CORONAVIRUS

Administration of aerosolized SARS-CoV-2 to K18-hACE2 mice uncouples respiratory infection from fatal neuroinvasion

Valeria Fumagalli[1,2]†, Micol Ravà[1]†, Davide Marotta[1,2]†, Pietro Di Lucia[1], Chiara Laura[1,2,3], Eleonora Sala[1,2], Marta Grillo[1], Elisa Bono[1], Leonardo Giustini[1], Chiara Perucchini[1], Marta Mainetti[1], Alessandro Sessa[4], José M. Garcia-Manteiga[3], Lorena Donnici[5], Lara Manganaro[5], Serena Delbue[6], Vania Broccoli[4,7], Raffaele De Francesco[5,8], Patrizia D'Adamo[4,9], Mirela Kuka[1,2], Luca G. Guidotti[1,2]*, Matteo Iannacone[1,2,10]*

The development of a tractable small animal model faithfully reproducing human coronavirus disease 2019 pathogenesis would arguably meet a pressing need in biomedical research. Thus far, most investigators have used transgenic mice expressing the human ACE2 in epithelial cells (K18-hACE2 transgenic mice) that are intranasally instilled with a liquid severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) suspension under deep anesthesia. Unfortunately, this experimental approach results in disproportionate high central nervous system infection leading to fatal encephalitis, which is rarely observed in humans and severely limits this model's usefulness. Here, we describe the use of an inhalation tower system that allows exposure of unanesthetized mice to aerosolized virus under controlled conditions. Aerosol exposure of K18-hACE2 transgenic mice to SARS-CoV-2 resulted in robust viral replication in the respiratory tract, anosmia, and airway obstruction but did not lead to fatal viral neuroinvasion. When compared with intranasal inoculation, aerosol infection resulted in a more pronounced lung pathology including increased immune infiltration, fibrin deposition, and a transcriptional signature comparable to that observed in SARS-CoV-2–infected patients. This model may prove useful for studies of viral transmission, disease pathogenesis (including long-term consequences of SARS-CoV-2 infection), and therapeutic interventions.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic is caused by the recently identified β-coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (*1*, *2*). Disease severity is variable, ranging from asymptomatic infection to multi-organ failure and death. Although SARS-CoV-2 primarily targets the respiratory system, some patients with COVID-19 can also exhibit extrarespiratory symptoms, including neurological manifestations such as loss of smell (anosmia) and taste (ageusia), headache, fatigue, memory impairment, vomiting, gait disorders, and impaired consciousness (*3*–*6*). SARS-CoV-2 can infect neurons in human brain organoids (*7*, *8*), and a few studies reported the presence of SARS-CoV-2 in olfactory sensory neurons and deeper areas within the central nervous system (CNS) in fatal COVID-19 cases (*8*–*12*). However, the neurotropism of SARS-CoV-2 and a direct role of CNS infection in the pathogenesis of neurological manifestations remain highly debated.

Despite the availability of effective vaccines against SARS-CoV-2, we still know little about COVID-19 pathogenesis. The availability of tractable animal models to mechanistically dissect virological, immunological, and pathogenetic aspects of the infection with SARS-CoV-2 and future human coronaviruses would provide major benefit. Wild-type laboratory mice are poorly susceptible to SARS-CoV-2 infection because the mouse angiotensin-converting enzyme (ACE) 2 does not act as a cellular receptor for the virus (*13*). Several transgenic mouse lineages expressing the human version of the SARS-CoV-2 receptor (hACE2) support viral replication and recapitulate certain clinical characteristics of the human infection (*13*). The most widely used model is the K18-hACE2 transgenic mouse (*14*), which expresses hACE2 predominantly in epithelial cells under the control of the cytokeratin 18 (*KRT18*) promoter (*15*). K18-hACE2 mice are typically infected by intranasally instilling liquid suspensions of SARS-CoV-2 under deep anesthesia. This results in disproportionate high CNS infection leading to fatal encephalitis (*16*–*20*), which rarely occurs in patients with COVID-19. Such viral neuroinvasion severely limits the usefulness of these mouse models, hampering studies on disease pathogenesis (including long-term consequences of SARS-CoV-2 infection) and on drug discovery. Here, we report the generation and characterization of an alternative COVID-19 platform based on controlled exposure of K18-hACE2 transgenic mice to aerosolized SARS-CoV-2.

RESULTS

Intranasal inoculation, but not aerosol exposure, of SARS-CoV-2 leads to fatal neuroinvasion

SARS-CoV-2 is mainly transmitted from person to person via respiratory droplets (*21*). In an attempt to mimic this transmission

[1]Division of Immunology, Transplantation, and Infectious Diseases, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy. [2]Vita-Salute San Raffaele University, 20132 Milan, Italy. [3]Center for Omics Sciences, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy. [4]Division of Neuroscience, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy. [5]INGM - Istituto Nazionale di Genetica Molecolare "Romeo ed Erica Invernizzi", Milan, Italy. [6]Department of Biomedical, Surgical and Dental Sciences, University of Milan, Italy. [7]National Research Council of Italy, Institute of Neuroscience, Italy. [8]Department of Pharmacological and Biomolecular Sciences (DiSFeB), University of Milan, Italy. [9]Center of Advanced Services for in-vivo testing–Animal behavior Facility, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy. [10]Experimental Imaging Centre, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy.
*Corresponding author. Email: iannacone.matteo@hsr.it (M.I.); guidotti.luca@hsr.it (L.G.G.)
†These authors contributed equally to this work.

route, we made use of a nose-only inhalation tower system that allows to expose unanesthetized mice to aerosolized virus under controlled pressure, temperature, and humidity conditions (see fig. S1, A to C, and Materials and Methods). Animals were located inside a restraint with a neck clip positioned between the base of the skull and the shoulders, thus avoiding thorax compression, keeping the airways completely unobstructed, and allowing for spontaneous breathing through the nose. K18-hACE2 transgenic mice were infected with a target dose of 1×10^5 tissue culture infectious dose 50 ($TCID_{50}$) of SARS-CoV-2 either through intranasal (IN) administration with 25 μl of diluted virus or through a 20- to 30-min exposure to aerosolized virus (AR) (Fig. 1, A and B and see Materials and Methods). Pulmonary function was measured during AR exposure using plethysmography. Frequency, tidal volume, minute volume, and accumulated volume of SARS-CoV-2–exposed mice were comparable with phosphate-buffered saline (PBS)–exposed mice (fig. S1D).

As expected (*19*, *20*), IN-infected animals exhibited significant body weight loss and a severe clinical score (see Materials and Methods for details), so that, by day 6 post infection (p.i.), ~80% of them had died and the remaining ones appeared lethargic (Fig. 1, C to E, and fig. S2). By contrast, AR-infected mice maintained stable body weight and did not show any signs of disease nor mortality, including at 20 days p.i. (Fig. 1, C to E, and figs. S2 and S3). The severe disease observed in IN-infected K18-hACE2 transgenic mice was associated with the detection of high viral RNA titers and infectious virus in the brain (Fig. 1, F to I). By contrast, neither SARS-CoV-2 RNA nor infectious virus was detected in the brain of mice exposed to aerosolized virus (Fig. 1, F to I). Immunohistochemical and immunofluorescence staining confirmed the presence of the SARS-CoV-2 nucleoprotein (N-CoV-2) in the brain of IN-infected, but not AR-infected, mice (Fig. 1, J and K). Specifically, diffuse staining for SARS-CoV-2 nucleoprotein was detected throughout the cerebrum with comparable staining in the different brain areas with the notable exception of the cerebellum, where most of its cells stained negative for viral antigens (Fig. 1K). Neurons were by far the most infected brain cells as shown by the costaining of the SARS-CoV-2 nucleoprotein with the pan-neuronal marker NeuN (~90% double-positive cells; Fig. 1L and fig. S4A). Nitric oxide has been implicated as a contributor to the host's innate defense against viral infections including those affecting the CNS (*22*). Accordingly, neurons in infected brains strongly up-regulated inducible nitric oxide synthase (iNOS) (*22*), which was undetectable in neuronal cells from control, uninfected mice (Fig. 1M). By contrast, only a minor fraction of astrocytes (~2%) and microglia (~4%) stained positive for the SARS-CoV-2 nucleoprotein (Fig. 1, N and O, and fig. S4, B and C). $Iba1^+$ myeloid cells in SARS-CoV-2–infected brains were activated as revealed by the characteristic morphology (swollen processes with reduced ramifications) and CD68 positivity (Fig. 1, P and Q). Consistent with the data on the recovery of infectious virus, viral RNA, and viral antigens, we found a significant immune cell recruitment (particularly of T cells, B cells, monocytes, and eosinophils) in the brains of IN-infected, but not AR-infected, mice (fig. S4, D and E). Together, these results demonstrate a profound viral neuroinvasion that correlates with the severe health deterioration in IN-infected mice. The high viral load and widespread viral distribution in the brain of IN-infected mice contrasts with the occasional localized detection of SARS-CoV-2 in the olfactory bulbs and/or the medulla of fatal COVID-19 cases (*10*, *23*–*27*) and caution against using this model to investigate the neurological complications of SARS-CoV-2 infection in humans.

We next investigated the potential SARS-CoV-2 entry portals to the CNS in IN-infected K18-hACE2 transgenic mice. One possibility is that the virus gains access to the CNS via the blood-brain barrier, which implies a viremic phase. However, no SARS-CoV-2 RNA was ever detected in the sera of infected mice (fig. S4, F and G), consistent with earlier reports (*18*–*20*). Alternatively, SARS-CoV-2 could enter the CNS by retrograde axonal transport upon olfactory sensory neuron infection. Indeed, and in line with previous studies (*18*–*20*), viral RNA and viral antigens were detected in the olfactory bulb of IN-infected, but not AR-infected, mice (Fig. 1, R and S, and fig. S5). Overall, the data indicate that IN, but not AR, infection of K18-hACE2 transgenic mice with SARS-CoV-2 results in lethal neuroinvasion likely via retrograde axonal transport after olfactory sensory neuron infection.

Aerosol exposure of K18-hACE2 transgenic mice to SARS-CoV-2 leads to efficient respiratory infection, anosmia, and fibrin deposition in the lung

We next analyzed viral replication in the upper respiratory tract of K18-hACE2 transgenic mice infected with SARS-CoV-2 via IN inoculation or AR exposure. We detected the presence of SARS-CoV-2 RNA in the nasal turbinates of both AR- and IN-infected mice at days 3 and 6 p.i. (Fig. 2, A and B). To examine whether viral replication within the upper respiratory tract induced anosmia, we subjected AR- and IN-infected mice to a social scent discrimination assay (Fig. 2C) (*19*). If olfaction is normal (as in PBS-treated controls), then male mice exposed to tubes containing male or female bedding preferentially spend time sniffing the female scent (Fig. 2, D to F). By contrast, both AR- and IN-infected mice spent significantly less time sniffing the female scent at day 3 p.i. (Fig. 2, D and E), indicative of hyposmia or anosmia. At day 3 p.i., the mobility of both AR- and IN-infected mice was normal, as there were no differences in the amount of time spent sniffing the male tube (Fig. 2D). At day 6 p.i., AR-infected mice still showed signs of hyposmia/anosmia, whereas IN-infected mice were completely lethargic preventing further analyses (Fig. 2, D to F). The data obtained in AR-infected mice are consistent with the hypothesis that hyposmia or anosmia occurs because of the infection of olfactory epithelium and in the absence of CNS infection or general malaise (*19*, *28*, *29*).

We next assessed viral replication in the lower respiratory tract of SARS-CoV-2–infected K18-hACE2 transgenic mice. We detected comparable amounts of SARS-CoV-2 RNA and infectious virus from the lungs of mice infected with the two different routes of administration at both days 3 and 6 p.i. (Fig. 2, G to J). Immunohistochemical and immunofluorescence staining for the SARS-CoV-2 nucleoprotein confirmed similar levels of viral antigens and similar staining patterns in the lungs of IN- and AR-infected mice (Fig. 2K). To gain insight into the impact of infection on lung physiology, pulmonary function was measured at days 3 and 5 p.i. via whole-body plethysmography (WBP). Consistent with previously published data (*30*, *31*), we confirmed that, when compared with control mice, IN-infected mice exhibited a significant loss in pulmonary function as indicated by changes in, e.g., respiratory frequency, tidal volume, Rpef (a measure of airway obstruction), and PenH [a controversial metric that has been used by some as an indirect measure of airway resistance and by others as a nonspecific assessment of breathing