of 675<sup>QTQTNSPRRR</sup>685  
6 sequence. This deletion encompasses the polybasic RRAR furin cleavage site situated at the junction of  
7 S1/S2 subunits, and harbors K<sup>986P</sup> and V<sup>987P</sup> consecutive proline substitutions in S2, within the hinge loop  
8 between heptad repeat 1 and the central helix (Figure S1A) (Walls et al., 2020). Both ILV::S<sub>ΔF2P</sub>- and  
9 ILV::S<sub>FL</sub>-primed (i.m.) and -boosted (i.n.) C57BL/6 mice possessed high serum titers of anti-S<sub>CoV-2</sub> IgG  
0 (Figure S1B) and high titers of anti-S<sub>CoV-2</sub> IgG and IgA in their lung extracts (Figure S1C), indicating that  
1 the modifications in the pre-fusion S<sub>ΔF2P</sub> form does not impact positively or negatively its capacity to induce  
2 Abs against native S<sub>CoV-2</sub>.

### 3 Sterilizing protection in hamster model by a single i.n. NILV::S<sub>ΔF2P</sub> administration

4 We recently showed that despite induction of high serum neutralizing activity, systemic immunization  
5 with ILV::S<sub>FL</sub> or NILV::S<sub>FL</sub> conferred significant but partial protection against SARS-CoV-2. In contrast,  
6 vaccination by systemic prime followed by i.n. boost with ILV::S<sub>FL</sub> or NILV::S<sub>FL</sub> induced almost sterilizing  
7 lung protection (Ku et al., 2021). Here, we first assessed the prophylactic effect of vaccination with only a  
8 single i.n. administration of NILV::S<sub>ΔF2P</sub> in the hamster model.

9 Hamsters ( $n = 6/\text{group}$ ) were: (i) primed i.m. at wk 0 with  $1 \times 10^8$  TU of NILV::S<sub>ΔF2P</sub> and boosted i.n.  
0 at wk 5 with the same amount of the vector, as a positive protection control, (ii) immunized i.n. with a  
1 single injection of  $1 \times 10^8$  TU of NILV::S<sub>ΔF2P</sub> at wk 0, or (iii) at wk 5 (Figure 1A). Sham-vaccinated controls  
2 received equivalent amounts of an NILV::GFP via i.n. at wks 0 and 5. Comparable and high titers of anti-  
3 S<sub>CoV-2</sub> IgG Abs were detected in the sera in the first two groups at wk 5 (Figure 1B). At wk 7, the serum  
4 Ab titer was maintained at a high level in the NILV::S<sub>ΔF2P</sub> i.m.-i.n. group while it was slightly decreased in  
5 some individuals of the “NILV::S<sub>ΔF2P</sub> i.n. wk 0” group. At this time point, in the “NILV::S<sub>ΔF2P</sub> i.n. wk 5”  
6 group, lower serum Ab titers were detected (Figure 1B). Although the virus neutralization activity was  
7 significantly lower in the sera of “NILV::S<sub>ΔF2P</sub> i.n. wk 5” hamsters compared to the NILV::S<sub>ΔF2P</sub> i.m.-i.n.  
8 group, the former had an equivalent neutralizing capacity in their lung homogenates (Figure 1C).

9 At wk 7, all animals were challenged i.n. with  $0.3 \times 10^5$  TCID<sub>50</sub> of a SARS-CoV-2. At 4 days post  
0 inoculation (dpi), only 2-3% weight loss was detected in the NILV::S<sub>ΔF2P</sub>-vaccinated groups, compared  
1 to 12% in sham-vaccinated hamsters (Figure 1D). At this time point, as determined by qRT-PCR detecting  
2 SARS-CoV-2 Envelop (E<sub>CoV-2</sub>) RNA, ~ 2-to-3 log<sub>10</sub> decreases were observed in NILV::S<sub>ΔF2P</sub>-vaccinated  
3 individuals of either i.m.-i.n. or single i.n. groups, compared to sham-vaccinated group (Figure 1E).

4 Assessment of lung viral load by a sub-genomic E<sub>CoV-2</sub> RNA (Esg) qRT-PCR, which was reported to be  
5 an indicator of active viral replication (Chandrashekar et al., 2020; Tostanoski et al., 2020; Wolfel et al.,  
6 2020) (Tostanoski et al., 2020), showed total absence of replicating virus in the three vaccinated groups  
7 versus a mean  $\pm$  SD of  $(1.24 \pm 0.99) \times 10^9$  copies of Esg RNA of SARS-CoV-2/lungs in the sham-  
8 vaccinated group (Figure 1E). Many publications use PFU counting to determine viral loads. We noticed  
9 that large amounts of NAbs in the lungs of vaccinated individuals, even though not necessarily spatially  
0 in contact with circulating viral particles in alive animal, can come to contact with and neutralize viral  
1 particles in the lung homogenates in vitro. In this case the PFU assay underestimates the amounts of  
2 cultivable viral particles (Figure S2A).

3 At 4 dpi, as evaluated by qRT-PCR in total lung homogenates, a substantial decreased in inflammation  
4 was detected in NILV::S<sub>ΔF2P</sub>-vaccinated hamsters compared to their sham-vaccinated counterparts,  
5 regardless of the immunization regimen, i.e., i.m.-i.n. prime-boost or single i.n. injection given at wk 0 or  
6 5 (Figure 2A). On lung histopathological examination, sham-vaccinated controls demonstrated lung  
7 infiltration and interstitial syndrome (Figure 2B-D), severe alveolo-interstitial inflammation leading to  
8 dense pre-consolidation areas (Figure 2E), accompanied by bronchiolar lesions, with images of  
9 bronchiolar epithelium desquamation into the lumen (Figure 2F-H). In vaccinated groups the lesions were  
0 minimal, although some degree of alveolar infiltration could be seen (Figure 2I).

1 These data collectively indicated that a single i.n. administration of NILV::S<sub>ΔF2P</sub> was as protective as a  
2 systemic prime and i.n. boost regimen, conferred sterilizing pulmonary immunity against SARS-CoV-2  
3 and readily prevented lung inflammation and pathogenic tissue injury in the susceptible hamster model.  
4 These data also showed the long-term feature of the conferred protection because 7 weeks after a single  
5 injection of the vaccine, the protection potential remained complete.

## 6 **Generation of new hACE2 transgenic mice with substantial brain permissibility to SARS-CoV-2** 7 **replication**

8 No hACE2 transgenic mice were available in Europe until September 2020. To set up a mouse model  
9 permissive to SARS-CoV-2 replication allowing assessment of our vaccine candidates, based on the  
0 previously produced B6.K18-ACE2<sup>2Pr<sup>l</sup>mn/JAX</sup> mice (McCray et al., 2007), we generated C57BL/6 transgenic  
1 mice with an LV (Nakagawa and Hoogenraad, 2011) carrying the *hACE2* gene under the human cytokeratin  
2 18 promoter, namely “B6.K18-hACE2<sup>1P-THV</sup>”. The permissibility of these mice to SARS-CoV-2 replication  
3 was evaluated after one generation backcross to WT C57BL/6 (N1). N1 mice with varying number of  
4 *hACE2* transgene copies per genome (Figure 3A) were sampled and inoculated i.n. with SARS-CoV-2  
5 (Figure 3B). At 3 dpi, the mean  $\pm$  SD of lung viral loads was as high as  $(3.3 \pm 1.6) \times 10^{10}$  copies of SARS-  
6 CoV-2 RNA/lung in permissive mice (Figure 3B). SARS-CoV-2 RNA copies per lung  $<1 \times 10^7$  correspond  
7 to the genetic material derived from the input in the absence of viral replication (Ku et al., 2021). We also

8 noted that the lung viral loads (Figure 3B) were not proportional to the *hACE2* transgene copy number per  
9 genome (Figure 3A). Remarkably, substantial viral loads, i.e.,  $(5.7 \pm 7.1) \times 10^{10}$  copies of SARS-CoV-2  
0 RNA, were also detected in the brain of the permissive mice (Figure 3B). Virus replication/dissemination  
1 was also observed, although to a lower extent, in the heart and kidneys.

2 We further compared the replication of SARS-CoV-2 in lungs and brain and the viral dissemination to  
3 various organs in B6.K18-hACE2<sup>IP-THV</sup> and B6.K18-ACE2<sup>2Prlmn/JAX</sup> mice (McCray et al., 2007) (Figure  
4 3C). The lung viral loads were slightly lower in B6.K18-hACE2<sup>IP-THV</sup> compared to B6.K18-ACE2<sup>2Prlmn/JAX</sup>  
5 mice. However, viral loads in the brain of B6.K18-hACE2<sup>IP-THV</sup> mice were substantially higher compared  
6 to their B6.K18-ACE2<sup>2Prlmn/JAX</sup> counterparts (Figure 3C). Measurement of brain viral loads by Esg qRT-  
7 PCR detected  $(7.55 \pm 7.74) \times 10^9$  copies of SARS-CoV-2 RNA in B6.K18-hACE2<sup>IP-THV</sup> mice and no copies  
8 of this replication-related RNA in 4 out of 5 B6.K18-ACE2<sup>2Prlmn/JAX</sup> mice. This substantial difference of  
9 SARS-CoV-2 replication in the brain of both transgenic strains was corroborated with significantly higher  
0 *hACE2* mRNA expression in the brain of B6.K18-hACE2<sup>IP-THV</sup> mice (Figure 3D). However, *hACE2* mRNA  
1 expression in the lungs of B6.K18-hACE2<sup>IP-THV</sup> mice was also higher than in B6.K18-ACE2<sup>2Prlmn/JAX</sup> mice,  
2 which does not account for the lower viral replication in the lungs of the former. A trend towards higher  
3 viral loads was also observed in the kidneys and heart of B6.K18-hACE2<sup>IP-THV</sup> compared to B6.K18-  
4 ACE2<sup>2Prlmn/JAX</sup> mice (Figure 3C).

5 In concordance with the lung viral loads, as evaluated by qRT-PCR applied to total lung homogenates,  
6 B6.K18-hACE2<sup>IP-THV</sup> mice displayed less pulmonary inflammation than B6.K18-ACE2<sup>2Prlmn/JAX</sup> mice  
7 (Figure 3E). Remarkably, this assay applied to total brain homogenates detected substantial degrees of  
8 inflammation in B6.K18-hACE2<sup>IP-THV</sup> — but not in B6.K18-ACE2<sup>2Prlmn/JAX</sup> — mice (Figure 3E). In  
9 addition, B6.K18-hACE2<sup>IP-THV</sup> mice reached the humane endpoint between 3 and 4 dpi and therefore  
0 displayed a lethal SARS-CoV-2-mediated disease more rapidly than their B6.K18-ACE2<sup>2Prlmn/JAX</sup>  
1 counterparts (Winkler et al., 2020).

2 Therefore, the large permissibility to SARS-CoV-2 replication at both lung and CNS, marked brain  
3 inflammation and rapid development of a lethal disease are major distinctive features offered by this new  
4 B6.K18-hACE2<sup>IP-THV</sup> transgenic model.

### 5 **Full protection of lungs and brain in LV::S<sub>ΔF2P</sub>-immunized B6.K18-hACE2<sup>IP-THV</sup> mice**

6 We then evaluated the vaccine efficacy of LV::S<sub>ΔF2P</sub> in B6.K18-hACE2<sup>IP-THV</sup> mice. In a first  
7 experiment with these mice, we used an integrative version of the vector. Individuals ( $n = 6$ /group) were  
8 primed i.m. with  $1 \times 10^7$  TU/mouse of ILV::S<sub>ΔF2P</sub> or an empty LV (sham) at wk 0 and then boosted i.n.  
9 at wk 3 with the same dose of the same vectors (Figure 4A). Mice were then challenged with SARS-CoV-  
0 2 at wk 5. A high serum neutralizing activity, i.e., EC50 mean  $\pm$  SD of  $5466 \pm 6792$ , was detected in

1 ILV::S<sub>ΔF2P</sub>-vaccinated mice [Figure 4B](#)). This vaccination conferred substantial degrees of protection  
2 against SARS-CoV-2 replication, not only in the lungs, but also in the brain ([Figure 4C](#)). Notably,  
3 quantitation of brain viral loads by Esg qRT-PCR detected no copies of this replication-related SARS-  
4 CoV-2 RNA in ILV::S<sub>ΔF2P</sub>-vaccinated mice *versus*  $(7.55 \pm 7.84) \times 10^9$  copies in the brain of the sham-  
5 vaccinated controls.

6 At 3 dpi, cytometric investigation of the lung innate immune cell subsets ([Figure 4D](#), [S3A](#)) detected  
7 significant decrease in the proportions of NK (CD11b<sup>int</sup> NKp46<sup>+</sup>) cells and neutrophils (CD11b<sup>+</sup> CD24<sup>+</sup>  
8 SiglecF<sup>-</sup> Ly6G<sup>+</sup>) among the lung CD45<sup>+</sup> cells in the ILV::S<sub>ΔF2P</sub>-vaccinated and protected B6.K18-  
9 hACE2<sup>IP-THV</sup> mice, compared to the sham-vaccinated and unprotected controls ([Figure 4D](#)). Frequencies  
0 of the other lung innate immune cells were not significantly distinct in the protected and unprotected  
1 groups ([Figure S3B](#)). At 3 dpi, as evaluated by qRT-PCR applied to brain homogenates, ILV::S<sub>ΔF2P</sub>-  
2 vaccinated B6.K18-hACE2<sup>IP-THV</sup> mice had significant decreases in the expression levels of IFN- $\alpha$ , TNF-  
3  $\alpha$ , IL-5, IL-6, IL-10, IL-12p40, CCL2, CCL3, CXCL9 and CXCL10, compared to the sham group ([Figure](#)  
4 [4E](#)). Furthermore, no noticeable changes in the lung inflammation were recorded between the two groups  
5 ([Figure S3C](#)).

6 Therefore, an i.m.-i.n. prime-boost with ILV::S<sub>ΔF2P</sub> prevents SARS-CoV-2 replication in both lung and  
7 CNS anatomical areas and inhibits virus-mediated lung infiltration and pathology and neuro-  
8 inflammation.

### 9 **Requirement of i.n. boost for full protection of brain in B6.K18-hACE2<sup>IP-THV</sup> mice**

0 To go further in characterization of the protective properties of LV, in the following experiments in  
1 B6.K18-hACE2<sup>IP-THV</sup> mice, similar to the hamster model, we used the safe and non-integrative version of  
2 LV. The observed protection of brain against SARS-CoV-2 may reflect the benefits of i.n. route of LV  
3 administration against this respiratory and neurotropic virus. To address this question, B6.K18-hACE2<sup>IP-</sup>  
4 <sup>THV</sup> mice were vaccinated with NILV::S<sub>ΔF2P</sub>: (i) i.m. wk 0 and i.n. wk5, as a positive control, (ii) i.n. wk 0,  
5 or (iii) i.m. wk 5. Sham-vaccinated controls received i.n. an empty NILV at wks 0 and 5 ([Figure 5A](#)). Mice  
6 were then challenged with SARS-CoV-2 at wk 7 and viral loads were determined in the brain by E- or Esg-  
7 specific qRT-PCR at 3dpi ([Figure 5B](#)). In this highly stringent pre-clinical model, even performant, a single  
8 i.n. or i.m. injection of NILV::S<sub>ΔF2P</sub> did not induce full protection in all animals of each group. Only i.m.  
9 prime followed by i.n. boost conferred full protection in all animals, showing the requirement of an i.n.  
0 boost to reach full protection of brain. The brain protection levels were proportional to plasma anti-S<sub>CoV-2</sub>  
1 IgG titers ([Figure 5C](#)). As analyzed by cytometry, composition of innate and adaptive immune cells in the  
2 cervical lymph nodes were unchanged in NILV::S<sub>ΔF2P</sub> i.m.-i.n. protected group, sham i.m.-i.n. unprotected  
3 group and untreated controls (data not shown). However, we detected increased proportion of CD8<sup>+</sup> T cells

4 in the olfactory bulb of NILV::S<sub>ΔF2P</sub> i.m.-i.n. protected group compared to the sham unprotected group  
5 (Figure 5D). CD4<sup>+</sup> T cells in the olfactory bulb had no distinctive activated or migratory phenotype, as assessed  
6 by their surface expression of CD69 or CCR7, respectively. In line with the absence of CCR7 expression on  
7 these T cells, and unlike the case of Murine Hepatitis Virus (MHV) infection (Cupovic et al., 2016), we did  
8 not detect up-regulation of CCL19 and CCL21 chemo-attractants (CCR7 ligands) in the brain and  
9 regardless of the mouse protection status (Figure 5E). Compared to NILV::S<sub>ΔF2P</sub> i.m.-i.n. protected group,  
0 increased amount of neutrophils (CD11b<sup>+</sup> Ly6C<sup>+</sup> Ly6G<sup>+</sup>) in the olfactory bulb (Figure 5F) and inflammatory  
1 monocytes (CD11b<sup>+</sup> Ly6C<sup>+</sup> Ly6G<sup>-</sup>) in the brain (Figure 5G) of unprotected mice, compared to NILV::S<sub>ΔF2P</sub>  
2 i.m.-i.n. protected group. The presence of these cells is a biomarker of inflammation and therefore,  
3 correlated with active viral replication.

4 Collectively, our data generated in the highly stringent B6.K18-hACE2<sup>IP-THV</sup> mouse model support the  
5 advantage of NILV::S<sub>ΔF2P</sub> i.n. boost in the immune protection of CNS from SARS-CoV-2 replication and  
6 the resulting infiltration and neuro-inflammation. Therefore, local induction and/or activation of mucosal  
7 immune response in the nasal cavity and olfactory bulbs, i.e. one of the potential entry points for the virus,  
8 emerges as a performant strategy.

## 9 Discussion

0 LV-based platform emerges recently as a powerful vaccination approach against COVID-19, notably  
1 when used in systemic prime followed by mucosal i.n. boost, able to induce sterilizing immunity against  
2 lung SARS-CoV-2 infection in preclinical animal models (Ku et al., 2021). In the present study, we first  
3 demonstrated that a single i.n. administration of an LV encoding the S<sub>ΔF2P</sub> prefusion form of S<sub>CoV-2</sub> confers,  
4 as efficiently as an i.m. - i.n. prime-boost regimen, full protection of lungs in the highly susceptible hamster  
5 model, as evaluated by virological, immunological and histopathological parameters. The hamster ACE2  
6 ortholog interacts efficaciously with S<sub>CoV-2</sub>, which readily allows host cell invasion by SARS-CoV-2 and  
7 its high replication rate. With rapid weight loss and development of severe lung pathology subsequent to  
8 SARS-CoV-2 inoculation, this species provides a sensitive model to evaluate the efficacy of drug or vaccine  
9 candidates, for instance compared to Rhesus macaques which develop only a mild COVID-19 pathology  
0 (Munoz-Fontela et al., 2020; Sia et al., 2020). The fact that a single i.n. LV administration, either seven or  
1 two weeks before SARS-CoV-2 challenge, elicits sterilizing lung protection in this susceptible model is  
2 valuable in setting the upcoming clinical trials with this LV-based vaccine and could provide remarkable  
3 socio-economic advantages for mass vaccination. The long-termed protection conferred, i.e., at least 7  
4 weeks after a single injection, is another major advantage of this vaccine strategy to be translated in humans.

5 To further investigate the efficacy of our vaccine candidates, we generated a new transgenic mouse  
6 model, using the LV-based transgenesis approach (Nakagawa and Hoogenraad, 2011). The ILV used in  
7 this strategy encodes for hACE2 under the control of the cytokeratin K18 promoter, i.e., the same promoter  
8 as previously used by Perlman's team to generate B6.K18-ACE2<sup>2PrImn/JAX</sup> mice (McCray et al., 2007), with  
9 a few adaptations to the lentiviral FLAP transfer plasmid. However, the new B6.K18-hACE2<sup>IP-THV</sup> mice  
0 have certain distinctive features, as they express much higher levels of hACE2 mRNA in the brain and  
1 display markedly increased brain permissibility to SARS-CoV-2 replication, in parallel with a substantial  
2 brain inflammation and development of a lethal disease in <4 days post infection. These distinct  
3 characteristics can arise from differential hACE2 expression profile due to: (i) alternative insertion sites of  
4 ILV into the chromosome compared to naked DNA, and/or (ii) different effect of the Woodchuck  
5 Posttranscriptional Regulatory Element (WPRE) *versus* the alfalfa virus translational enhancer (McCray et  
6 al., 2007), in B6.K18-hACE2<sup>IP-THV</sup> and B6.K18-ACE2<sup>2PrImn/JAX</sup> animals, respectively. Other reported  
7 *hACE2* humanized mice expressing the transgene under: (i) murine ACE2 promoter, without reported  
8 hACE2 mRNA expression in the brain (Yang et al., 2007), (ii) "hepatocyte nuclear factor-3/forkhead  
9 homologue 4" (HFH4) promoter, i.e., "HFH4-hACE2" C3B6 mice, in which lung is the principal site of  
0 infection and pathology (Jiang et al., 2020; Menachery et al., 2016), and (iii) "CAG" mixed promoter, i.e.  
1 "AC70" C3H × C57BL/6 mice, in which hACE2 mRNA is expressed in various organs including lungs and  
2 brain (Tseng et al., 2007). Comparison of AC70 and B6.K18-hACE2<sup>IP-THV</sup> mice could yield information to  
3 assess the similarities and distinctions of these two models. However, here we report much higher brain

4 permissibility of B6.K18-hACE2<sup>IP-THV</sup> mice to SARS-CoV-2 replication, compared to B6.K18-  
5 ACE2<sup>2PrImn/JAX</sup> mice. The B6.K18-hACE2<sup>IP-THV</sup> murine model not only has broad applications in COVID-  
6 19 vaccine studies, but also provides a unique rodent model for exploration of COVID-19-derived  
7 neuropathology. Based on the substantial permissibility of the brain to SARS-CoV-2 replication and  
8 development of a lethal disease, this pre-clinical model can be considered as a far more stringent than the  
9 golden hamster model.

0 The source of neurological manifestations associated with COVID-19 in patients with comorbid  
1 conditions can be: (i) direct impact of SARS-CoV-2 on CNS, (ii) infection of brain vascular endothelium  
2 and, (iii) uncontrolled anti-viral immune reaction inside CNS. ACE2 is expressed in human neurons,  
3 astrocytes and oligodendrocytes, located in middle temporal gyrus and posterior cingulate cortex, which  
4 may explain the brain permissibility to SARS-CoV-2 in patients (Song et al., 2020). Previous reports have  
5 demonstrated that respiratory viruses can invade the brain through neural dissemination or hematogenous  
6 route (Desforges et al., 2014). Besides that, the direct connection of olfactory system to the CNS via frontal  
7 cortex also represents a plausible route to invade the brain (Mori et al., 2005). Neural transmission of viruses  
8 to the CNS can occur as a result of direct neuron invasion through axonal transport in the olfactory mucosa.  
9 Subsequent to intraneuronal replication, the virus spreads to synapses and disseminate to anatomical CNS  
0 zones receiving olfactory tract projections (Koyuncu et al., 2013; Zubair et al., 2020; Berth, 2009; Koyuncu  
1 et al., 2013; Román et al., 2020). However, the detection of viral RNA in CNS regions without connection  
2 with olfactory mucosa suggests existence of another viral entry into the CNS, including migration of SARS-  
3 CoV-2-infected immune cells crossing the hemato-encephalic barrier or direct viral entry pathway via CNS  
4 vascular endothelium (Meinhardt et al., 2020). Although at steady state, viruses cannot penetrate to the  
5 brain through an intact blood-brain barrier (Berth, 2009), inflammation mediators which are massively  
6 produced during cytokine/chemokine storm, notably TNF- $\alpha$  and CCL2, can disrupt the integrity of blood-  
7 brain barrier or increase its permeability, allowing paracellular blood-to-brain transport of the virus or virus-  
8 infected leukocytes (Aghagoli et al., 2020; Hu et al., 2011).

9 The use of the highly stringent B6.K18-hACE2<sup>IP-THV</sup> mice demonstrated the importance of i.n. booster  
0 immunization for inducing sterilizing protection of CNS by the LV-based vaccine candidate developed  
1 against SARS-CoV-2. Olfactory bulb may control viral CNS infection through the action of local innate  
2 and adaptive immunity (Durrant et al., 2016). In line with these observations, we detected increased  
3 frequencies of CD8<sup>+</sup> T cells at this anatomically strategic area in i.m.-i.n. vaccinated and protected mice.  
4 In addition, substantial reduction in the inflammatory mediators was also found in the brain of the i.m.-i.n.  
5 vaccinated and protected mice, as well as decreased proportions of neutrophils and inflammatory  
6 monocytes respectively in the olfactory bulbs and brain. Regardless of the mechanism of the SARS-CoV-  
7 2 entry to the brain, we provide evidence of the full protection of the CNS against SARS-CoV-2 by i.n.  
8 booster immunization with NILV::S<sub>AF2P</sub>.

9 We recently demonstrated the strong prophylactic capacity of LV::S<sub>FL</sub> at inducing sterilizing protection  
0 in the lungs against SARS-CoV-2 infection (Ku et al., 2021). In the present study, moving toward a human  
1 clinical trial, we used LV encoding stabilized prefusion S<sub>ΔF2P</sub> forms of S<sub>CoV-2</sub>. The choice of S<sub>ΔF2P</sub> in this  
2 study was based on data indicating that stabilization of viral envelop glycoproteins at their prefusion forms  
3 improve the yield of their production as recombinant proteins in industrial manufacturing of subunit  
4 vaccines, and the efficacy of nucleic acid-based vaccines by raising availability of the antigen under its  
5 optimal immunogenic shape (Hsieh et al., 2020). The prefusion stabilization approach has been so far  
6 applied to S protein of several coronaviruses, including HKU1-CoV, SARS-CoV, and MERS-CoV.  
7 Stabilized S<sub>MERS-CoV</sub> has been shown to elicit much higher NAb responses and protection in pre-clinical  
8 animal models (Hsieh et al., 2020). We detected no difference between the capacity of S<sub>FL</sub> and S<sub>ΔF2P</sub> at  
9 inducing anti-S<sub>CoV-2</sub> IgG or IgA Ab responses in the sera or lung homogenates of LV-immunized animals.  
0 However, the possibility that the yield of LV::S<sub>ΔF2P</sub> production at industrial level is higher is likely.

1 The sterilizing protection of the lungs conferred by a single i.n. administration and the full protection of  
2 CNS conferred by i.n. boost is an asset of primary importance. The non-cytopathic and non-inflammatory  
3 LV encoding either full length, or stabilized forms of S<sub>CoV-2</sub>, from either ancestral or emerging variants of  
4 SARS-CoV-2 provides a promising COVID-19 vaccine candidate of second generation. Protection of the  
5 brain, so far not directly addressed by other vaccine strategies, has to be taken into the account, considering  
6 the multiple and sometimes severe neuropathological manifestations associated with COVID-19.

7

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## 4 **Author Contribution**

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

## 9 **Declaration of Interests**

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

4

5

## 6 **Figure Legend**

### 7 **Figure 1. Single i.n. injection of LV::S<sub>ΔF2P</sub> fully protects golden hamsters against SARS-CoV-2.**

8 **(A)** Timeline of the LV::S<sub>ΔF2P</sub> prime-boost vaccination regimen and SARS-CoV-2 challenge in hamsters. 9 **(B)** Serum anti-S<sub>CoV-2</sub> IgG responses expressed as mean endpoint dilution titers, determined by ELISA. **(C)** 0 Neutralization capacity of anti-S<sub>CoV-2</sub> Abs, expressed as EC50 neutralizing titers, determined in the sera and 1 lung homogenates of LV::S<sub>ΔF2P</sub>-immunized hamsters. Bars represent mean ± SD. **(D)** Percentages of weight 2 loss in LV::S<sub>ΔF2P</sub>- or sham-vaccinated hamsters at 4 dpi. **(E)** Lung viral loads quantitated by total E or Esg 3 qRT-PCR at 4 dpi. Statistical significance of the differences was evaluated by two tailed unpaired t test; \*\* = 4  $p < 0.01$ , \*\*\* =  $p < 0.001$ . Red lines indicate the limit of detection of each assay.

### 5 **Figure 2. Largely reduced infection-driven lung inflammation in LV::S<sub>ΔF2P</sub>-vaccinated hamsters.**

6 **(A)** Heatmap recapitulating relative log<sub>2</sub> fold changes in the expression of inflammation-related mediators 7 in S<sub>ΔF2P</sub>- or sham-vaccinated individuals, as analyzed at 4 dpi by use of RNA extracted from total lung 8 homogenates and normalized versus samples from untreated controls. Six individual hamsters per group 9 are shown in the heatmap. **(B)** Lung histological H&E analysis, as studied at 4 dpi. **(C)** Heatmap 0 recapitulating the histological scores, for: 1) inflammation score and 2) interstitial syndrome. **(D)** 1 Representative alveolo-interstitial syndrome and **(E)** severe inflammation in a sham-vaccinated and 2 infected hamster. Here the structure of the organ is largely obliterated, while remnants of alveolar spaces 3 and bronchiolar lumens can be seen. **(F-H)** bronchiolar lesions in sham-vaccinated animals. Shown are 4 epithelial cells and cell debris in the bronchiolar lumen (black arrows) **(F)**, papillary projections of the 5 bronchiolar epithelium into the lumen (star) **(G)** and degenerative lesions with effacement of the epithelium 6 (green arrow) **(H)**. **(I)** Mild alveolar infiltration in a vaccinated hamster. Some of the alveoli (arrow) are 7 partially or completely filled with cells and an eosinophilic exudate.

### 8 **Figure 3. Large permissibility of the lungs and brain of K18-hACE2<sup>IP-THV</sup> transgenic mice to**

9 **SARS-CoV-2 replication.** **(A)** Representative genotyping results from 15 N1 B6.K18-hACE2<sup>IP-THV</sup> mice 0 as performed by qPCR to determine their *hACE2* gene copy number per genome. Dots represent individual 1 mice. **(B)** Phenotyping of the same mice, inoculated i.n. with  $0.3 \times 10^5$  TCID<sub>50</sub> at the age of 5-7 wks and 2 viral loads determination in their various organs at 3 dpi by conventional E-specific qRT-PCR. **(C)** 3 Comparative permissibility of diverse organs from K18-hACE2<sup>IP-THV</sup> and B6.K18-ACE2<sup>2PrImn/JAX</sup> 4 transgenic mice to SARS-CoV-2 replication, as determined at 3 dpi by conventional E-specific or sub- 5 genomic Esg-specific qRT-PCR. Red lines indicate the qRT-PCR limits of detection. Statistical significance 6 of the difference was evaluated by Mann-Whitney test (\*=  $p < 0.01$ , \*\*=  $p < 0.001$ ). **(D)** Comparative 7 quantitation of *hACE-2* mRNA in the lungs and brain of B6.K18-hACE2<sup>IP-THV</sup> and B6.K18-ACE2<sup>2PrImn/JAX</sup> 8 transgenic mice. **(E)** Heatmap recapitulating log<sub>2</sub> fold change in cytokine and chemokine mRNA expression 9 in the lungs or brain of B6.K18-hACE2<sup>IP-THV</sup> and B6.K18-ACE2<sup>2PrImn/JAX</sup> transgenic mice at 3 dpi. Data

0 were normalized versus untreated controls. Statistical significance of the difference was evaluated by Mann-  
1 Whitney test (\*=  $p < 0.05$ , \*\*=  $p < 0.01$ ).

2 **Figure 4. Vaccination with LV::S<sub>ΔF2P</sub> protects both lungs and central nervous system from SARS-**  
3 **CoV-2 infection in K18-hACE2<sup>IP-THV</sup> transgenic mice. (A)** Timeline of prime-boost LV::S<sub>ΔF2P</sub>  
4 vaccination and SARS-CoV-2 challenge in K18-hACE2<sup>IP-THV</sup> mice. **(B)** Serum neutralization capacity of  
5 anti-S<sub>CoV-2</sub> Abs in LV::S<sub>ΔF2P</sub>-vaccinated mice. **(C)** Viral loads as determined in diverse organs at 3dpi by  
6 use of conventional E-specific or sub-genomic Esg-specific qRT-PCR. Red lines indicate the qRT-PCR  
7 detection limits. Statistical significance of the difference was evaluated by Mann-Whitney test (\*=  $p < 0.01$ ,  
8 \*\*=  $p < 0.001$ ). **(D)** Cytometric gating strategy determined to identify and quantify lung NK cells and  
9 neutrophils in the lungs of LV::S<sub>ΔF2P</sub>- or sham-vaccinated and SARS-CoV-2-challenged K18-hACE2<sup>IP-THV</sup>  
0 transgenic mice at 3 dpi. Percentages of NK and neutrophil subset were calculated among total lung CD45<sup>+</sup>  
1 cells. **(E)** Relative log<sub>2</sub> fold change in cytokine and chemokine mRNA expression in the brain of LV::S<sub>ΔF2P</sub>-  
2 or sham-immunized and SARS-CoV-2-challenged K18-hACE2<sup>IP-THV</sup> transgenic mice at 3 dpi. Data were  
3 normalized versus untreated controls. Statistical significance was evaluated by two tailed unpaired t test; \*  
4 =  $p < 0.05$ , \*\* =  $p < 0.01$ ).

5 **Figure 5. Booster vaccination with LV::S<sub>ΔF2P</sub> through i.n. route elicits full protection of CNS from**  
6 **SARS-CoV-2 infection. (A)** Timeline of various LV::S<sub>ΔF2P</sub> vaccination regimens and SARS-CoV-2  
7 challenge in B6.K18-hACE2<sup>IP-THV</sup> mice. **(B)** Viral loads in the brain at 3dpi determined by conventional E-  
8 specific or sub-genomic Esg-specific qRT-PCR. Red lines indicate the qRT-PCR detection limits. Statistical  
9 significance of the difference was evaluated by Mann-Whitney test (\*=  $p < 0.01$ ). **(C)** Plasma anti-S<sub>CoV-2</sub> Ab  
0 titers. **(D, F, G)** Cytometric analysis at 3 dpi performed on cells extracted from pooled olfactory bulbs or  
1 brain of LV::S<sub>ΔF2P</sub> i.m.-i.n. vaccinated and protected mice versus sham-vaccinated and unprotected mice.  
2 **(D)** Adaptive and **(F)** innate immune cells in the olfactory bulbs. **(E)** Expression of CCL19 or CCL21  
3 chemoattractant in the brain, as evaluated by qRT-PCR. **(G)** Innate immune cells in the brain.

## 4 **Methods**

### 5 **Construction and production of LV::S<sub>ΔF2P</sub>**

6 A codon-optimized S<sub>ΔF2P</sub> sequence (1-1262) (Table S1) was amplified from pMK-RQ\_S-2019-nCoV  
7 and inserted into pFlap by restriction/ligation between BamHI and XhoI sites, between the native human  
8 ieCMV promoter and a mutated Woodchuck Posttranscriptional Regulatory Element (WPRE) sequence.  
9 The *atg* starting codon of WPRE was mutated (mWPRE) to avoid transcription of the downstream truncated  
0 “X” protein of Woodchuck Hepatitis Virus for safety concerns (Figure S4). Plasmids were amplified and  
1 used to produce LV as previously described (Ku et al., 2021).

### 2 **Hamsters**

3 Male *Mesocricetus auratus* golden hamsters (Janvier, Le Genest Saint Isle, France) were purchased  
4 mature and weighed between 100 to 120 gr at the beginning of the experiments. Hamsters were housed in  
5 individually-ventilated cages under specific pathogen-free conditions during the immunization period. For  
6 SARS-CoV-2 infection they were transferred into individually filtered cages placed inside isolators in the  
7 animal facility of Institut Pasteur. Prior to i.m. or i.n. injections, hamsters were anesthetized by isoflurane  
8 inhalation or i.p. injection of Ketamine (Imalgene, 80 mg/kg) and Xylazine (Rompun, 5 mg/kg).

### 9 **Mice**

0 Female C57BL/6JRj mice (Janvier, Le Genest Saint Isle, France) were used between the age of 7 and  
1 12 wks. Transgenic B6.K18-ACE2<sup>2Pr1mn/JAX</sup> mice (JAX stock #034860) were from Jackson Laboratories  
2 and were a kind gift of Dr Jean Jaubert and Dr Xavier Montagutelli (Institut Pasteur). Transgenic B6.K18-  
3 hACE2<sup>IP-THV</sup> mice were generated and bred, as detailed below at the CIGM of Institut Pasteur. During the  
4 immunization period female or male transgenic mice were housed in individually-ventilated cages under  
5 specific pathogen-free conditions. Mice were transferred into individually filtered cages in isolator for  
6 SARS-CoV-2 inoculation at the Institut Pasteur animal facilities. Prior to i.n. injections, mice were  
7 anesthetized by i.p. injection of Ketamine (Imalgene, 80 mg/kg) and Xylazine (Rompun, 5 mg/kg).

### 8 **Mouse Transgenesis**

9 The human K18 promoter (GenBank: AF179904.1 nucleotide 90 to 2579) was amplified by nested PCR  
0 from A549 cell lysate, as described previously (Chow et al., 1997; Koehler et al., 2000). The “i6x7” intron  
1 (GenBank: AF179904.1 nucleotide 2988 to 3740) was synthesized by Genscript. The K18<sup>JAX</sup> (originally  
2 named K18i6x7PA) promoter includes the K18 promoter, the i6x7 intron at 5' and an  
3 enhancer/polyadenylation sequence (PA) at 3' of the *hACE2* gene. The<sup>K18 IP-ThV</sup> promoter, instead of PA,  
4 contains the stronger wild-type WPRE element at 3' of the *hACE2* gene. In contrast to K18<sup>JAX</sup> construct  
5 which harbors the 3' regulatory region containing a polyA sequence, the K18<sup>IP-ThV</sup> construct takes benefice  
6 of the polyA sequence already present within the 3' Long Terminal Repeats (LTR) of the lentiviral plasmid.

7 The i6x7 intronic part was modified to introduce a consensus 5' splicing donor and a 3' donor site sequence.  
8 The AAGGGG donor site was further modified for the AAGTGG consensus site. Based on a consensus  
9 sequence logo (Dogan et al., 2007), the poly-pyrimidine tract preceding splicing acceptor site  
0 (TACAATCCCTC in original sequence GenBank: AF179904.1 and TTTTTTTTTT in K18<sup>JAX</sup>) was  
1 replaced by CTTTTTCCTTCC to limit incompatibility with the reverse transcription step during  
2 transduction. Moreover, original splicing acceptor site CAGAT was modified to correspond to the  
3 consensus sequence CAGGT. As a construction facility, a ClaI restriction site was introduced between the  
4 promoter and the intron. The construct was inserted into a pFLAP plasmid between the MluI and BamHI  
5 sites. *hACE2* gene cDNA was introduced between the BamHI and XhoI sites by restriction/ligation.  
6 Integrative LV::K18-hACE2 was produced as described in (Ku et al., 2021) and concentrated by two cycles  
7 of ultracentrifugation at 22,000 rpm 1h 4°C.

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

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

6 Genomic DNA (gDNA) from transgenic mice was prepared from the tail biopsies by phenol-chloroform  
7 extraction. Sixty ng of gDNA were used as a template of qPCR with SYBR Green using specific primers  
8 listed in [Table S2](#). Using the same template and in the similar reaction plate, mouse *pkd1* (Polycystic Kidney  
9 Disease 1) and *gapdh* were also quantified. All samples were run in quadruplicate in 10  $\mu$ l reaction as  
0 follows: 10 min at 95°C, 40 cycles of 15 s at 95°C and 30 sec at 60°C. To calculate the transgene copy  
1 number, the  $2^{-\Delta\Delta C_t}$  method was applied using the *pkd1* as a calibrator and *gapdh* as an endogenous control.  
2 The  $2^{-\Delta\Delta C_t}$  provides the fold change in copy number of the *hACE2* gene relative to *pkd1* gene.

### 3 **Ethical Approval of Animal Studies**

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

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

0 As recently detailed elsewhere (Ku et al., 2021), T-splenocyte responses were quantitated by IFN- $\gamma$

1 ELISPOT and anti-S IgG or IgA Abs were detected by ELISA by use of recombinant stabilized S<sub>CoV-2</sub>.  
2 NAb quantitation was performed by use of S<sub>CoV-2</sub> pseudo-typed LV, as previously described (Anna et al.,  
3 2020; Sterlin et al., 2020). The qRT-PCR quantification of inflammatory mediators in the lungs and brain  
4 of hamsters and mice was performed in total RNA extracted by TRIzol reagent, as recently detailed (Ku et  
5 al., 2021). CCL19 and CCL21 expression were measured using the following primer pairs: forward primers  
6 were 5'-CTG CCT CAG ATT ATC TGC CAT-3' for CCL19 and 5'- AAG GCA GTG ATG GAG GGG-  
7 3' for CCL21; reverse primers were 5'- AGG TAG CGG AAG GCT TTC AC -3' for CCL19 and 5'- CGG  
8 GGT AAG AAC AGG ATT G -3' for CCL21.

### 9 **SARS-CoV-2 inoculation**

0 Hamsters or transgenic B6.K18-hACE2<sup>IP-THV</sup> or B6.K18-ACE2<sup>2PrImn/JAX</sup> were anesthetized by i.p.  
1 injection of Ketamine and Xylazine mixture, transferred into a biosafety cabinet 3 and inoculated i.n. with  
2  $0.3 \times 10^5$  TCID<sub>50</sub> of the BetaCoV/France/IDF0372/2020 SARS-CoV-2 clinical isolate (Lescure et al.,  
3 2020). This clinical isolate was a gift of the National Reference Centre for Respiratory Viruses hosted by  
4 Institut Pasteur (Paris, France), headed by Pr. van der Werf. The human sample from which this strain was  
5 isolated has been provided by Dr. Lescure and Pr. Yazdanpanah from the Bichat Hospital, Paris, France.  
6 Mice or hamsters were inoculated i.n. with 20  $\mu$ l or 50  $\mu$ l of viral inoculum, respectively. Animals were  
7 housed in an isolator in BioSafety Level 3 animal facilities of Institut Pasteur. The organs recovered from  
8 the infected animals were manipulated according to the approved standard procedures of these facilities.

### 9 **Determination of viral loads in the organs**

0 Organs from mice or hamsters were removed aseptically and immediately frozen at -80°C. RNA from  
1 circulating SARS-CoV-2 was prepared from lungs as recently described (Ku et al., 2021). Briefly, lung  
2 homogenates were prepared by thawing and homogenizing of the organs using lysing matrix M (MP  
3 Biomedical) in 500  $\mu$ l of ice-cold PBS in an MP Biomedical Fastprep 24 Tissue Homogenizer. RNA was  
4 extracted from the supernatants of lung homogenates centrifuged during 10 min at 2000g. These RNA  
5 preparations were used to determine viral loads by E-specific qRT-PCR.

6 Alternatively, total RNA was prepared from lungs or other organs by addition of lysing matrix D (MP  
7 Biomedical) containing 1 mL of TRIzol reagent and homogenization at 30 s at 6.0 m/s twice using MP  
8 Biomedical Fastprep 24 Tissue Homogenizer. Total RNA was extracted using TRIzol reagent  
9 (ThermoFisher). These RNA preparations were used to determine viral loads by Esg-specific qRT-PCR,  
0 hACE2 expression level or inflammatory mediators.

1 SARS-CoV-2 E gene (Corman et al., 2020) or E sub-genomic mRNA (Esg mRNA) (Wolfel et al., 2020),  
2 was quantitated following reverse transcription and real-time quantitative TaqMan® PCR, using  
3 SuperScript™ III Platinum One-Step qRT-PCR System (Invitrogen) and specific primers and probe  
4 (Eurofins) (Table S3). The standard curve of Esg mRNA assay was performed using in vitro transcribed

5 RNA derived from PCR fragment of “T7 SARS-CoV-2 Esg mRNA”. The in vitro transcribed RNA was  
6 synthesized using T7 RiboMAX Express Large Scale RNA production system (Promega) and purified by  
7 phenol/chloroform extraction and two successive precipitations with isopropanol and ethanol.  
8 Concentration of RNA was determined by optical density measurement, diluted to  $10^9$  genome  
9 equivalents/ $\mu\text{L}$  in RNase-free water containing  $100\mu\text{g/mL}$  tRNA carrier, and stored at  $-80^\circ\text{C}$ . Serial  
0 dilutions of this in vitro transcribed RNA were prepared in RNase-free water containing  $10\mu\text{g/ml}$  tRNA  
1 carrier to build a standard curve for each assay. PCR conditions were: (i) reverse transcription at  $55^\circ\text{C}$  for  
2 10 min, (ii) enzyme inactivation at  $95^\circ\text{C}$  for 3 min, and (iii) 45 cycles of denaturation/amplification at  $95^\circ\text{C}$   
3 for 15 s,  $58^\circ\text{C}$  for 30 s. PCR products were analyzed on an ABI 7500 Fast real-time PCR system (Applied  
4 Biosystems).

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

6 Isolation and staining of lung innate immune cells were largely detailed recently (Ku et al., 2021).  
7 Cervical lymph nodes, olfactory bulb and brain from each group of mice were pooled and treated with  $400$   
8 U/ml type IV collagenase and DNase I (Roche) for a 30-minute incubation at  $37^\circ\text{C}$ . Cervical lymph nodes  
9 and olfactory bulbs were then homogenized with glass homogenizer while brains were homogenized by  
0 use of GentleMacs (Miltenyi Biotech). Cell suspensions were then filtered through  $100\ \mu\text{m}$ -pore filters,  
1 washed and centrifuged at  $1200\ \text{rpm}$  during 8 minutes. Cell suspensions from brain were enriched in  
2 immune cells on Percoll gradient after 25 min centrifugation at  $1360\ \text{g}$  at RT. The recovered cells from  
3 lungs were stained as recently described elsewhere (Ku et al., 2021). The recovered cells from brain were  
4 stained by appropriate mAb mixture as follows. (i) To detect innate immune cells: Near IR Live/Dead  
5 (Invitrogen), Fc $\gamma$ II/III receptor blocking anti-CD16/CD32 (BD Biosciences), BV605-anti-CD45 (BD  
6 Biosciences), PE-anti-CD11b (eBioscience), PE-Cy7-antiCD11c (eBioscience), (ii) to detect NK,  
7 neutrophils, Ly-6C<sup>+/+</sup> monocytes and macrophages: Near IR DL (Invitrogen), Fc $\gamma$ II/III receptor blocking  
8 anti-CD16/CD32 (BD Biosciences), BV605-anti-CD45 (BD Biosciences), PE-anti-CD11b (eBioscience),  
9 PE-Cy7-antiCD11c (eBioscience), APC-anti-Ly6G (Miltenyi), BV711-anti-Siglec-F (BD), AF700-anti-  
0 NKp46 (BD Biosciences), FITC-anti-Ly6C (ab25025, Abcam) (iii) To detect adaptive immune cells: Near  
1 IR Live/Dead (Invitrogen), Fc $\gamma$ II/III receptor blocking anti-CD16/CD32 (BD Biosciences), APC-anti-  
2 CD45 (BD), PerCP-Cy5.5-anti-CD3 (eBioscience), FITC-anti-CD4 (BD Pharmingen), BV711-anti-CD8  
3 (BD Horizon), BV605-anti-CD69 (Biolegend), PE-anti-CCR7 (eBioscience) and VioBlue-Anti-B220  
4 (Miltenyi). Cells were incubated with appropriate mixtures for 25 minutes at  $4^\circ\text{C}$ , washed in PBS  
5 containing 3% FCS and fixed with Paraformaldehyde 4% by an overnight incubation at  $4^\circ\text{C}$ . Samples were  
6 acquired in an Attune NxT cytometer (Invitrogen) and data analyzed by FlowJo software (Treestar, OR,  
7 USA).

### 8 **Lung Histopathology**

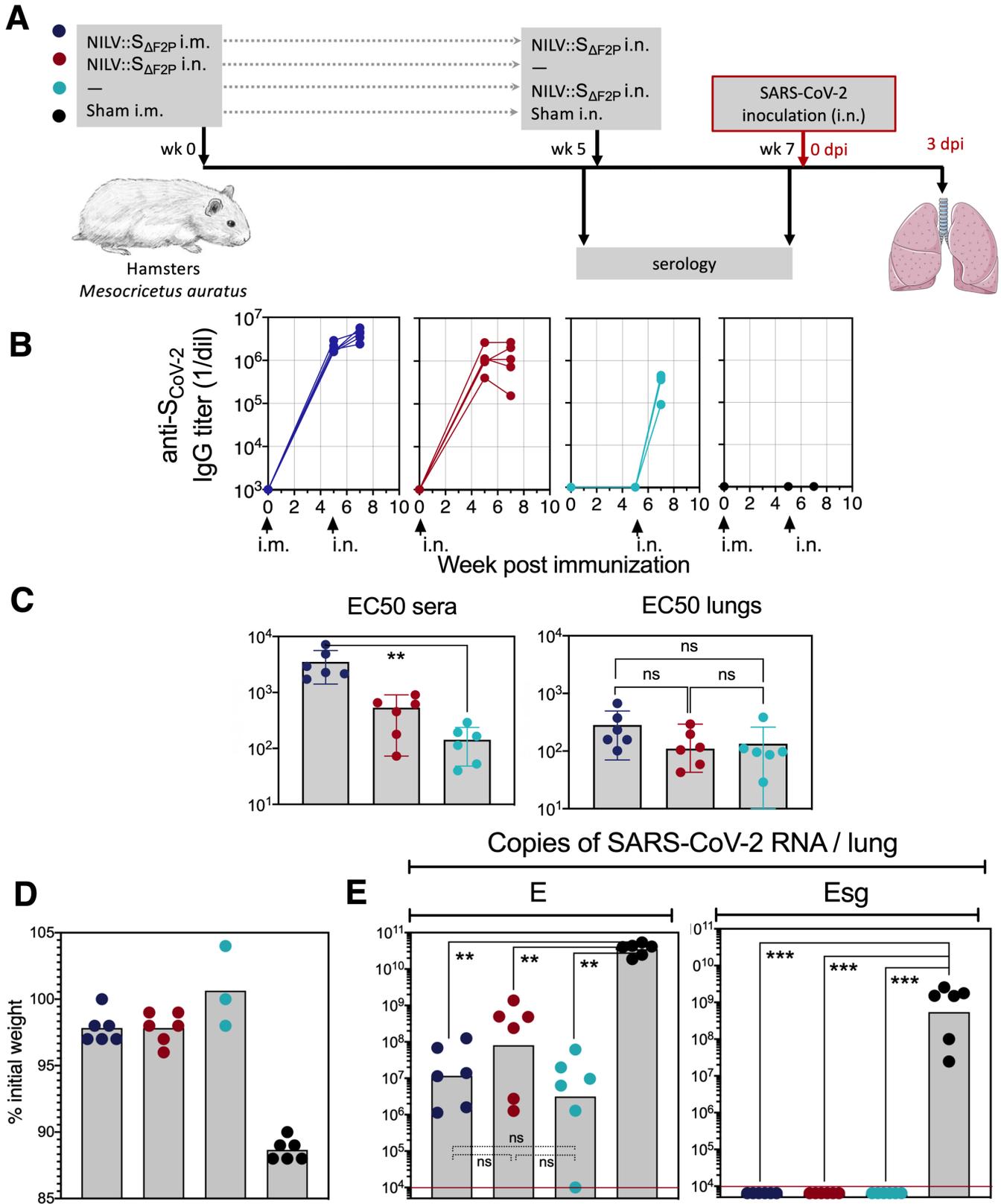
9 Samples from the lungs or brain of hamsters or transgenic mice were fixed in formalin for 7 days and  
0 embedded in paraffin. Paraffin sections (5- $\mu$ m thick) were stained with Hematoxylin and Eosin (H&E).  
1 Histopathological lesions were qualitatively described and when possible scored, using: (i) distribution  
2 qualifiers (i.e., focal, multifocal, locally extensive or diffuse), and (ii) a five-scale severity grade, i.e., 1:  
3 minimal, 2: mild, 3: moderate, 4: marked and 5: severe.

## 4 References

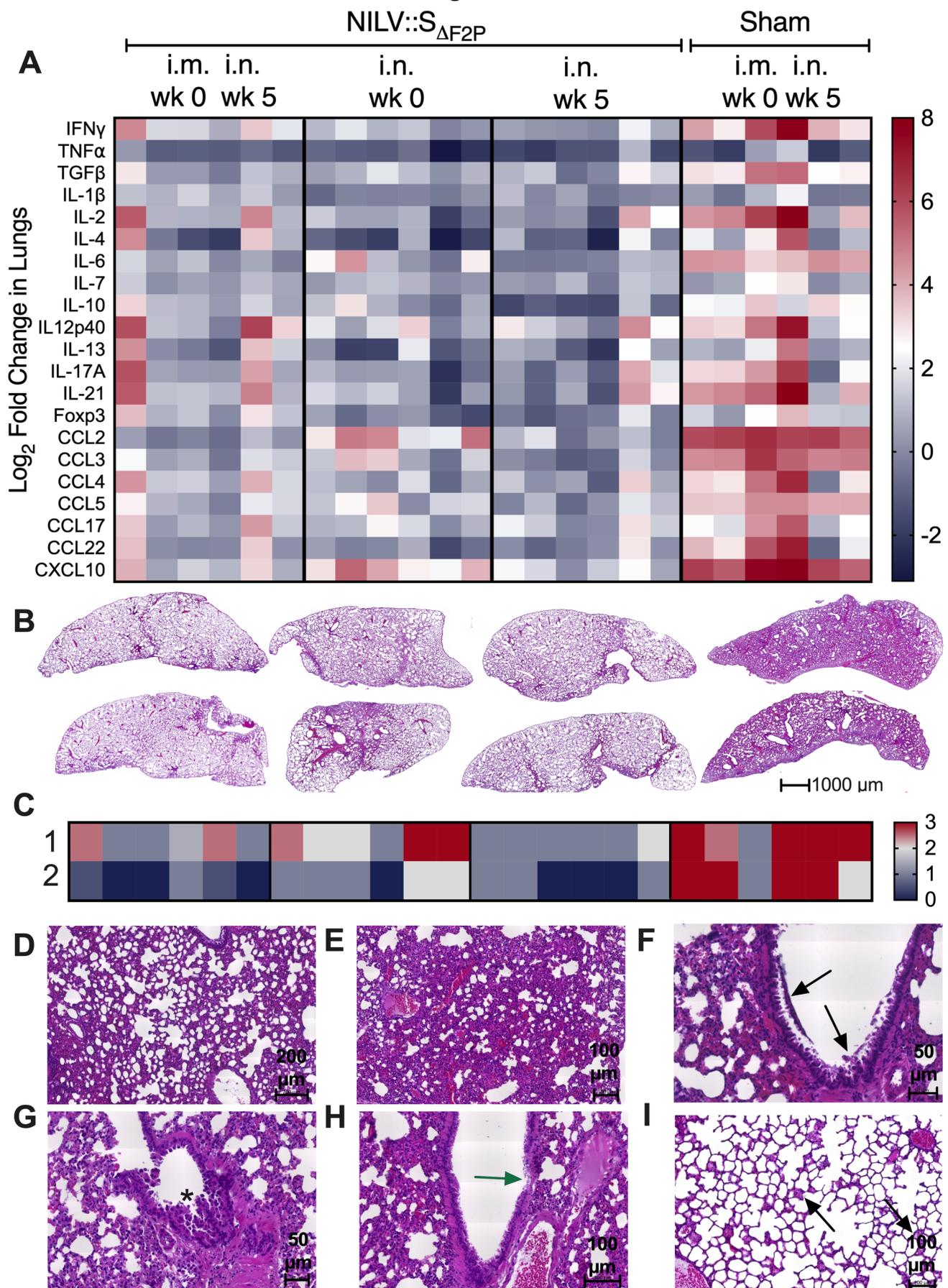
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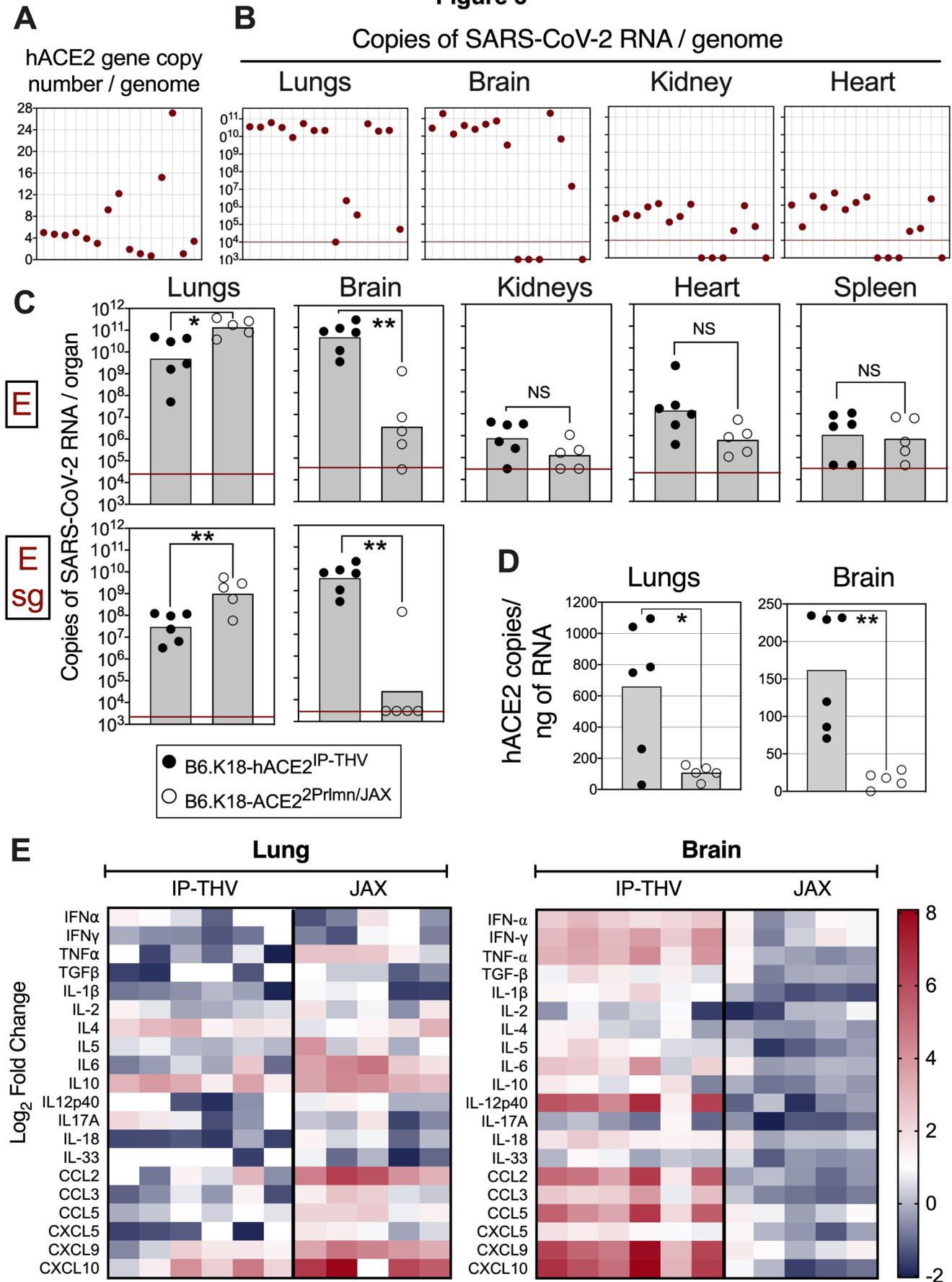
**Figure 1**



**Figure 2**



**Figure 3**



**Figure 4**

