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ORIGINAL RESEARCH PAPER

Angiotensin-(1–7) decreases inflammation and lung damage caused by betacoronavirus infection in mice

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Abstract

Objective Pro-resolving molecules, including the peptide Angiotensin-(1–7) [Ang-(1–7)], have potential adjunctive therapy for infections. Here we evaluate the actions of Ang-(1–7) in betacoronavirus infection in mice.

Methods C57BL/6J mice were infected intranasally with the murine betacoronavirus MHV-3 and K18-hACE2 mice were infected with SARS-CoV-2. Mice were treated with Ang-(1–7) (30 µg/mouse, i.p.) at 24-, 36-, and 48-hours post-infection (hpi) or at 24, 36, 48, 72, and 96 h. For lethality evaluation, one additional dose of Ang-(1–7) was given at 120 hpi. At 3- and 5-days post- infection (dpi) blood cells, inflammatory mediators, viral loads, and lung histopathology were evaluated.

Results Ang-(1–7) rescued lymphopenia in MHV-infected mice, and decreased airways leukocyte infiltration and lung damage at 3- and 5-dpi. The levels of pro-inflammatory cytokines and virus titers in lung and plasma were decreased by Ang-(1–7) during MHV infection. Ang-(1–7) improved lung function and increased survival rates in MHV-infected mice. Notably, Ang-(1–7) treatment during SARS-CoV-2 infection restored blood lymphocytes to baseline, decreased weight loss, virus titers and levels of inflammatory cytokines, resulting in improvement of pulmonary damage, clinical scores and lethality rates.

Conclusion Ang-(1–7) protected mice from lung damage and death during betacoronavirus infections by modulating inflammation, hematological parameters and enhancing viral clearance.

Keywords Covid-19 · Coronavirus · MHV-3 · SARS-CoV-2 · Angiotensin-(1–7)

Vivian V. Costa and Lirlândia P. Sousa contributed equally to this work as senior co-authors.

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Introduction

The global pandemic of coronavirus disease 2019 (COVID-19) caused by the extremely contagious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to nearly 700 million cases and 7 million deaths worldwide as of May of 2024 (https://www.worldometers.info/coronavirus/). In most cases, respiratory infection by SARS-CoV-2 are asymptomatic or cause mild respiratory symptoms. Yet, COVID-19 can cause acute respiratory distress syndrome (ARDS) and severe systemic inflammation leading to death [1]. Comprehensive studies using clinical and experimental approaches, have been employed to investigate the pathogenesis of betacoronavirus infection, which is associated with a systemic dysregulated host immune response with bystander tissue damage, increased morbidity and mortality [2]. Indeed, infection with SARS-CoV-2 can lead to compromise of alveolar structure, and local and systemic pro-inflammatory cytokines production [3, 4]. In addition, severe COVID-19 patients display a lower blood lymphocyte count compared to non-severe patients [5–7] indicating weakened immunity, and potentially worsening the prognosis [8, 9].

The main receptor for SARS-CoV-2 and other coronaviruses is the angiotensin converting enzyme 2. This enzyme is an important component of the renin-angiotensin system (RAS). More specifically, ACE-2 is a key enzyme for angiotensin-(1–7) [(Ang-(1–7)] production, a mediator involved in the RAS non-classical pathway [10] and possesses both anti-inflammatory and pro-resolving actions in the lungs [11]. The biological effects of Ang-(1–7) are mainly mediated by Mas receptor (MasR) and have been shown to antagonize the pro-inflammatory actions of Ang II [11, 12]. RAS dysregulation has been linked to inflammatory response exacerbation and development of severe acute respiratory syndrome, seen during severe pulmonary infections [13], such as influenza A virus (IAV) infection [14–16]. The impact of dysbalanced RAS responses in COVID-19 pathogenesis is yet to be explored [17–19].

Management of COVID-19 severe disease include antiviral, anticoagulant and anti-inflammatory steroids [20]. Indeed, regulation of the disbalanced inflammation evoked by SARS-CoV-2 has proved to be effective in protection of severe disease [21]. Over the years, the Ang-(1–7) hormone peptide has been associated with a number of pharmacological mechanisms in modulation of the inflammatory response by decreasing secretion of pro-inflammatory cytokine and leukocyte influx to the inflammatory sites and inducing key steps of resolution of inflammation, including apoptosis, efferocytosis and clearance of pathogen [11]. Despite the growing evidence supporting the anti-inflammatory and pro-resolving bioactions of Ang-(1–7), studies addressing the effect of the peptide during viral infection, especially in coronavirus infection in mice are lacking. Here, we have investigated the effect of Ang-(1–7) in inflammatory response triggered by murine and human betacoronaviruses and found that Ang-(1–7) modulates the inflammatory response during infection, while restores lymphopenia and decrease virus loads and lung damage, resulting in improvement of lung function and survival rates. These finds have a translational potential and reinforce clinical studies in SARS-CoV-2-infected patients suggesting that Ang-(1–7) could serve as an anti-inflammatory/pro-resolving molecule to be used as an adjunctive therapy along with antivirals in the treatment of COVID-19.

Materials and methods

Cell, virus, and plaque assay

L929 (ATCC CCL-1) and Calu-3 (ATCC HTB-55) cells were cultured at 37 °C with 5% CO_2 in high-glucose Dulbecco's Modified Eagle Medium (DMEM) for L929 cells or Minimal Essential Medium (MEM) for Calu-3 cells. Both media were supplemented with 7% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. The MHV-3 strain was provided and sequenced (GenBank accession number MW620427.1.) by Dr. Clarice Arns and Dr. Ricardo Durães-Carvalho from the State University of Campinas (UNICAMP, Brazil), and propagated in L929 cells. The SARS-CoV-2 gamma variant (P1 lineage; #EPI_ISL_1060902, hCoV-19/Brazil/AM-L70-71-CD1739/2020) was isolated from nasopharyngeal swabs of COVID-19 confirmed cases on Vero E6 cells. For viral titration, 100 µL of serially diluted virus suspensions, plasma samples, and lung and liver tissue homogenates were inoculated onto confluent monolayers of L929 cells (for MHV-3) or Calu-3 cells (for SARS-CoV-2) grown in 24-well plates. After gentle agitation for 1 h, the samples were harvested, and the culture medium was replaced by DMEM containing 1.6% carboxymethylcellulose, 2% FBS, and 1% penicillin-streptomycin-glutamine and maintained at 37ºC and 5% CO_2 for 2 days (for MHV-3) or 3 days (for SARS-CoV-2). The cells were fixed with 10% neutral-buffered formalin for 1 h and stained with 0.1% crystal violet. Viral titers were determined as plaque-forming units (PFU) expressed as Log PFU/g of tissue or Log PFU/mL of plasma.

Ethical statement and mouse models

For the experiments using MHV-3, male and female C57BL/6J mice aged six to eight weeks were obtained from the Central Animal House at UFMG and housed in the animal

facilities of the Biochemistry and Immunology Department at the same institution. In the SARS-CoV-2 experiments, we used male and female transgenic mice expressing the human ACE-2 receptor (K18-hACE2 mice from Jackson Laboratories) aged 10–12 weeks. These experiments were conducted in the Biosafety Level 3 (BSL-3) multiuser facility at the Institute of Biological Sciences, UFMG. All mice were kept under controlled conditions, with free access to food and water, at a temperature of 29–30 °C, following a 12-hour light/dark cycle, and maintained at 50–58% humidity. All the procedures performed in this study adhered to the Brazilian Guideline for the Care and Use of Animals in Teaching or Scientific Research Activities, with approval from the Animal Ethics Committees of UFMG (protocol number 159/2021, 191/2021 and CTNBio No 8.842/2023).

MHV-3 infection

Mice were anesthetized intraperitoneally with ketamine (50 mg/kg, Syntec, São Paulo, Brazil) and xylazine (5 mg/kg, Syntec). To induce a SARS-like disease, the mice received an intranasal inoculation of 10^3 PFU of MHV-3 in 30 µL of 0.9% sterile saline solution (Equiplex, São Paulo, Brazil) [22]. Control group mice were administered intranasally with the same volume of 0.9% sterile saline. If any mouse experienced a weight loss exceeding 25%, euthanasia was performed to prevent suffering. Three- and five-days post-infection, blood samples were collected from the abdominal vena cava of anesthetized mice using heparinized tubes. Euthanasia was then performed using an overdose of anesthetics (ketamine 240 mg/kg and xylazine 45 mg/kg, i.p.). Bronchoalveolar lavage fluid (BAL) was collected by instilling 1 mL of phosphate-buffered saline (PBS) through a tracheal catheter, withdrawing and re-instilling the fluid twice more. This process was repeated, and the lavages were pooled. The BAL was centrifuged (5 min, 300 × g, 4 °C), and the supernatant was collected for protein level analysis using the Bradford assay. Part of the resuspended cell pellet was used for total and differential leukocyte counting. Following the BAL collection, the lungs were harvested for subsequent viral titration, cytokine assays, and histopathological examination. To determination of survival curves mice were infected with 10^2 PFU of MHV-3 and continuously monitoring of body weight loss and clinical signs of disease for up to 10 days.

SARS-CoV-2 infection

Mice were anesthetized intraperitoneally using a mixture of ketamine (50 mg/kg, Syntec) and xylazine (5 mg/kg, Syntec) before being intranasally inoculated with 2×10^4 PFU of the SARS-CoV-2 gamma strain in 30 µL of 0.9% sterile saline solution [23, 24]. Control group mice were administered the same volume of 0.9% sterile saline intranasally. Over the next five days, mice were observed daily for changes in body weight and clinical score, encompassing ruffled fur, back arching, weight loss and activity level. Euthanasia was performed on any mice that experienced more than 25% weight loss to prevent undue suffering. Three and five days after inoculation, mice were anesthetized for blood cell collection from the vena cava. Afterwards, mice were euthanized using an overdose of anesthetics (ketamine 240 mg/kg and xylazine 45 mg/kg, i.p.). Lungs were then harvested and subjected to different analyses: routine histology, ELISA and virus titration.

Angiotensin-(1–7) treatment

Ang-(1–7) was sourced as a synthetic peptide from Bachem Inc., with its purity exceeding 99% as confirmed by high-performance liquid chromatography (HPLC). The peptide was diluted (saline + 0.02% DMSO) and administered intraperitoneally (i.p) at a dose of 30 µg/mouse, with the administration protocol varying based on the specific experiment, as detailed in the figures. The dose of Ang-(1–7) was based on previous studies in murine models of infection [25, 26]. For the 3-day post-inoculation (3dpi) treatment regimen, Ang-(1–7) administration started 24 h after MHV-3 or SARS-CoV-2 inoculation and continued at 36- and 48-hours later. In the 5-dpi treatment protocol, the peptide was administered at 24, 36, 48, 72, and 96 h post-inoculation. In survival curve studies, mice received the peptide at 24, 36, 48, 72, 96, and 120 h post-inoculation, with treatments continuing until death was observed. The vehicle group received saline + 0.02% DMSO. For SARS-CoV-2 experiments a group of infected mice was treated with Remdesivir (25 mg/kg, ip., 2x/day - GILEAD Sciences, São Paulo, Brazil), starting 6 h after virus inoculation, as a positive antiviral control group of the experiment.

Hematological evaluation

The numbers of total leucocytes, monocytes, granulocytes, lymphocytes, and circulating platelets were determined in blood samples using the Celltac MEK-6500 K hemocytometer (Nihon Kohden, Indaiatuba, São Paulo, Brazil).

BAL protein measurement

To evaluate edema formation and lung tissue damage, protein concentration BAL was quantified using the Bradford assay (Bio-Rad, Hercules, California, USA). In this procedure, the working reagent was diluted fivefold and incubated

with BSA standards or BAL samples. Following incubation at room temperature, absorbance was measured at 595 nm.

Cytokine assay

Lung homogenates were prepared by homogenizing lung samples in cold cytokine extraction buffer, containing 100 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM Triton X-100, 1% sodium deoxycholate, 0.5% NP-40, and a protease inhibitor cocktail (1%). The homogenate was centrifuged at 3,000× g for 10 min at 4 °C, and the supernatant was collected for further analysis. The concentrations of TNF, IFN-γ, IL-10, IL-6, CCL2, and CXCL1 in the lung homogenate supernatant and in plasma samples were measured using mouse ELISA kits (DuoSet ELISA System, R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Histopathology

Lung samples were fixed in 4% neutral buffered formalin for 48 h, followed by dehydration in ethanol and embedding in paraffin. Sections of 5 µm thickness were cut and stained with hematoxylin and eosin (H&E) for examination under light microscopy. A pathologist, blinded to the experimental groups, performed a histopathological assessment based on the criteria described by [22]. The evaluation included scoring of airway (0–4), vascular (0–4) and parenchymal inflammation (0–5), and polymorphonuclear (PMN) infiltrate (0–5), culminating in a total possible score of 18 points.

Immunohistochemistry

Paraffin-embedded MHV-3 lung samples were sectioned (5 µm thickness) and used for detecting viral double strand RNA (dsRNA) by immunohistochemistry. Sections were dewaxed, hydrated and submitted to antigen retrieval with boiling (95ºC) EDTA solution (EDTA 0,37 M, pH 8,0) for 20 min. The endogenous peroxidase activity was blocked with a 0.3% hydrogen peroxide solution, and then a protein block solution (Abcam ab64226) was used to inhibit non-specific reactions. Slides were then treated with Fc-blocking reagents from the Mouse-on-Mouse kit (Vector labs, California, USA), and incubated with the primary anti-dsRNA antibody (1:50, Merck MABE1134, Clone rJ2) overnight. Then, the secondary antibody (biotinylated goat anti-mouse IgG from the Mouse-on-Mouse kit) was applied for 30 min, followed by avidin-peroxidase treatment. The reaction was visualized using a DAB chromogenic solution and counterstained with hematoxylin.

Assessment of pulmonary function in MHV-3 infected mice

To evaluate lung function in mice infected with MHV-3, invasive forced spirometry was utilized. Mice were anesthetized with ketamine and xylazine and then tracheostomized. They were placed in a body plethysmograph and connected to a computer-controlled ventilator (Forced Pulmonary Maneuver System; Buxco Research Systems, Wilmington, NC, USA). This setup allowed for the measurement of various pulmonary parameters under mechanical ventilation, including flow respiratory, forced Expiratory volume at 20 milliseconds and lung resistance (Rl), as determined by resistance and compliance tests. To ensure the accuracy of the results, suboptimal maneuvers were discarded. Each mouse underwent at least three acceptable maneuvers for each test, providing reliable mean values for all the numerical parameters.

Calu-3 and A549 cells infection

The alveolar epithelial cells Calu-3 (Banco de Células do Rio de Janeiro- BCRJ) and A549-expressing human ACE-2 (BEI Resources), were infected with SARS-CoV-2 (Delta variant) at (multiplicity of infection) MOI of 1.0. Cells (5×10^4 cells/well in 96-well) were pre-treated by 30 min with Ang-(1–7) at different concentrations (1 and 10 µM) and then infected. Plates were incubated at 37 °C for adsorption. After 1 h, cell monolayers were washed, supernatant removed and 100 µl of DMEM supplemented or not with Ang-(1–7) was re-added to the wells. The control groups included uninfected cells treated with vehicle (saline + 0.02% DMSO) or with Ang-(1–7). After 24 h, the supernatants were collected, and viable virus titers determined via plaque assay in permissive Vero cells. Results are expressed as Log PFU/mL of supernatant. MTT assay was performed on the cell monolayer to assess cell metabolic activity. Cytokines were measured in cell free supernatants.

Statistical analysis

All results are presented as mean ± SEM. Data were analyzed by one-way ANOVA and differences between groups were assessed by Tukey post-test. When two groups were evaluated, the Student's *t* test was used. The log-rank test was used for the statistical analysis of survival curves. Results with $p < 0.05$ were considered statistically significant. Calculations were performed using Prism 8.0 software for Windows (GraphPad Software, San Diego, CA).

Results

Ang-(1–7) modulates inflammation and rescued lymphopenia induced by MHV-infection

To evaluate the therapeutic effect of Ang-(1–7) during coronavirus infection we first employed a model of murine coronavirus (MHV-3) infection [22] that resembles relevant clinical aspects of severe COVID-19, including lymphopenia and features of macrophage activation syndrome and cytokine storm [22]. Thus, mice were treated intraperitoneally as shown in (Fig. 1a), and euthanized at 3 dpi, the peak of lung disease in the model [22]. Intranasal infection of mice with MHV-3 led to leukopenia (Fig. 1b), primarily due to a decreased number of lymphocytes (Fig. 1c) in blood of infected mice. Of interest, Ang-(1–7) treatment restored leukocyte blood numbers, by increasing lymphocytes and granulocyte counts in the blood at 3 dpi (Fig. 1d). Thrombocytopenia was a feature of the infection and Ang-(1–7) did not modify the blood platelets counts in infected mice at this timepoint evaluated (Fig. 1e). Notably, treatment with Ang-(1–7) reduced leukocyte infiltration to the alveoli of infected mice (Fig. 1f-h), which was mainly characterized by monocytes/macrophages (Fig. 1g). Neutrophils were barely detected in the alveolar space of MHV-infected mice at 3 dpi (Fig. 1i). Akin to the anti-inflammatory effect of Ang-(1–7) in other models of viral infection [14], treatment of MHV-infected mice decrease the lung levels IL-6, TNF, CXCL-1 and IFN-γ (Fig. 1j). The levels of IL-10 and CCL2 were not changed by the infection at this time point (Fig. 1j).

To test whether serial administration of Ang-(1–7) could affect systemic signs seen at 5-day post-MHV infection, we applied a longer therapeutical protocol (scheme in Fig. 2a). In keeping with our previous findings, longer Ang-(1–7) treatment restored blood leukocytes/lymphocytes (Fig. 2b-c) and increased neutrophils (Fig. 2d) numbers. Notably, longer Ang-(1–7) treatment partially restored the virus-induced thrombocytopenia (Fig. 2e), decreased the numbers of monocytes/macrophages numbers into the airways (Fig. 2f-h). Neutrophils were barely detected in BAL (Fig. 2i). Ang-(1–7) decreased the lung and systemic levels of inflammatory cytokines/chemokines (Fig. 2j-k). Of note, treatment of mice with the peptide Ang-(1–7) alone did not influence blood and BAL counts (dotted lines in the graphs). Collectively, these results demonstrate that treatment with Ang-(1–7) modulates the local and systemic inflammatory response and rescued lymphopenia and thrombocytopenia throughout the MHV infection in mice.

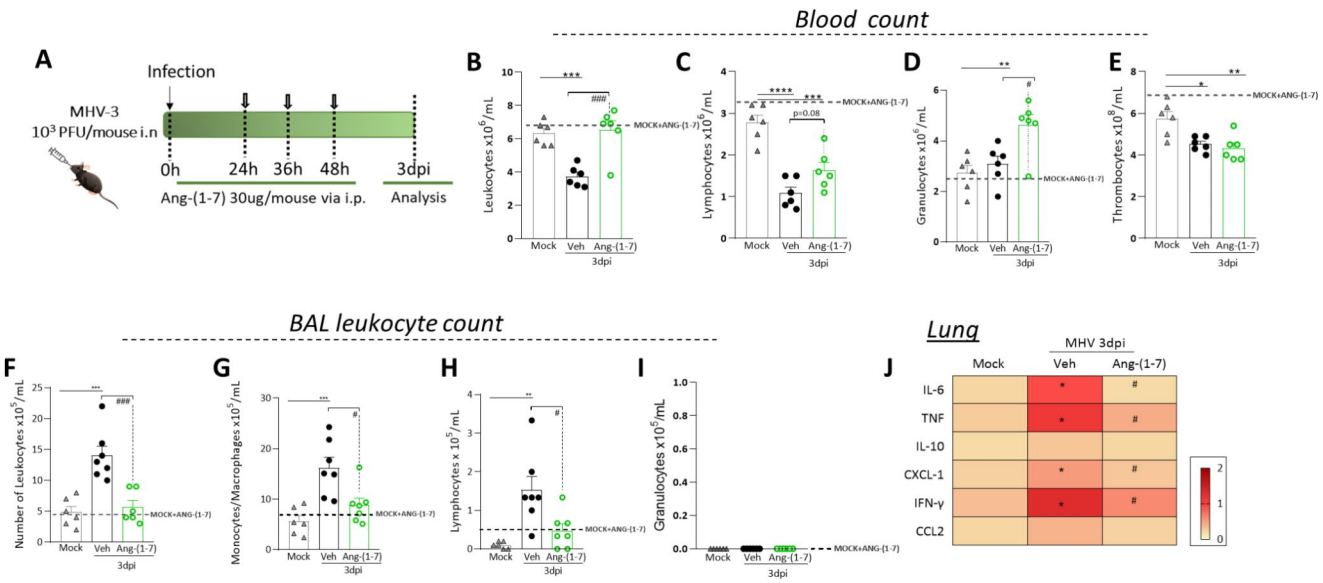

Fig. 1 Effect of Ang-(1–7) treatment in features of MHV-induced infection. **A** C57BL/6J mice were intranasally infected with MHV-3 (1×10^3 PFU/animal), followed by Ang-(1–7) treatments; **B** Blood counts of total leukocytes; **C** Lymphocytes; **D** Granulocytes and **E** Thrombocytes. BAL were harvested and evaluated for total and differential counting. **F** Total leukocytes; **G** Monocytes/macrophages; **H** Lymphocytes and **I** Granulocytes. **J** Heatmap of levels of cytokines and chemokines measured by enzyme-linked immunosorbent assay (ELISA) in lungs of mock controls, vehicle-treated MHV-3-infected mice, and Ang-(1–7)-treated MHV-3-infected mice. Differences between infection groups and the mock control were assessed by one-way ANOVA plus Tukey multiple-comparison test (SEM; $n = 6$–7). For $^{\#/*}$, $p < 0.05$; $^{\#\#/**}$, $p < 0.01$; $^{\#\#\#/***}$, $p < 0.001$; $^{\#\#\#\#/****}$, $p < 0.0001$ when compared to the mock group (*) or when compared vehicle group (#). The average of Ang-(1–7)-only treated group is presented as a dotted line in graphs

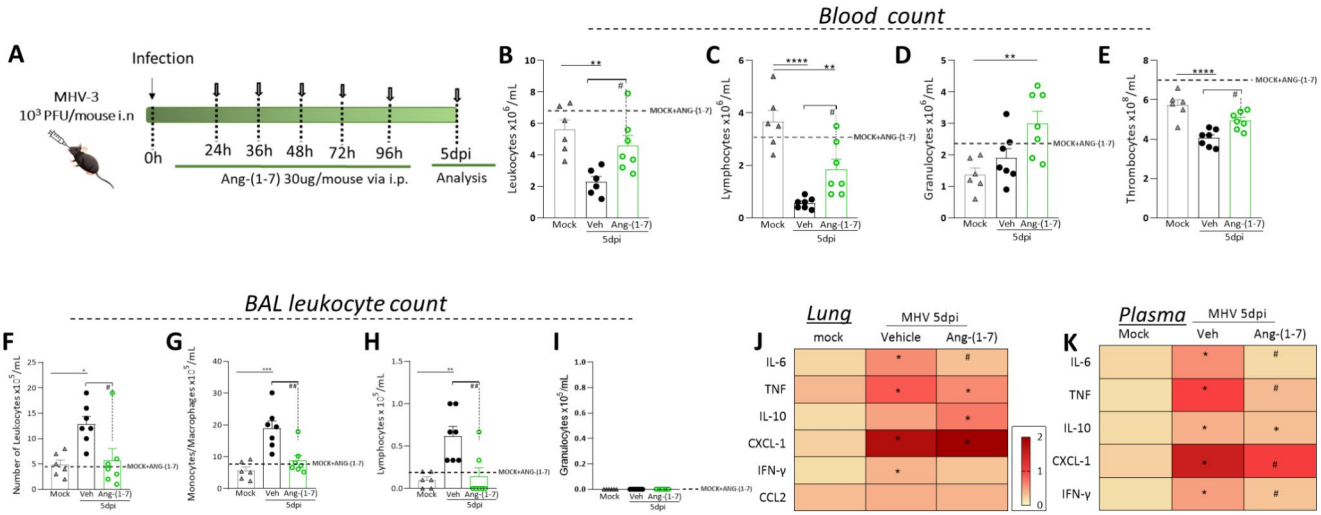

Fig. 2 Effect of delayed Ang-(1–7) treatment in features of MHV-induced infection. **A** C57BL/6J mice were intranasally infected with MHV-3 (1×10^3 PFU/animal), followed by Ang-(1–7) treatments. **B** Blood counts of total leukocytes; **C** Lymphocytes; **D** Granulocytes and **E** Thrombocytes. BAL were harvested and evaluated for total and differential counting. **F** Total leukocyte; **G** Monocytes/macrophages, **H** Lymphocytes and **I** Granulocytes. Heatmap of levels of cytokines and chemokines measured by enzyme-linked immunosorbent assay (ELISA) in **K** Lungs and **L** Plasma of mock controls, vehicle-treated MHV-3-infected mice, and Ang-(1–7)-treated MHV-3-infected mice. Differences between infection groups and the mock control were assessed by one-way ANOVA plus Tukey multiple-comparison test (SEM; $n = 6$–7). For $^{\#/*}$, $p < 0.05$; $^{\#\#/**}$, $p < 0.01$; $^{\#\#\#/***}$, $p < 0.001$; $^{\#\#\#\#/****}$, $p < 0.0001$ when compared to the mock group (*) or when compared to the vehicle group (#). Symbols inside the heatmaps represents statical difference ($p < 0.05$) in relation to the mock (*) or vehicle (#) group. The average of Ang-(1–7)-only treated group is presented as a dotted line in graphs

Ang-(1–7) decreases viral load and damage/mechanical dysfunction in lungs of MHV-3 infected mice

Next, we addressed whether Ang-(1–7) treatment could affect viral load in MHV-infected mice. Viral double-strand RNA (dsRNA) was detected by immunostaining of lung sections at 3 dpi (Fig. 3a). immunostaining of uninfected (Mock) lung sections was used as control. Importantly, in lung slices from Ang-(1–7)-treated mice the staining was lower (Fig. 3a). In agreement with the immunohistochemistry analysis, plaque-forming unit (PFU) assay showed reduced viral titers in the lungs, liver, and plasma of Ang-(1–7)-treated mice at 3 dpi and 5 dpi (Fig. 3b-e) when compared to vehicle-treated infected-animals.

Histopathological scores of lungs of infected mice evaluated at 3 dpi showed pronounced tissue damage that was significantly reduced after Ang-(1–7) treatment (Fig. 4a-b). In addition, total protein levels in BAL, an indirect measurement of edema, were reduced after Ang-(1–7) treatment compared to the vehicle group (Fig. 4c). Moreover, Ang-(1–7) improved lung function of infected mice as evaluated by flow parameters, forced expiratory volume, and pulmonary resistance (Fig. 4d-f). In summary, these data suggest that Ang-(1–7) treatment reduced viral loads while protecting mice from inflammation-related lung damage and dysfunction caused by MHV-infection.

Ang-(1–7) decreases lethality of MHV-infected mice

Given the protective effect of Ang-(1–7) during MHV infection, we questioned if Ang-(1–7)-mediated immunomodulation and viral control could result in improvement of lethality in mice. To test that, infected mice were treated for 5 days with Ang-(1–7) (scheme in Fig. 5a) and were monitored daily for weight loss and lethality. Infected animals began to lose weight progressively after 2–3 dpi (Fig. 5b) and succumbed to the infection between 6 and 8 dpi (Fig. 5d). Treatment with Ang-(1–7) prevented the weight loss observed from the 5 dpi (Fig. 5c). Of note, treated animals exhibited progressive weight gain until the end of the experimental analysis. Notably, Ang-(1–7) partially rescued mice from MHV-induced lethality (100% *versus* 40%, vehicle to Ang-(1–7) respectively - Fig. 5d).

Ang-(1–7) treatment improves the outcome of SARS-CoV-2 infection in mice

Next, we caried out experiments with SARS-CoV-2-infected mice in BSL-3 conditions. To that, transgenic mice expressing the human angiotensin I-converting enzyme 2 (ACE2) receptor (K18-hACE2 mice) were infected with SARS-CoV-2 and further treated with Ang-(1–7) (scheme in Fig. 6a). As for the MHV infection, blood lymphocyte counts were significantly reduced with SARS-CoV-2 infection (Fig. 6b). Ang-(1–7) treatment promoted a modest

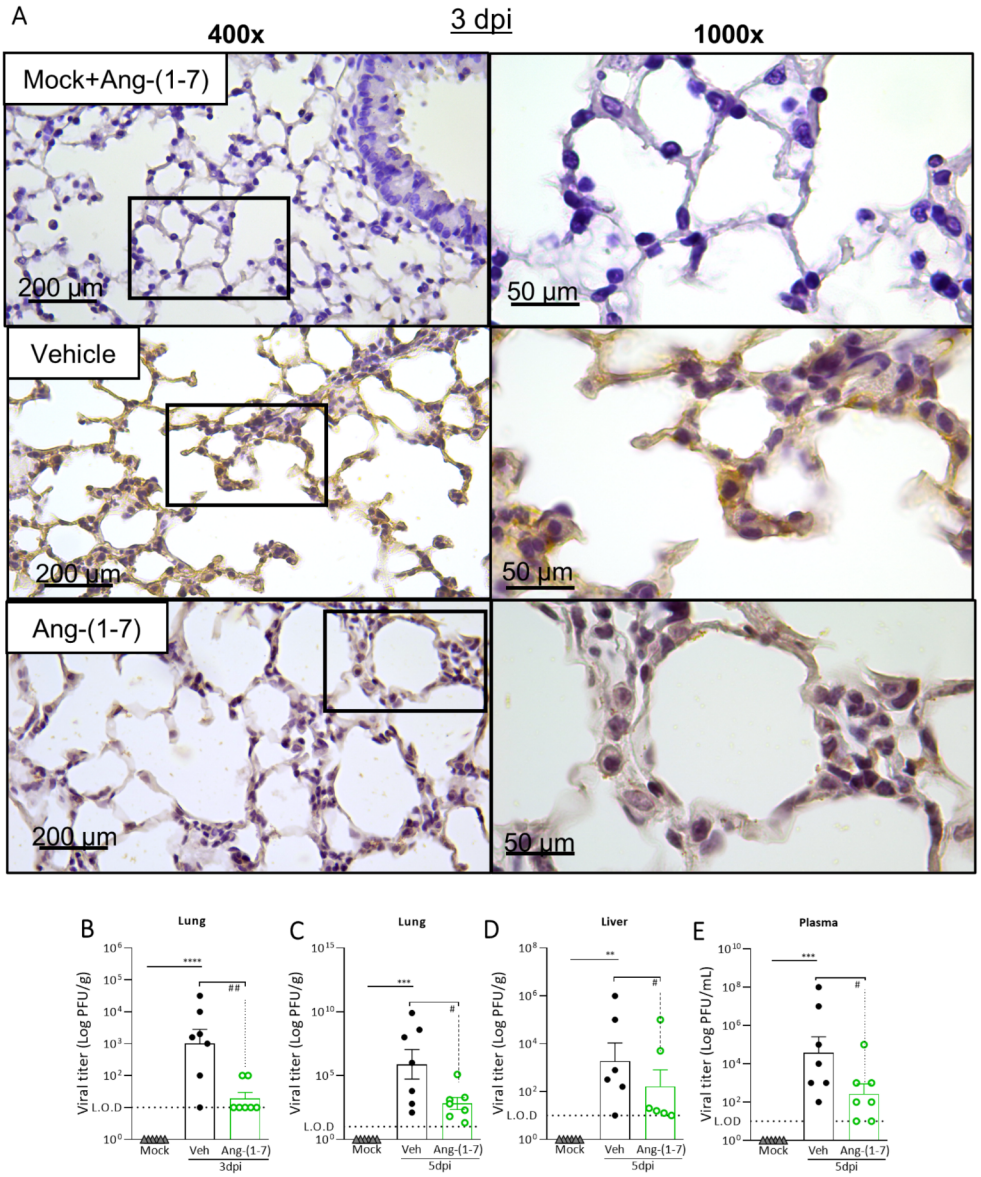

Fig. 3 Impact of the Ang-(1–7) treatment in viral loads of MHV-infected mice. C57BL/6J mice were infected with MHV-3 (1×10^3 PFU/animal), followed by Ang-(1–7) treatments. Mice were euthanized at 3 dpi or 5 dpi and samples were collected for virus detection. Immunohistochemistry (IHC) analysis of viral double-stranded RNA (dsRNA) assessed at 3 dpi. **A** Scale = 200 µm (lower magnification) and 50 µm (higher magnification) at 3 dpi; **B-E** Viral load determined in organs (lung and liver) and plasma of MHV-3-infected mice by plaque assay. Differences between infection groups and the mock control were assessed by one-way ANOVA plus Tukey multiple-comparison test (SEM; $n = 6–7$). LOD: limit of detection. For $^{\#/*}$, $p < 0.05$; $^{\#\#/**}$, $p < 0.01$; $^{\#\#\#/***}$, $p < 0.001$; $^{\#\#\#\#/****}$, $p < 0.0001$ when compared to the mock group (*) or when compared to the vehicle group (#). LOD, limit of detection

recovery in blood lymphocyte, starting at 3 dpi (Fig. 6b) and continued treatment led to a significant recovery of lymphocytes in the blood at 5 dpi (Fig. 6b). In agreement with previous studies [22], SARS-CoV-2 infection in K18-hACE2 mice led to intense weight loss of mice (Fig. 6c). Notably, treatment with Ang-(1–7) promoted significant recovery of the weight of infected mice on days 4–5 after infection compared to the vehicle-treated group (Fig. 6c-d). In addition, SARS-CoV-2 infected mice treated with Ang-(1–7) showed improved clinical scores at 5 dpi (Fig. 6e). Importantly, viral loads in the lungs were significantly reduced at 3 dpi after Ang-(1–7) treatment, and they were even lowers at 5 dpi, but with similar titters between vehicle and Ang-(1–7), as evidenced by plaque-forming assays (Fig. 6f). Of importance, no direct antiviral effect was observed after treating SARS-CoV-2-infected lung epithelial cells Calu-3 or A549 with Ang-(1–7) (supplementary Fig. 1). Lung levels of IL-6, TNF, CXCL1 and IFN-γ were significantly reduced in Ang-(1–7)-treated mice in comparison to the vehicle-treated mice (Fig. 6g). The IL-10 levels were not significantly changed by the infection at this time point (Fig. 6g). In addition, Ang-(1–7) significantly decreased the levels of IL-8 in A549 cells infected with SARS-CoV-2 (Fig. 6h). Although IL-6 levels seem to decrease in Ang-(1–7)-treated cells, it did not reach statistic significance (Fig. 6h).

Lungs histopathological analysis of SARS-CoV-2 infected mice showed tissue damage that was decreased by Ang-(1–7) treatment (Fig. 7a-b). Considering that Ang-(1–7) treatment improved several features of inflammation and improved pulmonary pathology, we next investigated whether Ang-(1–7) treatment could influence survival of SARS-CoV-2-infected mice. For this purpose, 24 h-infected

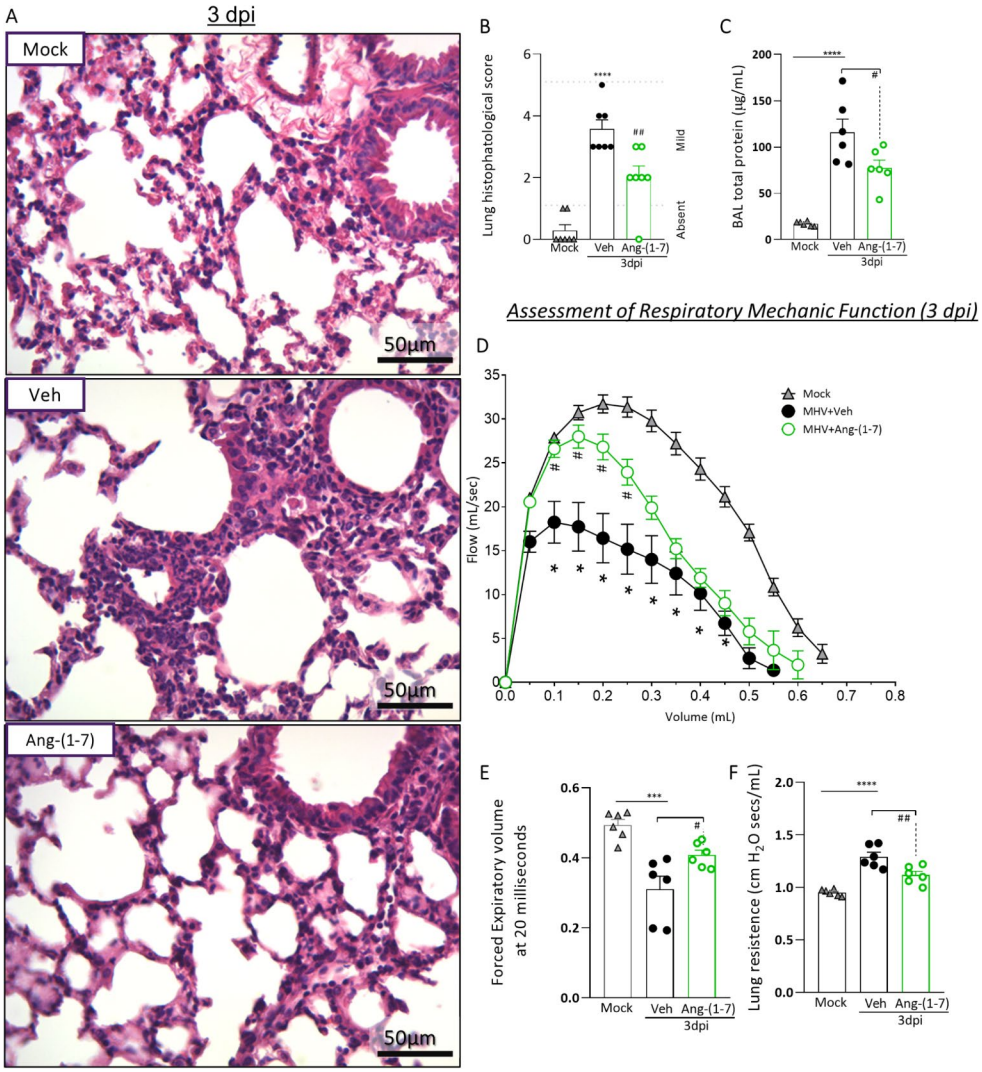

Fig. 4 Impact of Ang-(1–7) treatment on MHV-3-induced lung damage. **A** C57BL/6J mice were infected with MHV-3 (1×10^3 PFU/animal/intranasally), treated with Ang-(1–7) or vehicle at 24, 36 and 48 h hpi with Ang-(1–7) (30 µg intraperitoneally) and euthanized at 72 hpi for lung mechanics and histopathological analysis. Hematoxylin and eosin (H&E) staining of lung sections; Bars, 50 µm (high magnification) assessed at 3 dpi; **B** The histopathological score evaluated airway, vascular and parenchymal inflammation, neutrophilic infiltration, and epithelial lesion. **C** Total protein in BALF; **D-F** Assessment of respiratory mechanic function by analyses of flow, forced expiratory volume, and resistance at 3 dpi. Differences between infection groups and the mock control were assessed by one-way ANOVA plus Tukey multiple-comparison test (SEM; $n = 6–7$). For $^{\#/*}$, $p < 0.05$; $^{\#\#/**}$, $p < 0.01$; $^{\#\#\#/***}$, $p < 0.001$; $^{\#\#\#\#/****}$, $p < 0.0001$ when compared to the mock group (*) or when compared to the vehicle group (#)

mice were treated twice a day during 5 days and the survival rates were evaluated. All vehicle treated mice died by day 7, while treatment with Ang-(1–7) resulted in 22% of mice surviving (Fig. 7c). Of Note, Remdesivir treatment recued 38% of mice from virus-induced lethality (Fig. 7c). Taken together, our results indicate that treatment with Ang-(1–7) protects mice against SARS-CoV-2 by taming inflammation and damage at the same time it reduces viral titers resulting in improved survival of mice.

Discussion

RAS activation results in the production of angiotensin II, which upon binding to AT1 receptor evoke effects as vasoconstriction, inflammation, and oxidative stress [10]. Conversely, angiotensin-converting enzyme (ACE)-2 counteracts these effects by degrading angiotensin II into Ang-(1–7), which, upon activation of the Mas receptor, elicits anti-inflammatory, antioxidative, vasodilatory, and pro-resolving responses [11, 12, 14, 26]. Early in COVID-19 pandemic several reviews claimed a putative benefic effect of Ang-(1–7) [27–29], given the known actions of this peptide. However, while higher systemic levels of Ang II were consistently documented in COVID-19 patients [30, 31] compared to healthy donors, divergent findings of Ang-(1–7) measurement were reported in severely ill hospitalized COVID-19 patients, with some exhibiting elevated Ang-(1–7) concentrations while others show decreased levels [32, 33]. In addition, two randomized clinical trials using synthetic Ang-(1–7) (TXA-127) and an angiotensin II type 1 receptor-biased ligand (TRV-027) showed no clinical benefit for patients with severe COVID-19 [34], as initially hypothesized. Nevertheless, some concerns regarding the administration protocol have been raised by experts in the field [35]. In this regard, recent results from a clinical trial using a Mas-receptor activation by 20-hydroxyecdysone (BIO101) has shown positive results in severe COVID-19

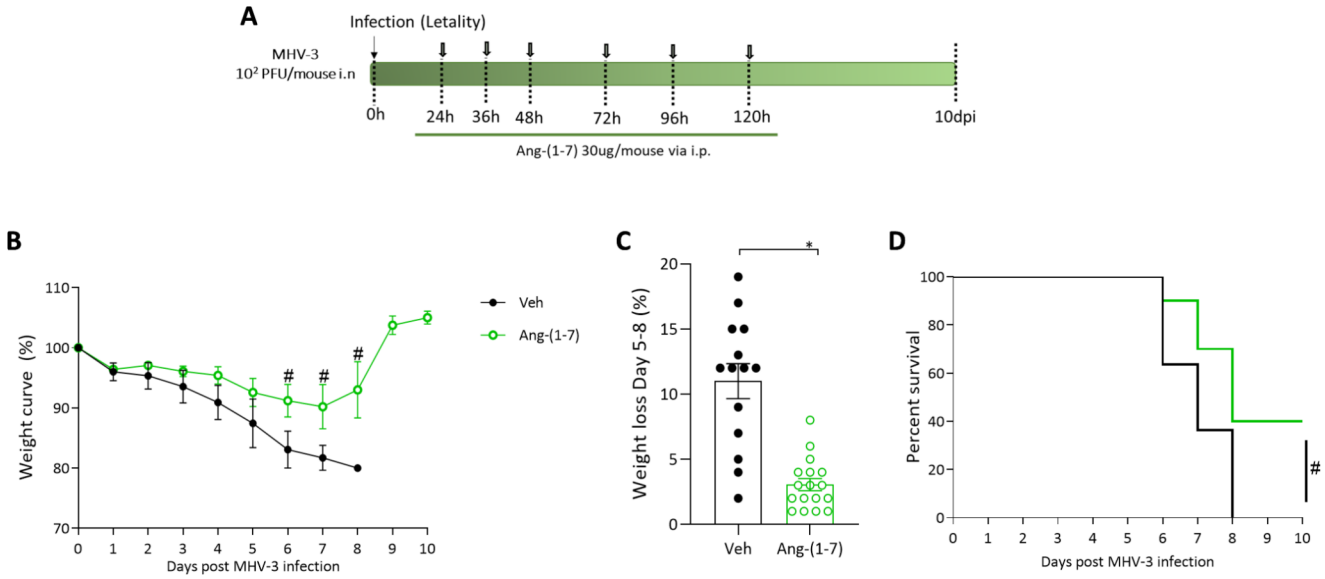

Fig. 5 Ang-(1–7) rescue mice from MHV-induced lethality. **A** C57BL/6J mice were intranasally infected with MHV-3 (1×10^2 PFUs/animal), followed by Ang-(1–7) treatments. **B** Percentage of weight of infected mice treated or not with Ang-(1–7); **C** Weight Loss in polled mice from Day 5–8 in % **C**; **D** Survival curve of infected mice treated or not with Ang-(1–7). Survival rates were monitored for 10 days and those animals that reached the criteria were humane euthanasia ($n = 10$) by group. The difference between the vehicle and treated groups is indicated in the graphs by # for $p < 0.05$ or the p-value by t-test. Log-rank test was used to compare survival curves

by significantly reducing the risk of death or respiratory failure and supporting the use of MasR activators/agonists during disease [36]. The protective bioactions of the ACE2/Ang (1–7)/MasR axis in pre-clinical models of lung injury, including IAV [13–16] and the lack of specific pre-clinical date regarding the role Ang-(1–7) in coronavirus diseases, emphasize the urgent need to understand the effects of Ang-(1–7) during betacoronavirus infection for better planning of novel clinical studies in humans. Here, we have investigated the effect of the delayed administration of Ang-(1–7) on the immunopathology evoked by two betacoronavirus, MHV-3 and SARS-CoV-2, in mice. Importantly, this pro-resolving peptide afforded significant protection in infected mice by taming the inflammatory response, restoring lymphopenia, and decreasing viral loads and lung damage. Notably, Ang-(1–7) improved lung function and partially rescued mice from morbidity and lethality.

MHV-3 is a betacoronavirus that infects mice and can be used in BSL-2 safety conditions. Infection with MHV-3 caused severe acute respiratory syndrome in C57BL/6J mice with efficient viral replication in the lungs, tissue damage associated with inflammation, and compromised respiratory function [22]. SARS-CoV-2 triggered similar host inflammatory responses, characterized by an overabundance of pro-inflammatory cytokines, leading to the infiltration of immune cells into both the bronchioalveolar space and lung interstitial compartment [1–3]. These events contribute to lung tissue damage, ultimately leading to reduced lung compliance, and impaired lung function [37]. In this context, mice infected with MHV-3 exhibited elevated edema and heightened infiltration of inflammatory cells to the airways, along with hyperplasia and disruption of tissue architecture, characteristics associated with acute respiratory distress syndrome [22]. In the present study, the treatment with Ang-(1–7) improved clinical parameters and protected mice from death, resulting in higher percentage of survival in Ang-(1–7)-treated mice. Mechanistically, Ang-(1–7) treatment decreased monocyte/macrophage infiltration to the airways coupled to decreased levels of cytokines, viral RNA and PFUs in the lungs of MHV-infected mice compared to vehicle-treated group. The effects of Ang-(1–7) taming inflammation and promoting viral clearance were associated with improvement of lung function and respiratory mechanics, in agreement with the data obtained from Ang-(1–7)-treated IAV-infected mice [14].

In addition to its beneficial role on lung inflammation and function, Ang-(1–7) reduced virus titers in plasma, when evaluated at the time point of viremia (5dpi) in this model [22]. Therefore, our findings suggest that administration of Ang-(1–7) is protective during coronavirus infection by reducing inflammation without causing immunosuppression. Of importance, these pro-resolving effects are also observed for Ang-(1–7) in the settings of bacterial infection [26, 38]. Ang-(1–7) treatment rescued the number of leukocytes in MHV-infected mice in the blood, and thus maintaining the host's ability to fight infections [11]. Here, no direct effect of Ang-(1–7) in virus replication in human airway epithelial cell lines were observed. However, we found

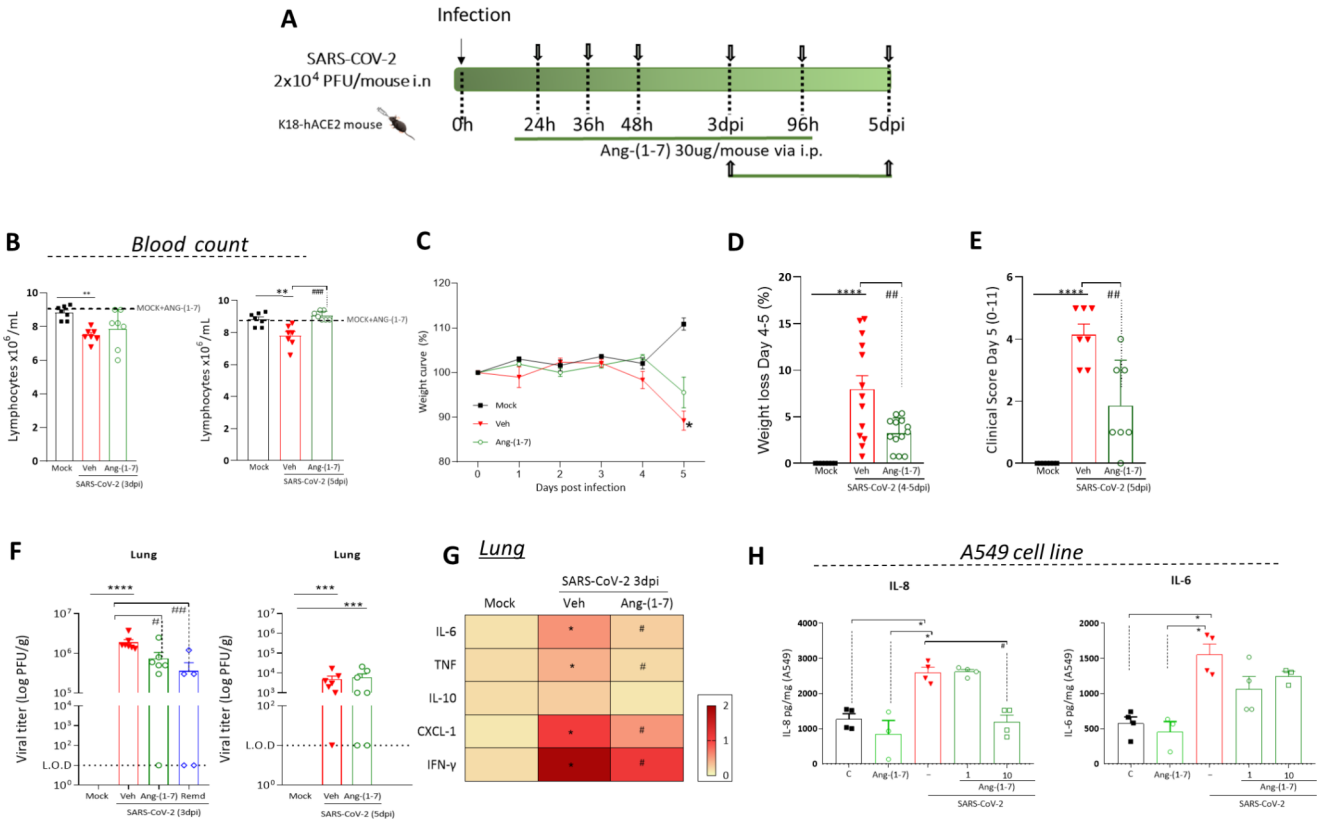

Fig. 6 Ang-(1–7) is protective in a model of SARS-CoV-2 infection. **A** hK18ACE2 Mice infected with (2×10^4 PFUs/animal i.n), followed by Ang-(1–7) treatments. **B** Lymphocytes 3dpi and 5dpi; **C** % body weight curve of infected mice treated or not with Ang-(1–7); **D** Weight loss in polled mice from day 4–5 in %; **E** Clinical score (day 5); **F** Viral load, and **G** cytokines/chemokines in the lungs of Mock, vehicle-treated SARS-COV-2 infected mice, and Ang-(1–7)-treated SARS-CoV-2 infected mice; **H** Cytokine in cell free supernatants of A549 cells infected with SARS-CoV-2 and treated with Ang-(1–7). Differences between infection groups and the mock control were assessed by one-way ANOVA plus Tukey multiple-comparison test (SEM; $n = 5$–7). For $^{\#/*}$, $p < 0.05$; $^{\#\#/**}$, $p < 0.01$; $^{\#\#\#/***}$, $p < 0.001$; $^{\#\#\#\#/****}$, $p < 0.0001$ when compared to the mock group (*) or when compared to the vehicle group (#). LOD, limit of detection. The average of Ang-(1–7)-only treated group is presented as a dotted line in graphs

that Ang-(1–7) decreased inflammatory cytokine release in these in vitro settings, suggesting that the reduction of virus titers in vivo is most probably associated to overall better control of the inflammatory response, and enhancing host resilience to infection. Of note, Ang-(1–7) inhibits the phosphorylation of JAK/STAT proteins [39], which are essential regulators of local and systemic response to viral infections [40]. Indeed, JAK inhibitors such as baricitinib has antiviral properties against SARS-CoV-2 infection [41] suggesting that adjunctive anti-viral and anti-inflammatory/pro-resolving based strategies as potential therapies in the settings of infectious diseases [11, 12]. Whether Ang-(1–7) is regulating the JAK/STAT pathway and contributing for the observed effect in viral load remain to be investigated. Further studies will fully establish the role of Ang-(1–7) in promoting pathogen control during coronavirus infection.

Transgenic mice expressing human ACE2 (K18-hACE2) are highly susceptible to SARS-CoV-2 infection [1]. In these animals, the infection results in intense production of cytokines locally and systemically, increased weight loss, leukopenia, and sustained viral replication in the nasal turbinates, lungs, and brains resulting in a viral sepsis-like disease [2]. Similarly to the results obtained during MHV-3 infection, significant improvements were observed after Ang-(1–7) treatment in SARS-CoV-2 infection. Ang-(1–7) notably attenuated weight loss, rescue lymphopenia reduced lung inflammation/injury and viral loads, and improved the overall clinical score when compared with the vehicle-treated mice. All this Ang-(1–7) actions led to a better outcome in SARS-CoV-2-infected mice and improving survival.

The role of the ACE2/Ang-(1–7)/MasR axis in balancing actions that attenuate inflammation, oxidative stress, apoptosis, and fibrosis are well described [10]. Of interest, the membrane-bound ACE2 was shown to serve as a viral receptor for SARS-CoV-2 and other coronaviruses in the host cells. After virus binding and adsorption, ACE2 is internalized and degraded [18]. Thus, the enzymatic production of Ang-(1–7) decreases, preventing inflammation resolution and perpetuating tissue damage and coagulopathy, features of severe COVID-19 [42]. In this regard, administration

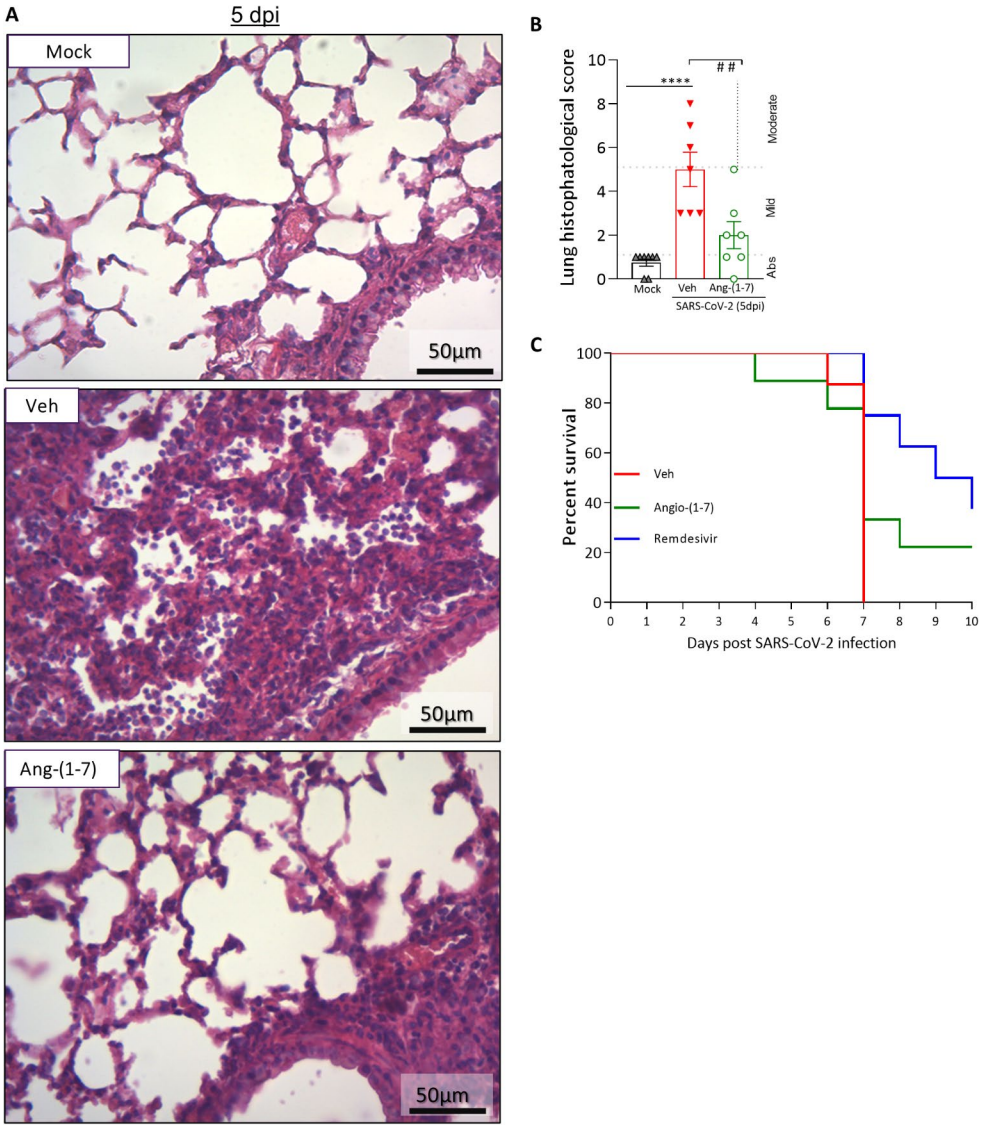

Fig. 7 Effect of Ang-(1–7) treatment on lung damage triggered by SARS-CoV-2. hK18ACE2 Mice infected with (2×10^4 PFUs/animal i.n., $n = 7$), were euthanized after 120 h for lung histological analysis. **A** Hematoxylin and eosin (H&E) staining of lung sections assessed at 5 dpi; Bars, 50 µm (high magnification); **B** The histopathological score evaluated airway, vascular and parenchymal inflammation, neutrophilic infiltration, and epithelial lesion; **C** Survival curve of infected mice treated or not with Ang-(1–7) or remdesivir. For $^{\#/*}$, $p < 0.05$; $^{\#\#/**}$, $p < 0.01$; $^{\#\#\#/***}$, $p < 0.001$; $^{\#\#\#\#/****}$, $p < 0.0001$ when compared to the mock group (*) or when compared to the vehicle group (#)

of an ACE2 recombinant protein is effective in protecting mice from ALI induced by the systemic injection of SARS-CoV-2 spike protein [43].

Increased levels of pro-inflammatory cytokines in serum have been associated with pulmonary inflammation and severe lung damage in patients with COVID-19 [6, 44]. Of note, SARS-CoV-2 infection leads to an over activation of macrophages with exacerbated secretion of pro-inflammatory mediators and associated damage [45]. Here, high levels of pro-inflammatory cytokines/chemokine such as IL-6, TNF, CXCL1, and IFN-γ were found in the plasma and lungs of coronavirus infected animals, associated to increased infiltration of macrophages. Ang-(1–7) reduced infiltration of these leukocytes to the airways, tamed the production of pro-inflammatory cytokines and prevented lung damage and dysfunction. Similarly, we have shown that in a pre-clinical model of IAV infection [14] and allergic inflammation [46], Ang-1-7 reduced cytokine production that was associated with the attenuation of the overall lung damage. In addition, Ang-(1–7) shifts macrophage phenotype towards anti-inflammatory and pro-resolving profiles [38, 47]. Of note, Ang-(1–7) decreased the release of IL-8 by human alveolar epithelial cells infected with SARS-CoV-2, in agreement with previous findings using the spike protein of SARS-CoV-2 as stimuli [48]. These in vitro data obtained in human cells, reinforce our in vivo data in betacoronavirus-infected mice, in which these cytokines are increased during infection and decreased in lungs and plasma following Ang-(1–7) treatment, demonstrating the important translational potential of our findings.

We have shown that Ang-(1–7) induces IL-10 release in vitro by macrophages [38]. Herein, we detected a significant increase of IL-10 levels only in plasma at 5 days post-MHV infection, with no further changes following

Ang-(1–7) treatment. In keeping with that, IAV-infection in mice lead to pro-inflammatory cytokines production in BAL that are modulated by Ang-(1–7) treatment, without significant changes in IL-10 levels [14]. In addition, Ang-(1–7) showed no effect in IL-10 levels in polymicrobial sepsis in rats [49] and sheep [50], while it reduced pro-inflammatory cytokines levels. On the other hand, treatment with Ang-(1–7) increased lung levels of IL-10 and other markers of resolution in a model of allergy [46]. One can argue that the modulatory effect of Ang-(1–7) in IL-10 production seems to be dependent on the pre-clinical model studied.

Lymphocyte count is a biomarker for monitoring the severity and mortality of COVID-19 disease [51]. Indeed, a notable reduction in T lymphocytes is positively associated with in-hospital mortality and the severity of illness [9]. In our study, we have observed reduced leukocyte levels in the peripheral blood of animals infected with coronavirus, alongside heightened number of these cells in bronchoalveolar lavage. This suggests an accumulation of inflammatory cells in lung tissue, indicative of leukocyte trafficking from the bloodstream to the viral-infected respiratory tract [52]. Our findings demonstrate that treatment with Ang-(1–7) regulates leukocyte numbers in the airways and attenuates lymphopenia induced by coronavirus infection. Curiously, analog drugs of Ang-(1–7) are widely recognized for their capacity to stimulate hematopoiesis and accelerate the replenishment of circulating cells [53]. Thus, Ang-(1–7) could be acting in bone marrow to restore lymphopenia caused by coronavirus infection [54, 55]. Yet, further studies will clarify the associated mechanism.

In conclusion, this study offers evidence supporting Ang-(1–7) as a crucial regulator of the immune response during coronavirus infection. Our findings demonstrate for the first time that Ang-(1–7) regulates both local and systemic immune responses, mitigates pulmonary inflammation, improves clinical outcomes, reduces viral load, and enhances survival in mice infected with MHV-3 or SARS-CoV-2. Altogether, these findings support the hypothesis that Ang-(1–7) could serve as an interesting therapeutic strategy for critical illnesses such as COVID-19 [56], and reinforce the need for new clinical trial using Ang-(1–7) based peptides or other MasR agonists.

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Author contributions L.P.S., and E.B.S.L. analyzed data and wrote the paper. E.B.S.L., A.F.S.C, I.Z., A.H.A.M., C.C, E.S.L., F.S.C., L.C.O, F.R., F.R.S.S., L.P.S.C, C.M.Q-J., performed the experiments and analyzed data. I.M.C., performed in vitro analyses. R.A.S.S. provided expertise. R.C.R. performed the lung mechanical assessment. M.M.T., V.V.C., L.P.S and L.P.T conception and design, analysis, and interpretation of data, drafting, editing and revising manuscript and project funding. All authors approved the final version of the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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