

Peer Review File

Manuscript Title: COVID-19 Treatments and Pathogenesis Including Anosmia in K18-hACE2 mice

Reviewer Comments & Author Rebuttals.**Reviewer Reports on the Initial Version:**

Referee #1 (Remarks to the Author):

This manuscript by Zheng and co-workers describes the use of K18-hACE2 transgenic mice to investigate the pathogenesis of SARS-CoV-2. These investigators previously generated K18-hACE2 transgenic mice and used them to study the pathogenesis of SARS-CoV (McCray et al., 2006 and Netland et al., 2008). In their previous studies, they found that infection with 2.3×10^4 PFU of SARS-CoV resulted in lethal disease, and the mice died of neuronal death in the absence of encephalitis. Here, they show that infection with SARS-CoV-2 causes a dose-dependent disease, with lethal disease at 1×10^5 PFU, and mostly sublethal disease at 1×10^4 PFU. They detected virus in both the lung and the brain of infected mice, and documented evidence of thrombosis and vasculitis in mice with severe pneumonia. Importantly, they show that treatment with convalescent plasma prevented significant clinical disease (death). Therefore, this mouse model system is an important tool for evaluating countermeasures to SARS-CoV-2.

The most interesting and unique part of this manuscript is that the authors evaluated the mice for anosmia, the loss of the sense of smell. Humans infected with SARS-CoV-2 report anosmia, even if other symptoms are relatively mild, or sometimes patients report anosmia prior to the onset of pneumonia. Here, the authors tested the mice for their ability to find hidden food, and for male mice to detect dander from female mice in a social scent discrimination test (Fig 4). Interestingly, they found that mice infected with SARS-CoV-2 took a significantly longer time to find the hidden food, and that the male mice didn't exhibit the same level of social scent discrimination as the uninfected mice. Furthermore, this anosmia was observed in the SARS-CoV-2-infected mice treated with the convalescent plasma. By developing a mouse model system for anosmia, the authors may be able to gain insights into the specific pathogenesis of SARS-CoV-2, and learn how it may differ from the pathogenesis of SARS-CoV. Therefore, this K18-hACE2 transgenic mouse model system provides a unique tool to dissect the differences in the pathogenesis of SARS-CoV-2 and SARS-CoV in terms of upper respiratory/nasal pathogenesis. The authors highlight the importance of this transgenic mouse model system for studying the pathological underpinnings of both mild and lethal COVID-19.

Comments for the authors' consideration:

1. This is a well-written manuscript. Additional discussion of the differences in the pathogenesis of SARS-CoV versus SARS-CoV-2 in this model would enhance this manuscript. Both viruses use ACE-2 as a receptor, but there seems to be a difference in the upper airway infection. Why?
2. How does this model compare with hACE-2 transgenic mouse systems recently described by Bao et al, Nature published 7 May 2020, Jiang et al, Cell July 9, 2020 and Sun et al., Cell Host & Microbe, July 8, 2020? Discussion of the similarities and differences in these transgenic model systems would strengthen this manuscript.

Minor comments:

1. Figure 1e is very small and difficult to read.
2. Consider making a separate figure of the nasal and sinus tissues (Fig 3), as this is likely important for future studies of the pathogenesis of anosmia. Day 2 (e-j) and day 5 (k-o) are interesting, but it would be better if the pictures were organized with day 2 in one column and day 5 in an adjacent column (or row). Is there any pathology information from SARS-CoV on nasal and sinus tissues? If not, indicate that this analysis should be done in future studies.
3. Is there statistical differences in the groups in 5a and 5b?

Referee #2 (Remarks to the Author):

Perlman and colleagues report that K18-hACE2 mice are a useful model of COVID-19 including anosmia. Infection of these mice resulted in severe pneumonia and infection in the brain, as well as thrombosis and vasculitis. Convalescent plasma protected against lethal disease. The authors also argue that mice developed anosmia early after infection.

Overall this is an interesting report of an animal model that is accessible and will be useful to the field. The question is how unique is this model compared with other reports of hACE2 transgenic mouse models. A concern is that mice had substantial encephalitis, with nearly as much virus in brain as in lung on day 6, raising the question of how comparable this is with humans. Did mice die of progressive pneumonia and respiratory failure, or of encephalitis and neurologic disease?

The data on infection of olfactory epithelium is important and convincing. However, the behavioral studies on anosmia are less convincing, since there are many reasons why infected mice that are clinically ill with pneumonia and weight loss may perform less well on various tasks. There is nothing that is truly convincing for the sensation of anosmia. Textual modifications to remove specific conclusions of anosmia would be appropriate.

The studies on convalescent plasma to prevent fatal disease are important. The authors should speculate on why convalescent plasma failed to prevent "anosmia". Is blockade of virus in the olfactory epithelium a higher bar over lung? How does blockade of virus in the olfactory epithelium compare with nasal tissue?

Referee #3 (Remarks to the Author):

In this study, the authors used a K18-hACE2 mouse model developed previously by Dr Perlman for studies of SARS CoV, to investigate the pathogenesis of SARS-CoV-2. Although at high doses, they reported severe lung disease and lesions resembling some aspects reported in severe COVID-19 patients, all mice also developed infection of the brain (Fig 1b). This aspect does not appear to reflect the scenario for most patients with severe COVID-19 and should be discussed further including potentially how the virus reaches the brain if ACE2 is not expressed in the olfactory sensory neurons as stated (l.166). Do these mice develop viremia? The innovative aspects of the study include the development of the model to study anosmia and their demonstration that passive high antibody titer convalescent plasma (CP, from a recovered COVID-19 patient), if given 24 hr pre-challenge (but not 24hr post-challenge) prevented lethal infection. Also CP given 24 hr post-challenge (not tested pre-challenge?) did not prevent anosmia.

1. Multiple studies suggest that SARS-CoV-2 infects endothelial cells in humans which could contribute to pulmonary vascular endothelialitis, etc (Varga et al 2020). This model appears to be limited to infection of epithelial cells, so presumably this important aspect of COVID-19 would not be reflected in this mouse model. This should also be noted and discussed.

2. Although the authors showed that SARS-CoV-2 infects sustentacular cells that could contribute to the anosmia observed, infection of sustentacular cells was reported previously in a comprehensive study in a hamster model of SARS-CoV-2 (ref 29), so this is not an original finding. However assessment of anosmia in the mouse model and the inability of the CP given post-challenge to prevent anosmia provide new perspectives on the potential cell targets involved and the inability of passive circulating antibodies to prevent anosmia (suggesting local immunity may

be needed), respectively.

Specific comments.

3. Mice. Were male and female mice used in the study and was there any difference in their susceptibility or disease including anosmia? What were the ages of the mice used?

Were there any attempts to infect mice using aerosolized virus versus the less natural route used of IN inoculation of anesthetized mice? Importantly to control for the artificial infection protocol, did the authors examine if the infected mice could transmit virus to contacts and if the contacts also developed anosmia? How was the CP administered to the mice?

4. Abstr I 27. In Fig 1b it appears that all mice had virus detected in the brain.

I 30 Please qualify sentence ...if administered pre-challenge.

5. Disc I 210 Please qualify—"some features..."

There is no discussion of the cytokine/chemokine/ innate responses relative to those detected in COVID-19 patients and their possible role in disease in the mice.

6. Figs. For all figures where not stated, please clarify the dose of virus for each of the figs shown.

For Fig 5 in the legend, please provide the definitions for each of the tissues on the X axis

Referee #4 (Remarks to the Author):

In this manuscript, Zheng et al describe the infection of K18-hACE2 mice with SARS-CoV-2. The authors observe a series of very serious effects including lung damage, brain infection, inflammation, vasculitis, as well as clotting disorders that appear in a dose dependent manner with the amount of virus injected. These serious effects are a much better model of severe COVID-19 in humans than currently used animal models (ferret, macaque).

The authors explore further the widely reported loss of smell in COVID-19. They determine that the loss of smell happens before the invasion of the olfactory bulb by SARS-CoV-2 and might relate to damage in the olfactory epithelium. Using two behavioral measurements, they show a reduction in olfactory acuity, consistent with anosmia reported in humans.

The topic is timely and this model would be a valuable resource for interventions but also for understanding the long-term neurological consequences of SARS-CoV-2 infection in the brain.

Main concerns

1) The social scent discrimination is done only with male mice. COVID-19 has a more severe presentation in men than women, which remains unexplained. How does the sex of the K18-hACE2 mice affect the severity of their disease? The authors should divide the data in Figure 1A between male and female mice. This would help interpreting the results of the male mice data shown in figures 4B. The authors should report the percentage weight loss in male mice on 2dpi and 3 dpi.

2) For the social scent discrimination assay, the authors report the time exploring the two odors. Not surprisingly, the time exploring any odor goes down in the infected animals, probably due to malaise. However, what is relevant is the preference for the female odor over the male odor. For each animal, the authors should calculate a preference index = (female time - male time) / (female time + male time). The preference index should be larger than zero for uninfected animals. The authors then would compare the distributions of preference indexes for the uninfected animals with the 2DPI group and the 3 DPI using a t-test. This statistical test would tell us if there is a reduction in preference due to hyposmia. The authors could also compare the distributions of preference index against zero. If, for instance, the distribution at 3 DPI had a zero mean, this would suggest real anosmia. These differences should be addressed in the discussion. This task, properly analyzed, would be enough to report olfactory loss

3) The food buried task has the interpretation problem that mice are losing weight during the infections. Do 2/3 dpi animals eat less food? Could the authors provide the weights of the animals to show that they are not different than the uninfected animals? These caveats should be

addressed and discussed.

Minor concerns

- 1) What is the titer of the infection used for the animals doing the behavioral task?
- 2) Were the uninfected animals manipulated and inoculated with saline? The authors should explain the procedure better.

Author Rebuttals to Initial Comments:

Referee #1 (Remarks to the Author):

.....The most interesting and unique part of this manuscript is that the authors evaluated the mice for anosmia, the loss of the sense of smell. Humans infected with SARS-CoV-2 report anosmia, even if other symptoms are relatively mild, or sometimes patients report anosmia prior to the onset of pneumonia. Here, the authors tested the mice for their ability to find hidden food, and for male mice to detect dander from female mice in a social scent discrimination test (Fig 4). Interestingly, they found that mice infected with SARS-CoV-2 took a significantly longer time to find the hidden food, and that the male mice didn't exhibit the same level of social scent discrimination as the uninfected mice. Furthermore, this anosmia was observed in the SARS-CoV-2-infected mice treated with the convalescent plasma. By developing a mouse model system for anosmia, the authors may be able to gain insights into the specific pathogenesis of SARS-CoV-2, and learn how it may differ from the pathogenesis of SARS-CoV. Therefore, this K18-hACE2 transgenic mouse model system provides a unique tool to dissect the differences in the pathogenesis of SARS-CoV-2 and SARS-CoV in terms of upper respiratory/nasal pathogenesis. The authors highlight the importance of this transgenic mouse model system for studying the pathological underpinnings of both mild and lethal COVID-19.

Comments for the authors' consideration:

1. This is a well-written manuscript. Additional discussion of the differences in the pathogenesis of SARS-CoV versus SARS-CoV-2 in this model would enhance this manuscript. Both viruses use ACE-2 as a receptor, but there seems to be a difference in the upper airway infection. Why?

Response: We agree that this is an important question that when answered, may provide insight into the differences in transmissibility exhibited by the two viruses. We will begin these analyses shortly. Rather than speculate on why SARS-CoV and SARS-CoV-2 cause differences in pathogenesis and given space limitations, we prefer to wait to discuss this topic until our ongoing analyses are complete.

2. How does this model compare with hACE-2 transgenic mouse systems recently described by Bao et al, Nature published 7 May 2020, Jiang et al, Cell July 9, 2020 and Sun et al., Cell Host & Microbe, July 8, 2020? Discussion of the similarities and differences in these transgenic model systems would strengthen this manuscript.

Response: We agree that a comparison of the different hACE2 transgenic mice models would be very useful. However, this is difficult, given space limitations. Instead, we have noted the variability in disease that is noted in these various models (line 202-204).

Minor comments:

1. Figure 1e is very small and difficult to read.

Response: This figure is now Extended Figure Data 1d and is easier to read.

2. Consider making a separate figure of the nasal and sinus tissues (Fig 3), as this is likely important for future studies of the pathogenesis of anosmia. Day 2 (e-j) and day 5 (k-o) are interesting, but it would be better if the pictures were organized with day 2 in one column and day 5 in an adjacent column (or row). Is there any pathology information from SARS-CoV on nasal and sinus tissues? If not, indicate that this analysis should be done in future studies.

Response: As suggested by the reviewer, Figure 2 is now focused on nasal and sinus tissues. We are also interested in pathological changes after SARS-CoV-infection as, to our knowledge, this has not been studied.

3. Is there statistical differences in the groups in 5a and 5b?

Response: Statistical analyses are now included in these figures. These are Fig. 3a and Extended Data Fig. 6b, respectively, in the revised manuscript.

Referee #2 (Remarks to the Author):

.....Overall this is an interesting report of an animal model that is accessible and will be useful to the field. The question is how unique is this model compared with other reports of hACE2 transgenic mouse models. A concern is that mice had substantial encephalitis, with nearly as much virus in brain as in lung on day 6, raising the question of how comparable this is with humans. Did mice die of progressive pneumonia and respiratory failure, or of encephalitis and neurologic disease?

Response: As we noted in the text, some mice succumb to the infection even in the absence of brain involvement. Since the initial submission, we obtained additional data supporting this conclusion and added these data to Figures 1a-b. This is also evident in mice that received diluted convalescent plasma (1:9 dilution). These mice die, without evidence of brain infection (Fig. 3b). These findings are emphasized in the text (lines 71-73, 187-189).

The data on infection of olfactory epithelium is important and convincing. However, the behavioral studies on anosmia are less convincing, since there are many reasons why infected mice that are clinically ill with pneumonia and weight loss may perform less well on various tasks. There is nothing that is truly convincing for the sensation of anosmia. Textual modifications to remove specific conclusions of anosmia would be appropriate.

Response: This issue was also raised by other reviewers. Reviewer 4 suggested calculating a preference index to address this issue, since this approach will compensate for issues of malaise and decreased mobility. Preference indices are shown in Extended Data Fig. 5 and are discussed in the text (lines 173-175). We also added weight curves for the mice being studied (Fig. 2b, 2e). The preference indices showed statistically significant differences consistent with hyposmia/anosmia and the weight curves showed minimal weight loss over the duration of the experiment.

The studies on convalescent plasma to prevent fatal disease are important. The authors should speculate on why convalescent plasma failed to prevent "anosmia". Is blockade of virus in the olfactory epithelium a higher bar over lung? How does blockade of virus in the olfactory epithelium compare with nasal tissue?

Response: Our results suggest that blockade of virus in the nasal and olfactory epithelium is a higher bar than the lungs. As shown in Figure 3, neither nasal or olfactory epithelium were protected from virus infection by convalescent plasma, while virus loads in the lungs were reduced at the same time. We discuss the implications of olfactory epithelium infection on

the development of anosmia (line 216-225) but because of space limitations have not speculated on why CP failed to prevent anosmia .

Referee #3 (Remarks to the Author):

In this study, the authors used a K18-hACE2 mouse model developed previously by Dr Perlman for studies of SARS CoV, to investigate the pathogenesis of SARS-CoV-2. Although at high doses, they reported severe lung disease and lesions resembling some aspects reported in severe COVID-19 patients, all mice also developed infection of the brain (Fig 1b). This aspect does not appear to reflect the scenario for most patients with severe COVID-19 and should be discussed further including potentially how the virus reaches the brain if ACE2 is not expressed in the olfactory sensory neurons as stated (l.166). Do these mice develop viremia? The innovative aspects of the study include the development of the model to study anosmia and their demonstration that passive high antibody titer convalescent plasma (CP, from a recovered COVID-19 patient), if given 24 hr pre-challenge (but not 24hr post-challenge) prevented lethal infection. Also CP given 24 hr post-challenge (not tested pre-challenge?) did not prevent anosmia.

Response: Not all mice that succumb to the infection develop encephalitis. We have added additional data and clarified this in the revised manuscript (lines 71-73, 187-189). Other groups have reached the same conclusion (ref 11, line 68). Mice do not develop detectable viremia so while the rapidity of the overwhelming brain infection is striking and consistent with hematogenous spread via disruption of the blood brain barrier as well as spread through the olfactory bulb and its connections, further work will be required to ascertain the details. We did not determine whether CP given 24 hr post challenge prevented or decreased anosmia since CP given pre-challenge had no effect on the development of anosmia.

1. Multiple studies suggest that SARS-CoV-2 infects endothelial cells in humans which could contribute to pulmonary vascular endothelialitis, etc (Varga et al 2020). This model appears to be limited to infection of epithelial cells, so presumably this important aspect of COVID-19 would not be reflected in this mouse model. This should also be noted and discussed.

Response: While this is possibly true, we have occasionally seen thrombi in lungs, brains and other tissues (Extended Data Fig. 1c, 2a, 4c). Whether thrombi result from direct virus infection or inflammatory molecule expression in COVID-19 patients is currently not known. We chose not to discuss these issues in more detail because they require greater discussion than is possible given space limitations.

2. Although the authors showed that SARS-CoV-2 infects sustentacular cells that could contribute to the anosmia observed, infection of sustentacular cells was reported previously in a comprehensive study in a hamster model of SARS-CoV-2 (ref 29), so this is not an original finding. However assessment of anosmia in the mouse model and the inability of the CP given post-challenge to prevent anosmia provide new perspectives on the potential cell targets involved and the inability of passive circulating antibodies to prevent anosmia (suggesting local immunity may be needed), respectively.

Response: We thank the reviewer for this comment.

Specific comments.

3. Mice. Were male and female mice used in the study and was there any difference in their susceptibility or disease including anosmia? What were the ages of the mice used? Were there any attempts to infect mice using aerosolized virus versus the less natural route used of IN inoculation of anesthetized mice? Importantly to control for the artificial infection

protocol, did the authors examine if the infected mice could transmit virus to contacts and if the contacts also developed anosmia? How was the CP administered to the mice?

Response: Male and female mice were used, with no substantial differences in outcomes. Mice inoculated with 10^3 PFU survive, 2/3 female and 3/7 male mice inoculated with 10^4 PFU survive and all mice inoculated with 10^5 PFU die (lines 58-61). Mice were 6-9 weeks of age in this study. We did not attempt to deliver virus by aerosolization because we do not have this capability in our BSL3 laboratory. We have not yet performed transmission studies but plan to do so. CP was administered intravenously (line 184).

4. Abstr I 27. In Fig 1b it appears that all mice had virus detected in the brain. I 30 Please qualify sentence ...if administered pre-challenge.

Response: We studied additional mice in the revised manuscript and identified several more mice that died without evidence of brain infection. These data coupled with results from CP-treated mice show that mice succumb to the pulmonary infection even in the absence of brain infection. We modified the abstract so that it is clear that plasma was delivered pre-challenge (line 31).

*5. Disc I 210 Please qualify—"some features..."
There is no discussion of the cytokine/chemokine/ innate responses relative to those detected in COVID-19 patients and their possible role in disease in the mice.*

Response: We moved these data to Extended Data Fig. 3. We now briefly discuss the similarity of these responses to those observed in COVID-19 patients (line 95-99).

6. Figs. For all figures where not stated, please clarify the dose of virus for each of the figs shown. For Fig 5 in the legend, please provide the definitions for each of the tissues on the X axis.

Response: We used 10^5 virus in all experiments except Figure 1a. We modified the figure legends as suggested and provide the definitions for each of the tissues in the revised Figure 3c legend.

Referee #4 (Remarks to the Author):

.....The topic is timely and this model would be a valuable resource for interventions but also for understanding the long-term neurological consequences of SARS-CoV-2 infection in the brain.

Main concerns

1) The social scent discrimination is done only with male mice. COVID-19 has a more severe presentation in men than women, which remains unexplained. How does the sex of the K18-hACE2 mice affect the severity of their disease? The authors should divide the data in Figure 1A between male and female mice. This would help interpreting the results of the male mice data shown in figures 4B. The authors should report the percentage weight loss in male mice on 2dpi and 3 dpi.

Response: We are interested in sex-specific differences as well since we found substantial differences in SARS-CoV-infected male and female mice in terms of morbidity. For the purposes of this manuscript, we saw no substantial differences in male and female mice in disease severity (lines 58-61). We also added data demonstrating anosmia in infected female mice and also show weight changes over the first few days of infection for both sexes in revised Figure 2. Infected female mice developed at least as much anosmia as male mice. Mice did not lose significant amounts of weight over the course of the measurement.

2) For the social scent discrimination assay, the authors report the time exploring the two odors. Not surprisingly, the time exploring any odor goes down in the infected animals, probably due to malaise. However, what is relevant is the preference for the female odor over the male odor. For each animal, the authors should calculate a preference index = $(\text{female time} - \text{male time}) / (\text{female time} + \text{male time})$. The preference index should be larger than zero for uninfected animals. The authors then would compare the distributions of preference indexes for the uninfected animals with the 2DPI group and the 3 DPI using a t-test. This statistical test would tell us if there is a reduction in preference due to hyposmia. The authors could also compare the distributions of preference index against zero. If, for instance, the distribution at 3 DPI had a zero mean, this would suggest real anosmia. These differences should be addressed in the discussion. This task, properly analyzed, would be enough to report olfactory loss.

Response: We thank the reviewer for this suggestion and now include preferential indices in Extended Data Fig. 5 and discussed the results in the revised manuscript (line 173-177). The data support the conclusion that the mice develop hyposmia/anosmia.

3) The food buried task has the interpretation problem that mice are losing weight during the infections. Do 2/3 dpi animals eat less food? Could the authors provide the weights of the animals to show that they are not different than the uninfected animals? These caveats should be addressed and discussed.

Response: Weight loss is minimal at these days post infection (new Fig. 2b, 2e). We have added more discussion of these issues to the revised manuscript (line 170-173).

Minor concerns

1) What is the titer of the infection used for the animals doing the behavioral task?

Response: 10^5 PFU. This is noted in the legend in the revised manuscript.

2) Were the uninfected animals manipulated and inoculated with saline? The authors should explain the procedure better.

Response: The mice were inoculated with PBS (added to Fig. 2 legend and methods (lines 508, 515, 526)).

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

In this revised manuscript, the authors addressed all the concerns of the reviewers.

Referee #2 (Remarks to the Author):

The revised manuscript successfully addressed the concerns of this reviewer.

Referee #3 (Remarks to the Author):

The authors have addressed my major concerns, added additional relevant data and improved their manuscript including the figures.

A minor correction is needed for Fig 3 legend. OE should be olfactory epithelium?

Author Rebuttals to First Revision:

We thank the editor and reviewers for their comments and have responded as follows:

Referee 3 (Remarks to the Author):

The authors have addressed my major concerns, added additional relevant data and improved their manuscript including the figures.

A minor correction is needed for Fig 3 legend. OE should be olfactory epithelium?

Response: We corrected the legend.