Lethal Infection of K18-\textit{hACE2} Mice Infected with Severe Acute Respiratory Syndrome Coronavirus

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Severe acute respiratory syndrome (SARS) was first identified in Guangdong Province in China (28). Over the ensuing 9 months, more than 8,000 cases were identified throughout the world, with a ~10% case fatality rate. A novel coronavirus, SARS coronavirus (SARS-CoV), was identified as the causative agent (6, 17, 29, 32). Initial investigations indicated that the virus spread to humans from infected exotic animals such as Himalayan palm civets (\textit{Paguma larvata}) and Chinese ferret badgers (\textit{Melogale moschata}) (12); more recent work has suggested that the natural reservoirs for the virus are wild bat populations in China (19, 24). Although SARS has not recurred in human populations to a significant extent since 2003, the potential severity of such a recurrence has spurred interest in developing an animal model for the human disease.

SARS-CoV infects and replicates in mice, ferrets, hamsters, and several species of nonhuman primates (cyonolgous and rhesus macaques, African green monkeys, and common marmosets) (reviewed in reference 37). However, none of these animals develop a clinical disease that is reproducible and equivalent in severity to that observed in SARS patients. A mouse model would be useful for answering many questions about SARS pathogenesis and for testing vaccine efficacy, in developing a more robust murine model, we generated transgenic (Tg) mice in which expression of \textit{hACE2} (human angiotensin-converting enzyme 2) was targeted to epithelial cells. While human ACE2 and murine ACE2 (mACE2) molecules are very homologous, mACE2 does not support SARS-CoV binding as efficiently as \textit{hACE2} (22). Here we show that the transgenic expression of \textit{hACE2} in epithelia converts a mild SARS-CoV infection into a rapidly fatal disease.

MATERIALS AND METHODS

**Mice.** All animal studies were approved by the University of Iowa and the Veterans Administration Institutional Animal Care and Use committees. Mice transgenic for expression of \textit{hACE2} (K18-\textit{hACE2} mice) were generated as follows (see Fig. 1A). The \textit{hACE2} coding sequence was PCR amplified from IMAGE consortium clone ID 5243048 (ATCC, Manassas, VA) and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). The lacZ coding sequence in the previously described pK18mTElacZ-K18i6x7pA construct (16) (a kind gift from Jim Hu, Hospital for Sick Children, Toronto, Canada) was then replaced by the \textit{hACE2} coding sequence to create pK18-\textit{hACE2}. 5’ of the \textit{hACE2} coding sequence, this plasmid contains 2.5 kb of upstream genomic sequence, the promoter, and the first intron (with a mutation in the 3’ splice acceptor site to reduce exon skipping) of the human cytokeratin 18 (K18) gene as well as a translational enhancer sequence from alfalfa mosaic virus. Downstream of the \textit{hACE2} coding sequence are exon 6, intron 6, exon 7, and the poly(A) signal of the human K18 gene. These elements were found to be necessary for high-level expression and epithelial cell specificity (4, 16). The purified 6.8-kb DNA fragment generated from an HpaI and XbaI double digest of pK18-\textit{hACE2} was used as the transgene for injection into pronuclei of fertilized (C57BL/6J × SJL/J)F₂ mouse eggs to generate transgenic embryos. Mice used in this study were backcrossed two to three times onto a C57BL/6 background. Tail DNA was obtained from mice using an Extract-N-Amp tissue PCR kit (Sigma-Aldrich, St. Louis, MO). Mice transgenic for \textit{hACE2} expression were detected by PCR analysis using forward primer ACCGCTGTAAGCAAGACAAAG and reverse primer primate AATTAG CCACAAGCAAATCACTAGGACACAGCATCC.

**Determination of \textit{hACE2} copy number.** Genomic DNA from each founder line of transgenic mouse and from wild-type mice was isolated from the liver using DNAzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. One nanogram of genomic DNA and three consecutive 1:1 dilutions
DNA was determined to equal 4.5/1,100,000. The titer of virus used for all studies, as determined by a plaque assay, was W. Bellini and T. Ksiazek at the Centers for Disease Control, Atlanta, GA. The H2O by adding 0.5 ml at a time (total lavage volume, approximately 4 ml). The inserted into the exposed trachea. Lungs were inflated with sterile saline to 25 cm opened by midline incision, and lungs were lavaged in situ via PE-90 tubing was determined as described previously (36).

To determine the hACE2 gene copy number in each transgenic mouse, the 2^-DeltaCT method was performed using the PKD1 gene as an endogenous calibrator (1). The 2^-DeltaCT method indicates the n-fold change in copy number of the hACE2 gene relative to the PKD1 gene, which is a single-copy gene in mice.

Infection with SARS-CoV. The Urbani strain of SARS-CoV was obtained from W. Bellini and T. Ksiazek at the Centers for Disease Control, Atlanta, GA. The virus was propagated and titrated on Vero E6 cells in a biosafety level 3 laboratory. The titer of virus used for all studies, as determined by a plaque assay, was 7.6 x 10^6 PFU/ml. Mice were lightly anesthetized with halothane and infected intranasally with the indicated dosage of SARS-CoV in 30 µl of Dulbecco’s modified Eagle medium. Infected mice were examined and weighed daily. To obtain specimens for virus titers, animals were sacrificed and organs were aseptically removed into sterile phosphate-buffered saline. In some cases, blood was obtained via cardiac puncture, and the entire organs were collected in 1 ml of RNAlater (E-Bioscience, San Diego, CA) followed by a biotinylated goat anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA). Samples were developed by sequential incubation with a streptavidin-horseradish peroxidase conjugate (Jackson ImmunoResearch) and diaminobenzidine (Sigma-Aldrich).

RNA protection assays for cytokines and chemokines. Five micrograms of total RNA obtained from lungs or brains was analyzed by RNase protection assays using a custom set of probes purchased from BD PharMingen (San Diego, CA). After electrophoresis, gels were exposed to a phosphorimaging screen and analyzed using Bio-Rad Quantity One 4.4.0 software. Levels of RNA were normalized to those of a housekeeping gene (L32) in order to allow interanimal comparison of cytokine/chemokine mRNA levels.

Treatment with a neutralizing antibody. In some cases, mice were treated intravenously with 25 mg of an anti-S neutralizing human MAB (MBL SARS-201; supplied by Donna Ambrosino, Massachusetts Biologic Laboratories) (10) per kg of body weight or a control humanized anti-respiratory syncytial virus MAB (palivizumab; Medimmune, Gaithersburg, MD) 1 day prior to infection.

RESULTS

Development and characterization of K18-hACE2 mice. hACE2 is expressed in human airway and alveolar epithelia (13), and SARS-CoV infects primary airway epithelial cells in vitro (14, 34). Since studies with nonhuman primates have shown that SARS-CoV infection began in airway epithelia (25), we generated transgenic mice in which hACE2 expression was driven by the K18 promoter as described in Materials and Methods (Fig. 1A). The K18 promoter confers efficient transgene expression in airway epithelial cells (but not in alveolar epithelia), as well as in epithelia of other internal organs, including the liver, kidney, and gastrointestinal tract (4). We generated three founder lines that transmitted the hACE2 gene to their progeny. Levels of transgene DNA in founder lines ranged from 4 to 10 copies per genome as determined by quantitative PCR (Fig. 1B). We detected hACE2 mRNA in several tissues, including the lung, colon, liver, and kidney (Fig. 2A and C), whereas endogenous mACE2 was most abundantly expressed in the ileum (8). Notably, very low but measurable levels of ACE2 were detected in the brains of both non-Tg and K18-hACE2 mice (Fig. 2B and C). Using lung sections and an antibody that detects both hACE2 and mACE2 in immunofluorescence assays, we detected ACE2 in airway epithelia in both non-Tg and K18-hACE2 mice, with no obvious differences in distribution (data not shown).

SARS-CoV-infected K18-hACE2 mice develop severe clinical disease. It was shown previously that intranasal inoculation of BALB/c or C57BL/6 mice with SARS-CoV resulted in minimal clinical disease, although C57BL/6 mice exhibited reduced weight gain after inoculation (9, 36). In agreement with these data, infection of non-Tg littermates resulted in no mortality or clinical disease, and mice gained weight over the course of the experiment (Fig. 3A and B). In marked contrast, K18-hACE2 mice inoculated intranasally with SARS-CoV began to lose weight by days 3 to 5 postinfection (p.i.). Concomitant with weight loss, mice became lethargic, with labored breathing. As shown in Fig. 3A, mice from all founder lines were dead by day 7 p.i., and nearly all mice from lines 1 and 2 were moribund by 4 days p.i. As noted above, mice from lines 1 and 2 contained the greatest number of hACE2 transgene copies. Since line 1 and line 2 mice exhibited nearly identical phenotypes, we used line 2 mice as representative of this more susceptible phenotype in the studies reported here.
Virus titers were 0.5 to 1 log unit higher in the lungs of K18-hACE2 mice than in those of their non-Tg littermates at day 2 p.i. and were higher in the lungs of K18-hACE2 mice that exhibited a more rapid disease course (line 2) than in those of mice surviving a few days longer (line 3) (Fig. 3C). While virus was partially cleared from the lungs of all mice by days 3 to 4 p.i., titers were 3 log units higher in K18-hACE2 mice than in non-Tg mice (line 3, day 4 p.i.; \( P < 0.0005 \)). These results were confirmed by quantitative real-time RT-PCR, with higher levels of viral RNA present in the lung at 2 days p.i. than at 4 days p.i. (Fig. 4). Together, these data suggest that enhanced virus replication played a key role in the more severe disease observed in K18-hACE2 mice.

Although the K18 promoter is active in the epithelia of multiple organs, virus was not detected in the liver, kidney, or small intestine (ileum) at either 2 or 4 days p.i. (Fig. 3D). We also analyzed the brain for evidence of SARS-CoV, since the virus has been detected in patient brains in some studies (5, 11, 42) (Fig. 3D). Virus was never detected in the brains of non-Tg mice at days 2 to 4 p.i. In line 2 mice, virus was not detected in the brain at day 1 p.i. but was present at 2 days p.i. and was present at very high levels by 3 days p.i. Virus was also detected at low levels on day 2 and at high levels on day 4 p.i. in the brains of line 3 mice, even though levels of hACE2 mRNA in the brains of these mice were barely above background (Fig. 2B). Levels of viral RNA in the brain also increased dramatically from day 2 to day 4 p.i. (Fig. 4). Of note, SARS-CoV infects the brains of C57BL/6 mice at later times (9 days) p.i. (9), showing that the central nervous system (CNS) is a secondary site of infection even in non-Tg mice.

Mice were inoculated intranasally with \( 2.3 \times 10^4 \) PFU in
these initial experiments. In subsequent experiments, we showed that virus was lethal at even lower dosages, since 3/3 and 5/6 mice (line 2) died after infection with 2.3 × 10^3 and 2.3 × 10^2 PFU, respectively. Thus, the 50% lethal dose of SARS-CoV for K18-hACE2 mice was less than 230 PFU after intranasal inoculation. SARS-CoV was not transmitted from moribund mice to uninfected K18-hACE2 mice (n = 4) housed in the same cages. This was not surprising, however, since mice do not cough or sneeze, and virus was not detected in the gastrointestinal tract or kidney.

**Inflammatory changes and viral antigen in the lungs and brains of K18-hACE2 mice infected with SARS-CoV.** To better understand the pulmonary lesions associated with the virulent phenotype of K18-hACE2 mice, we performed histologic analysis of the lungs. At day 2 p.i., both nontransgenic and K18-hACE2 mice showed evidence of perivascular and peribronchiolar inflammation (Fig. 5C and D). We observed more widespread inflammatory cell infiltrates, increased inflammatory cell margination through vessels, more epithelial cell sloughing, and more signs of lung injury in infected K18-hACE2 mice (Fig. 5D) compared to their nontransgenic littermates (Fig. 5C). Staining for viral antigen revealed similar localization of SARS-CoV in the airway epithelia of the two groups of mice (Fig. 5E and F). By day 4 p.i., nontransgenic mice showed near-complete resolution of the pulmonary findings, with minimal evidence of inflammatory changes (Fig. 5I). In contrast, K18-hACE2 mice showed continued perivascular and peribronchial inflammation, hemorrhage, and congestion each time point. Significantly more virus was detected in line 2 lungs at day 2 p.i. than in the lungs of non-Tg mice (P < 0.005). More virus was detected in line 3 lungs at day 4 p.i. (P < 0.0004), but not at day 2 p.i., than in the lungs of non-Tg mice.

**FIG. 3.** SARS-CoV causes lethal disease in K18-hACE2 mice. (A and B) K18-hACE2 mice (lines 1 [n = 15], 2 [n = 11], and 3 [n = 15]) and 15 non-Tg mice were infected intranasally with 2.3 × 10^3 PFU of SARS-CoV and were monitored daily for mortality (A) and weight (B). (C and D) Tissues were harvested from infected mice and assayed for infectious virus as described in Materials and Methods. Virus was detected only in the brains and lungs of K18-hACE2 mice and only in the lungs of non-Tg mice. Tissues from 3 to 6 mice were analyzed at each time point. Significantly more virus was detected in line 2 lungs at day 2 p.i. than in the lungs of non-Tg mice (P < 0.02). More virus was detected in line 3 lungs at day 4 p.i. (P < 0.0004), but not at day 2 p.i., than in the lungs of non-Tg mice.

**FIG. 4.** Quantitative RT-PCR for the N gene of SARS-CoV. RNA was prepared from the lungs and brains of K18-hACE2 (line 3) and non-Tg mice at days 2 and 4 p.i. RNA levels were detected by quantitative RT-PCR as described in Materials and Methods. Viral RNA levels parallel levels of infectious virus (Fig. 1). RNAs from six mice were analyzed in all groups, except that three brains from each group were analyzed at day 2. Significantly more viral RNA was detected in K18-hACE2 lungs than in non-Tg lungs at days 2 and 4. For K18-hACE2 lungs, significantly more viral RNA was detected at 2 days p.i. than at 4 days p.i. (P < 0.005).
of alveolar septa (Fig. 5J). Staining for viral antigen was negative for both infected K18-hACE2 and non-Tg mice at day 4 p.i. (data not shown). These findings for the K18-hACE2 mouse share some features with the pulmonary lesions described for SARS patients, including modest mixed inflammatory cell infiltrates (11, 39), the detection of virus in conducting airway epithelia (11), alveolar septal thickening (39), and epithelial shedding and proliferation (7, 27). We saw no evidence of diffuse alveolar damage or acute respiratory distress syndrome, but it should be noted that patients with such findings commonly received assisted ventilation and supplemental oxygen, which complicate postmortem pulmonary findings.

FIG. 5. Pulmonary disease in SARS-CoV-infected K18-hACE2 and non-Tg mice. (A through J and L) K18-hACE2 and non-Tg mice were either left uninfected (A and B) or infected with 2.3 × 10⁶ PFU of SARS-CoV. Lungs were fixed in zinc formalin and stained with hematoxylin and eosin. Non-Tg mice showed mild perivascular and peribronchiolar inflammation in response to SARS-CoV 2 (C) and 4 (I) days following infection. K18-hACE2 mice demonstrated more-extensive disease 2 days following infection, characterized by epithelial sloughing (D, arrowheads) and more-extensive areas of mixed inflammatory cell infiltrates within and around airways, blood vessels, and the alveolar parenchyma. At day 2 p.i., viral antigen was localized to conducting airway epithelia in non-Tg (E) and K18-hACE2 (F) mice. Cells recovered from BAL specimens of infected K18-hACE2 mice (H) included macrophages with more vacuoles, consistent with activation, as well as enhanced neutrophilia and lymphocytosis compared to non-Tg mice (G). By 4 days p.i., inflammation in infected non-Tg lungs was resolving (I), while perivascular and peribronchiolar infiltrates and hemorrhage (arrowhead) were detected in K18-hACE2 mice (J). In some animals, bronchioles were completely occluded by neutrophils with marked intra-alveolar edema and without vasculitis (L), consistent with aspiration. BAL specimens were obtained from uninfected and infected K18-hACE2 and non-Tg mice and results pooled for 3 and 4 days p.i. Bars, 50 μm. (K) BAL analysis. Means (standard errors) are shown. K18-hACE2 Tg mice exhibited increased numbers of lymphocytic and neutrophilic cells in BAL specimens compared to non-Tg mice. n ≥ 6 for all conditions except for naïve non-Tg mice (n = 3). *, P < 0.05 for comparison to naïve mice. Mac, macrophages; L, lymphocytes; PMN, neutrophils.
In addition to the findings described above, patchy, intense neutrophilic infiltrates were noted in the lungs of some K18-hACE2 mice (Fig. 5L). These lesions obstructed the bronchioles with degenerate neutrophil aggregates and were associated with foci of necrotizing bronchopneumonia and alveolar flooding with seroproteinaceous fluid. In some areas, the neutrophilic inflammation was centered on foreign material (identical to esophageal contents), consistent with aspiration pneumonia (data not shown). We suspect that these aspiration events are neurogenic in nature, a consequence of pharyngeal and laryngeal dysfunction that may occur secondary to the spread of the virus to the CNS. Aspiration pneumonia has also been noted in mice models of influenza infection (33) and occasionally for patients with SARS (26).

To characterize the inflammatory cell infiltrates observed in infected lungs, we obtained bronchoalveolar lavage (BAL) specimens from K18-hACE2 and non-Tg mice as described in Materials and Methods (Fig. 5G, H, and K). Total cell numbers were increased in lavage fluid from infected K18-hACE2 and non-Tg mice, and, as with SARS-CoV-infected patients (7, 26, 27), large numbers of macrophages were recovered in BAL specimens from macrophages. Macrophages from infected K18-hACE2 mice were larger than non-Tg macrophages and contained more vacuoles and cell debris in their cytoplasm, consistent with activation (Fig. 5G and H). Of note, we detected greater numbers of lymphocytes in BAL samples obtained from K18-hACE2 mice than in those from non-Tg mice. Low levels of neutrophils were also present in infected K18-hACE2 mice; neutrophils were not detected in infected human lungs, but no tissue samples were obtained prior to 5 day p.i. in any published report.

In agreement with the high levels of virus assayed in the brains of infected K18-hACE2 mice at day 4 p.i., we also detected viral antigen in large numbers of neurons throughout the cerebrum, thalamus, and brainstem, with relative sparing of the olfactory bulb and cerebellum (Fig. 6A and C). Although cytokeratin 18 is an epithelial cell protein, K18-based expression of a LacZ reporter in cortical and brainstem neurons has been reported previously (4). Infection of the CNS was accompanied by relatively minimal meningeal and perivascular infiltration (Fig. 6B), suggesting that mice died prior to a substantial cellular host immune response in the brain. No virus antigen was detected in the brains of non-Tg mice at day 4 p.i. (Fig. 6E) or in those of any mice at day 2 p.i.

**Uprgulation of proinflammatory cytokines and chemokines in SARS-CoV-infected K18-hACE2 mice.** Elevated levels of several cytokines and chemokines, including interleukin-1 (IL-1), IL-6, IL-12, CXCL8, CXCL10, and CCL2, were detected in the serum of SARS patients and may have contributed to clinical disease (15, 38, 40, 41, 43). Similarly, levels of several proinflammatory cytokine and chemokine mRNAs, including gamma interferon (IFN-γ), CXCL9, CXCL10, CCL2, and CCL7, were elevated in the lungs of K18-hACE2 mice and, to a lesser extent, in those of non-Tg mice at 2 days p.i.; in parallel with virus levels, levels of these cytokine and chemokine mRNAs were greatly diminished by 4 days p.i. (Fig. 7A and B). Conversely, no cytokine or chemokine mRNAs were elevated in the brain at day 2 p.i., but several, most notably IL-6, IFN-γ, CCL2, and CCL12, were detected at high levels in the infected K18-hACE2 CNS at day 4 p.i. (Fig. 7C). Remarkably, no IFN-α/β mRNA was detected in infected lungs, and only low levels of IFN-β mRNA were detected in the brain, consistent with the observation that SARS-CoV does not induce type 1 IFN in fibroblasts, macrophages, or dendritic cells (3, 20, 35).

**Pretreatment of K18-hACE2 mice with a human anti-SARS-CoV MAb prevents clinical disease.** To determine whether K18-hACE2 mice will be useful for evaluating anti-SARS-CoV therapy, animals were treated, as proof of principle, with a human MAb that binds to the hACE2 receptor binding domain of the SARS-CoV surface glycoprotein (MAb 201). MAb 201 has previously been shown to diminish virus replication and the severity of pathological changes in SARS-CoV-infected mice and hamsters (10, 31). Intravenous administration of MAb 201 (25 mg/kg of body weight), but not of a control antibody, to K18-hACE2 mice 1 day prior to SARS-CoV infection completely prevented death (Fig. 8), clinical disease, and weight loss (data not shown).

**DISCUSSION**

We show here that transgenic expression of hACE2 behind an epithelial cell-specific promoter, with no other modifica-
tions of the virus or murine host, is sufficient to convert a mild infection with SARS-CoV to a lethal infection. This finding contrasts markedly with the findings of a study of mice infected with another strain of human coronavirus, HCoV-229E, in which only immunodeficient (STAT1−/−) mice transgenically expressing the human receptor developed mild disease, and even this required the use of a mouse-adapted strain of the virus (18). Our results are consistent with the notion that SARS-CoV, unlike HCoV-229E, has the ability to “jump” species, requiring adaptation to the host ACE2 receptor for enhanced virus replication. Since we could not detect ACE2 expression in novel sites in the lungs of K18-hACE2 mice, we favor the conclusion that hACE2 facilitates more efficient viral entry into, and replication in, the airways, resulting in a prolonged infection and a greater virus burden (Fig. 3C).

Like findings for infected nonhuman primates (25), our results suggest that SARS-CoV infection of K18-hACE2 mice began in airway epithelial cells. Viral antigen is detected primarily in pneumocytes and alveolar macrophages in human autopsy material (11, 26). However, this observation may reflect the difficulty of obtaining specimens from patients with SARS at early times; virus may be cleared from infected airways by the time that samples are obtained. Enhanced virus replication in airway cells results in increased inflammatory cell infiltration, more epithelial cell sloughing and proliferation (consistent with repair), and augmented cytokine and chemokine production in the lungs of K18-hACE2 mice compared to non-Tg mice (Fig. 5D and J and 7A and B).

FIG. 7. Detection of proinflammatory cytokine and chemokine mRNAs in the lungs and brains of infected K18-hACE2 and non-Tg mice. Infected K18-hACE2 (line 3) and non-Tg mice were sacrificed at day 2 p.i. (6 mice each) (A) and day 4 p.i. (6 mice each) (B and C). RNAs were prepared from lungs (A and B) and brains (C) and assayed for cytokine and chemokine mRNA levels by using an RNase protection assay as described in Materials and Methods. Data are shown as levels of RNA normalized to the level of a housekeeping gene (L32). (A and B) There were significant differences (P < 0.05) in pulmonary mRNA levels of CCL7, CCL12, CXCL10, and IL-12p35 between K18-hACE2 and non-Tg mice at day 2 (A). Differences in levels of tumor necrosis factor alpha and IL-6 were nearly significant (P < 0.06). At day 4 p.i., there was a significant difference in IL-1β levels between K18-hACE2 and non-Tg mice (B). There was a statistically significant decrease (P < 0.05) in the levels of all cytokines and chemokines when infected K18-hACE2 mice at days 2 and 4 p.i. were compared, except for CXCL10 (P = 0.06) and IL-1β (P = 0.47). IL-2, IL-4, IL-10, IL-12p40, IFN-β, IFN-α, CCL3, and CXCL2 (MIP-2) were not detected in lungs. (C) Levels of all cytokine and chemokine mRNAs in infected K18-hACE2 and non-Tg brains were indistinguishable from those of naive brains at day 2 p.i. By day 4 p.i., all cytokine and chemokine mRNA levels were statistically higher in K18-hACE2 mice than in non-Tg mice (P < 0.02). IL-2, IL-4, IL-10, IFN-α, CCL3, and CXCL2 were not detected in brains. Stippled bars, naive mice; open bars, non-Tg mice; solid bars, K18-hACE2 mice.

FIG. 8. Treatment with an anti-SARS-CoV neutralizing antibody protects K18-hACE2 mice against clinical disease. K18-hACE2 mice (line 2) received 25 mg of MAb 201 (9 mice) or a control antibody (7 mice)/kg 1 day prior to infection with 2.3 × 10⁴ PFU of SARS-CoV. Mice were monitored for survival and weight loss. All infected K18-hACE2 mice that received MAb 201 survived and exhibited no weight loss (data not shown).
One consequence of elevated cytokine and chemokine production is increased blood–brain barrier permeability, which likely facilitated widespread entry of SARS-CoV into the K18-hACE2 CNS. In agreement with this hypothesis, we could detect low levels of virus in the blood of some mice (titers, <500 TCID<sub>50</sub>/ml; two, two, and none of three mice were positive at days 1, 2, and 3 p.i., respectively). Neurotropic coronaviruses generally enter the CNS via olfactory neurons, with subsequent transneuronal spread to other sites within the brain. However, it is unlikely that SARS-CoV spread to the brain via the olfactory system, because we detected no virus in the olfactory bulb (Fig. 6A). Further, virus was detected throughout the brain, without preferential infection of sites transneuronally connected to the olfactory bulb.

Our results show that the CNS is an important target for SARS-CoV in K18-hACE2 mice, even in line 3 mice, which express very low levels of hACE2 in the brain (Fig. 2C). While the CNS is not considered a major site of infection in infected humans, SARS-CoV has been detected in the brains of infected patients (5, 11, 42). Also, some SARS survivors have neurological/psychological sequelae that are not well understood (21, 42); our results support the notion that direct virus infection contributes to the CNS dysfunction that is observed in these patients. Disease in the K18-hACE2 CNS may also be partially immunopathological, as evidenced by the high levels of proinflammatory cytokines and chemokines detected in infected brains (Fig. 7C). While patients with SARS generally die from pulmonary failure, it is likely that infection of the CNS is a major factor contributing to the fatal outcome observed for SARS-CoV-infected K18-hACE2 mice.

We anticipate that these mice will be useful for studies of pathogenesis, especially for examining the role of proinflammatory chemokines and cytokines in pulmonary and CNS disease and the basis of viremia and extrapulmonary spread. Furthermore, the lack of cellular infiltration into the CNS is unexpected, given the levels of SARS-CoV (Fig. 3D, 4, and 6), raising the possibility that the virus specifically inhibits inflammatory cell migration into this organ. In addition to their utility in studies of SARS pathogenesis, K18-hACE2 mice will also be very useful in vaccine and other therapeutic studies, especially those directed against human strains of SARS-CoV. We have shown that treatment with a fully human anti-SARS-CoV neutralizing MAb 1 day prior to infection prevented clinical disease. These results suggest that treatment of exposed persons with this antibody could be completely protective, even against severe disease.

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