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# Relevance of angiotensin-(1-7) and its receptor Mas in pneumonia caused by influenza virus and post-influenza pneumococcal infection

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ABSTRACT

Resolution failure of exacerbated inflammation triggered by Influenza A virus (IAV) prevents return of pulmonary homeostasis and survival, especially when associated with secondary pneumococcal infection. Therapeutic strategies based on pro-resolving molecules have great potential against acute inflammatory diseases. Angiotensin-(1–7) [Ang-(1–7)] is a pro-resolving mediator that acts on its Mas receptor (MasR) to promote resolution of inflammation. We investigated the effects of Ang-(1–7) and the role of MasR in the context of primary IAV infection and secondary pneumococcal infection and evaluated pulmonary inflammation, virus titers and bacteria counts, and pulmonary damage. Therapeutic treatment with Ang-(1–7) decreased neutrophil recruitment, lung injury, viral load and morbidity after a primary IAV infection. Ang-(1–7) induced apoptosis of neutrophils and efferocytosis of these cells by alveolar macrophages, but had no direct effect on IAV replication *in vitro*. MasR-deficient ($MasR^{-/-}$) mice were highly susceptible to IAV infection, displaying uncontrolled inflammation, increased viral load and greater lethality rate, as compared to WT animals. Ang-(1–7) was not protective in $MasR^{-/-}$ mice. Interestingly, Ang-(1–7) given during a sublethal dose of IAV infection greatly reduced morbidity associated with a subsequent *S. pneumoniae* infection, as seen by decrease in the magnitude of neutrophil influx, number of bacteria in the blood leading to a lower lethality. Altogether, these results show that Ang-(1–7) is highly protective against severe primary IAV infection and protects against secondary bacterial infection of the lung. These effects are MasR-dependent. Mediators of resolution of inflammation, such as Ang-(1–7), should be considered for the treatment of pulmonary viral infections.

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## 1. Introduction

Influenza A virus (IAV) infections are important public health threat leading to substantial numbers of hospitalizations. Seasonal or pandemic influenza can be associated with bacterial coinfections that contribute significantly to the increase of morbidity and death [1]. Indeed, approximately 25 % of deaths associated with influenza are due to second bacterial infection. *S. pneumoniae* is the main cause of community-acquired pneumonia [2] and one of the most common infecting bacteria following Influenza virus infection [3,4].

Inflammation triggered by IAV or pneumococcal infections is necessary for viral and bacterial clearance but must be tightly regulated to prevent lung injury, viral replication and bacterial dissemination [5,6]. Indeed, infection caused by either Influenza virus or *S. pneumoniae* may result in compromise of alveolar structure, local and systemic production of cytokines and vigorous recruitment of neutrophils into the airways [7,8]. Combined infections result in even more serious pulmonary injury when compared with single infection by either of these pathogens [9]. It is believed that modulation of inflammation during infection with these pathogens may decrease pulmonary inflammatory response and injury, hence being useful in the adjunctive treatment of severe pneumonia [5,6].

The production of pro-resolving molecules during the inflammatory process is necessary to resolve inflammation adequately and facilitate the return to homeostasis [10]. In addition, it has been suggested that these molecules may be useful in the context of lung infection by decreasing lung inflammation and injury and by facilitating immune responses against certain infections [10]. Angiotensin-(1–7) [Ang-(1–7)] is a biologically active heptapeptide of the Renin-Angiotensin System (RAS) known for its anti-inflammatory and anti-fibrotic effect in different acute and chronic models of inflammatory disease, including asthma [11], dyslipidemia [12], renal injury [13,14], arthritis [15] and lung fibrosis [16]. Ang-(1–7) plays a pro-resolving role by inducing monocyte recruitment, promoting apoptosis and efferocytosis of neutrophils and leading to tissue repair [17,18]. The major pathway for Ang-(1–7) formation is *via* ACE2 [19,20] and most of its actions are mediated through binding to its Mas receptor (MasR) [21]. Here, we studied the relevance of Ang-(1–7) and its receptor in the context of IAV infection.

## 2. Material and methods

### 2.1. Study design, ethical statement and mice

This study was randomized, blinded and controlled by vehicle during the experimental and analysis stages. All experimental procedures described here were conducted according to Brazilian guidelines on animal work and approved by the local Animal Ethics Committee from Universidade Federal de Minas Gerais (CETEA - UFMG - protocol number 381/2015). Wild type (WT) and MasR knockout ($MasR^{-/-}$) mice (male C57BL/6; 8–11 weeks old) were maintained in a controlled environment with free access to filtered water and standard laboratory chow *ad libitum*.

### 2.2. Influenza A virus and S. Pneumoniae infection

Influenza A virus (IAV) strain A/WSN/33 H1N1 (WSN/33) was produced in chicken eggs and then cultured in MDCK cells as described previously [22]. WT and $MasR^{-/-}$ mice were anesthetized with 10 mg/kg of xylazine plus 70 mg/kg of ketamine and received $10^4$ PFU for the moderate infection (lethal dose, 50 %) or $5 \times 10^2$ PFU for the mild infection (sublethal dose) of IAV WSN/33, as previously described [23]. For pneumococcal infections, WT mice were anesthetized by inhaling isoflurane [3–5 % inhalation and 1–2 % maintenance (dose mg / kg)]; and immediately received $10^3$ CFU of *Streptococcus pneumoniae* to mild infection (sublethal dose). At day 16, mice were euthanized to access inflammation and bacterial loads. The inoculum was prepared in sterile phosphate buffered saline (PBS) or just PBS for Mock group, 30 or 40 μL intranasally. Mice survival was monitored for 21 days after infection.

### 2.3. Angiotensin-(1–7) treatment

Ang-(1–7) oral formulation was produced as previously described [24]. The oligosaccharide (hydroxypropyl-β-cyclodextrin – HPβCD) protects Ang-(1–7) from inactivation by digestive tract enzymes [25]. The duration of the administration the Ang-(1–7) / HPβCD formulation varied according to experiment, but always starting 3 days after infection and given twice a day, every 12 h. In lethality experiments, mice were treated from day 3 to day 10 or till death was noticed. For experiments evaluating cytokines and inflammation, the formulation was given from day 3 till day 5 or 7, as shown in each experiment. In experiments to evaluate the effects of the formulation on secondary pneumococcal infection, mice were infected with a low inoculum of IAV ($5 \times 10^2$ PFU), treated from days 3–10 (8 days of treatment) and infected with *S. pneumoniae* on day 14. The start of treatment on day 3 was based on the window of treatment opportunity for oseltamivir [26,27] and also on the period of inflammatory response and viral load already observed in studies by our and other research groups [22,28]. For oral administration, the infected groups received vehicle (92 μg/Kg of HPβCD in filtered water, 100 μl, by gavage) and Ang-(1–7) group received Ang-(1–7) / HPβCD [60 μg/Kg of Ang-(1–7) and 92 μg/Kg of HPβCD in distilled water, 100 μl, by gavage]. Mock group received 100 μl of filtered water. The dose used was based on previous studies using murine models of asthma [18], arthritis [17], emphysema [29] and pulmonary fibrosis [16].

### 2.4. Western blot analysis

For *in vitro* infection, human pulmonary adenocarcinoma Cell line (A549) and Madin Darby Canine Kidney Cell line (MDCK) were maintained at 37 °C and 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum (Gibco) $3 \times 10^5$ cells / well were seeded and incubated for 24 h. To analyze viral proteins by Western blot, A549 cells were infected with IAV WSN/33 (MOI 5) and treated with medium or Ang-(1–7) 1 μM/well. The time points were collected 2, 4, 6, and 8 h post infection. To analyze viral titrations, A549 cells were infected with IAV WSN/33 (MOI 0.1), adsorbed for 30 min and then treated with medium or Angiotensin-(1–7) 30, 100 or 300 nM/well. Time points were collected 8, 24 and 48 h after infection and samples were analyzed by plaque assay in MDCK cells.

To evaluate the expression of MasR, we collected, weighed and homogenized 30 mg of lung tissue in 1 mL of lysis solution. The protein content of the lysate was determined by Bradford assay (Bio-Rad). Extracts (70 μg) were separated by electrophoresis on 10 % SDS-PAGE and electro transferred to nitrocellulose membranes. Membranes were blocked overnight at 4 °C with PBS containing 5% (w/v) nonfat dry milk and 0.1 % Tween-20, washed with PBS containing 0.1 % Tween-20, and then incubated with anti-rabbit to Mas1L (1:500 – Abcam), anti-mouse β-actin (1:3000) and secondary anti-mouse or anti-rabbit peroxidase conjugate antibody (1:3000).

### 2.5. Bronchoalveolar lavage fluid (BALF) and total/differential cell count

BALF was performed and tissues taken for further analysis. For the BALF, two aliquots of 1 mL of PBS were flushed three times into the lungs to collect leukocytes of the airways of mice. Then, BALF samples (2 mL each) were centrifuged at 600 x g for 10 min at 4 °C. Total number of leukocytes was determined by counting leukocytes in a modified Neubauer chamber. Differential counts were obtained from cytospin (Shandon III) preparations to evaluate the percentage of each leukocyte on a slide stained with May–Grunwald–Giemsa, based on morphological

criteria. Each slide was counted three times and the percentage was used to calculate the absolute number of each leukocyte type.

### 2.6. Assessment of neutrophil apoptosis

Apoptosis was assessed morphologically, as previously described [30,31]. Briefly, cells in BALF were collected from mice on day 5 and 7 post moderate IAV infection. Leukocytes were cytocentrifuged, fixed and stained with May-Grünwald-Giemsa and counted using oil immersion microscopy (x100 objective) to determine the proportion of neutrophils with distinctive apoptotic morphology (cell retraction, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies out or inside macrophages). At least 500 cells were counted per slide and results are expressed as the median of percentage of cells with apoptotic morphology.

Assessment of apoptosis was also performed by flow cytometry using FITC-labeled annexin-V and 7AAD as an index of loss of nuclear membrane integrity (FITC Annexin V Apoptosis Detection Kit, BD Pharmingen™, US).

### 2.7. Flow cytometry

Cells present in the BALF were staining with fluorescent antibodies against F4/80 (25−4801-82, PEcy7, eBioscience, USA), and Ly6 G (562737, BV421, BD Biosciences). After being stained for surface markers, cells were stained with annexin-V and 7AAD according to manufacturer's instructions (556547, FITC Annexin V Apoptosis Detection Kit, BD Pharmingen™, US). Stained cells were acquired in BD FACSCanto II cell analyzer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

### 2.8. Plaque assay

To determine viral load in lungs, we collected, weighted and homogenized 100 mg of lung tissue in 1 mL of sterile PBS. Plaque assay was performed as previously described [22]. For a viral adsorption, samples were diluted and incubated for 1 h on the MDCK cell monolayer. Then, 1% of agarose in DMEM was added to the cells followed by an incubation of 72 h. Cells were fixed and stained with violet crystal for plaque counts. The virus titer was expressed as number of plaque-forming units (PFU) per gram of lung tissue (PFU/100 mg of lung).

### 2.9. Lung MPO and NE

After performing BALF, lungs were perfused with 5 mL of PBS to remove circulating blood and frozen. The right lung of mice was collected for indirect quantification of neutrophils recruitment into the tissue (MPO – myeloperoxidase assay). As previously described [32], 50 mg of lung tissue were homogenized in a buffered solution containing antiproteases. MPO levels were performed using 25 µl of the supernatant of the homogenized and 25 µL of a solution of 1.6 mM of 3, 39−5,59-tetramethylbenzidine (TMB; Sigma—dissolved in dimethyl sulfoxide) and 0.01 mM of $H_2O_2$, diluted in phosphate buffer (pH 5.4) containing HTAB. The homogenate supernatant (50 µl) was also used to measure neutrophilic elastase activity. To this end, the supernantant was incubated with 50 µl substrate MeO-Suc-AA-Pro-Val-pNA (M4765 - Sigma) in a 96-well microplate at 37 °C for 1 h, as previously described [33].

### 2.10. Histological analysis

The left lungs of mice were formalin-fixed, dehydrated gradually in ethanol and processed as described [22]. 4 µm sections of the tissue were stained with Hematoxylin & Eosin and analyzed by a pathologist blinded to the experiment. The scoring method used here has been described in detail elsewhere [22]. Briefly, a score of 18 points evaluated airway inflammation (up to 4 points), vascular inflammation (up to 4 points), parenchymal inflammation (up to 5 points) and general neutrophil infiltration in the lungs (up to 5 points).

### 2.11. Phagocytosis assay

Neutrophils were differentiated from cell line progenitors by culturing them with differentiation medium (Optimem 1% Penicillin/Streptomycin, 10 % FCS, 30 µM β-Mercaptoethanol, 500 µL SN SCF) for 4 days. For inducing apoptosis, neutrophils were incubated with 25 µM Roscovitin for 12 h and then stained with 5 µM CellTrackerTM Red CMTPX (C34552, Thermo-Fisher). Alveolar macrophages were harvested by bronchoalveolar lavage (BALF) from mice and incubated in proliferation medium (RPMI, 10 % FBS, 1% Glutamine, 1%Penicillin/Streptomycin, 1% Pyruvate, 5 ng/mL of mouse GM-CSF) for 7 days. Alveolar macrophages were treated with 300 nM of Ang-(1–7) (A9202, Sigma-Aldrich) during 24 h to then perform the phagocytosis assay by adding apoptotic red-stained neutrophils and incubating in co-culture for 15 and 30 min. Alveolar macrophages were stained with FITC anti-mouse CD11c (553801, BD Pharmingen™, US) diluted 1:200 and slides were mounted with mounting medium containing DAPI. Several random images were taken from each experiment and time point. With ImageJ, quantification was performed by counting the apoptotic neutrophils that were adhered and phagocytosed by macrophages.

### 2.12. Measurement of cytokines and chemokines

BALF supernatants were used to assess concentrations of cytokines and chemokines (IL-10, IFN-γ, CXCL1, and CCL5) measurements by ELISA DuoSet kits according to the manufacturer's instructions (R&D Systems, USA).

## 3. Results

### 3.1. Administration of Angiotensin-(1–7) prevents lethality and controls inflammation after IAV infection

Initial experiments evaluated the effects of Angiotensin-(1–7) treatment, an agonist of the receptor Mas, in IAV-induced pneumonia. To this end, wild type (WT) mice were infected with an inoculum of IAV that corresponds to 1 LD50 ($LD_{50}$ = 50 % of lethality – $10^4$ PFU) intranasally on day 0 and then treated with Ang-(1–7) from the third to tenth day after IAV infection and then followed for 21 days (Fig. 1A). Ang-(1–7) treatment of IAV-infected mice decreased lethality from 82 % to 53 % in vehicle-treated mice (Fig. 1B) and both surviving groups recovered weight loss (Fig. 1C). Moreover, to better understand the therapeutic effects of Ang-(1–7), animals given Ang-(1–7) or vehicle were culled and tissues examined at 3, 5 and 7 days after infection. Treatment with Ang-(1–7) decreased the total number of leukocytes (Fig. 1D) and neutrophils (Fig. 1F) in the airways. There was no difference in the number of mononuclear cells recruited when compared to Ang-(1–7) and vehicle groups (Fig. 1E), even when we differentiate the lymphocyte and macrophage count (data not shown). On the fifth day post infection, Ang-(1–7) treatment was associated with decreased levels of CXCL1 and IFN-γ in BALF (Fig. 1G,H). No difference in levels of CCL5 and IL-10 was detected in the time points evaluated. Consistent with the lower neutrophil infiltrate and CXCL1 levels into the airways, mice treated with Ang-(1–7) showed less myeloperoxidase (MPO) activity in the lungs (Fig. 1I). There was an increase in neutrophil elastase activity, a surrogate marker of NET formation [34,35], after infection, but this was not altered by treatment with Ang-(1–7) (Supplementary Fig. 1A). Interestingly, although inflammatory responses tended to decrease in treated mice, the viral titer in the lungs was decreased during viral pneumonia (Fig. 1J). Ang-(1–7) treatment had no direct antiviral effect, since it was not able to decrease virus replication in A549 cells

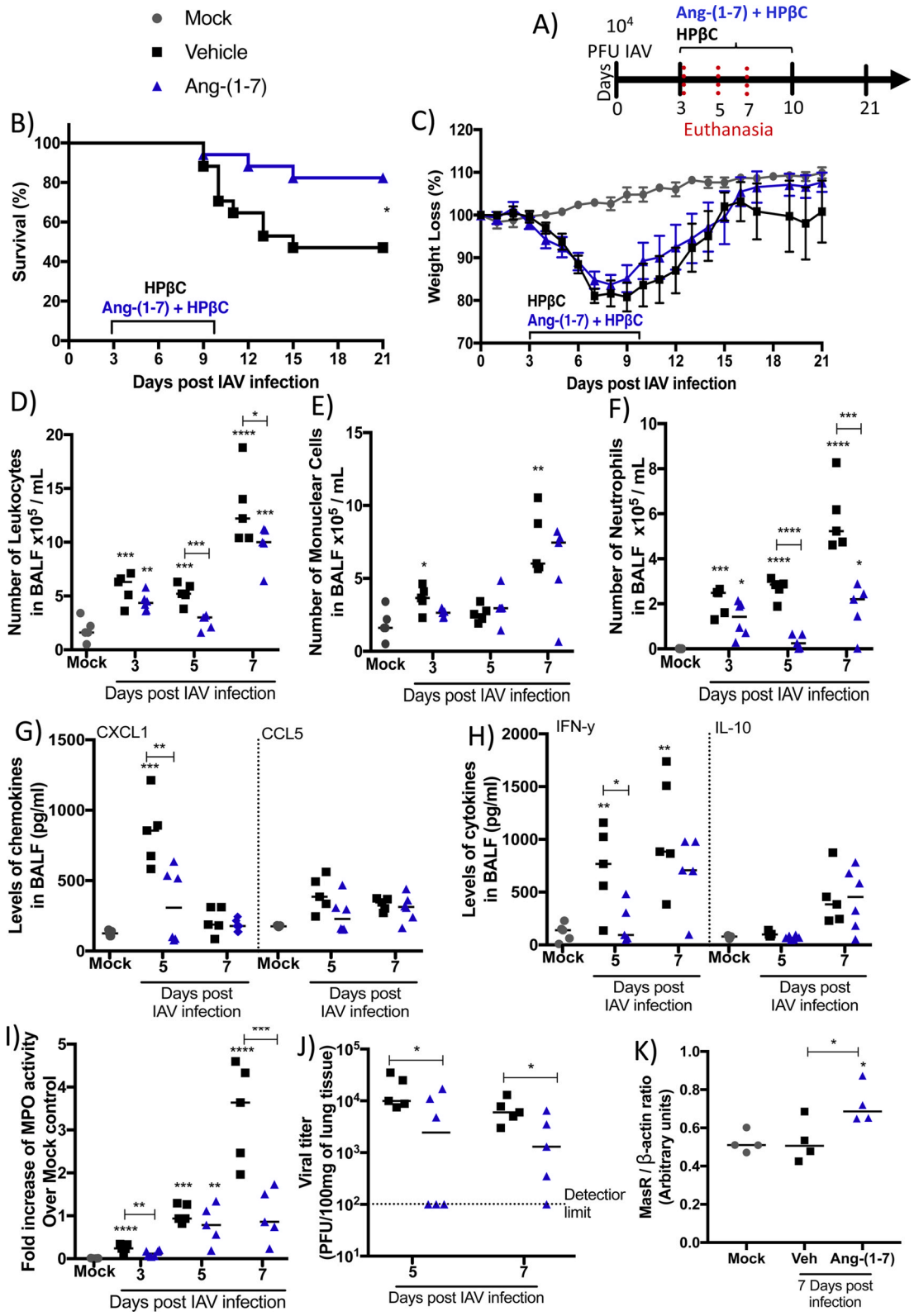


**Fig. 1. Angiotensin-(1-7) protects from lethality and controls inflammation during IAV pneumonia.** (A) WT mice were infected intranasally with $10^4$ PFU and treated twice a day, from day three to seven post-infection, with Vehicle (92 μg/Kg of HPβCD) or Angiotensin-(1-7) [60 μg/Kg of Ang-(1-7) plus 92 μg/Kg of HPβCD], twice a day by gavage. (B—C) Survival and weight loss were monitored daily (* for $p < 0.05$ by Log Rank test, $n = 16$ mice). On days 3, 5 and 7 post-IAV, BALF from infected mice was evaluated for (D) total leukocyte, (E) mononuclear cell and (F) neutrophil numbers. (G—H) Levels of CXCL1, CCL5, IFN-γ and IL-10 were also evaluated in BALF. Lungs from infected mice were used for (I) MPO analysis showed by fold increase, (J) viral loads and (K) MasR expression by western blot. PBS instilled mice (Mock) was used as controls. Data are presented as Median, $n = 5$-6 of three independent-experiments. *, ** and *** for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, when compared to Mock or indicated groups by one-way ANOVA.

(Supplementary Fig. S2A) and there was no change in the production of viral proteins after infection (Supplementary Fig. S2B). In addition, there was an increase in the expression of MasR in the lung tissue after IAV infection (Fig. 1K). These results suggest that Ang-(1–7) improves viral pneumonia and prevents mortality.

### 3.2. Angiotensin-(1–7) induces apoptosis and increases binding and phagocytosis of apoptotic neutrophils

Ang-(1–7) has been associated with resolution of inflammation by inducing neutrophil apoptosis and efferocytosis (phagocytosis of apoptotic neutrophils) and macrophage reprogramming [17,18,36]. Ang-(1–7) treatment of infected mice increased the frequency of apoptotic neutrophils into the airways, as seen by apoptotic morphology counting 5 and 7 days post IAV infection (Fig. 2A) and flow cytometric analyzes 7 days post IAV infection (Fig. 2B).

In addition, in an *ex vivo* experiment, Ang-(1–7) directly increased the interaction of apoptotic neutrophils with alveolar macrophages. There was increased binding and efferocytosis of apoptotic neutrophils (Fig. 2C). In agreement with the decrease in the inflammatory infiltrate observed in mice treated with Ang-(1–7), histological analyzes showed less vascular, airways and parenchyma inflammation of the lung (Fig. 3A,B). These results suggested that Ang-(1–7) treatment was able to control the viral-induced inflammatory response by increasing apoptosis and subsequent binding and efferocytosis of neutrophils.

### 3.3. Absence of receptor Mas is associated with worsening of viral pneumonia

As shown in our previous results, administration of Ang-(1–7) prevented death, controlled inflammation and promoted resolution of neutrophilic inflammation. Our next step was to assess whether MasR would have a role during IAV infection. To this end, MasR$^{-/-}$ mice were infected with IAV ($10^4$ PFU) intranasally. Our results showed that MasR$^{-/-}$ mice succumbed on the ninth day after infection (Fig. 4A) while 53 % of WT group survived. However, no difference in weight loss of WT and MasR$^{-/-}$ group was observed (Fig. 4B). The immune response triggered by IAV was also evaluated to better understand the role of MasR during infection. 7 days after IAV infection, there was an intense recruitment of leukocytes into the airways (Fig. 4C), mainly mononuclear cells (Fig. 4D) and a trend to increased neutrophil recruitment in MasR$^{-/-}$ mice, as compared to WT mice (Fig. 4E). IAV infection induced and increase in the concentration of CXCL1, CCL5 (Fig. 4F), IL-10 (Fig. 4G) and IFN- γ (Fig. 4H) in BALF of infecgted mice. Levels of CCL5 were higher in MasR$^{-/-}$ than WT mice (Fig. 4F), but there was no significant increase of other cytokines in comparison to infected WT mice. In addition, viral titers were greater in the lungs of MasR$^{-/-}$ than WT mice after IAV infection (Fig. 4I). Therefore, we can suggest that absence of MasR worsens lung injury and outcome following infection.

Next, we investigated whether the actions of exogenous Ang-(1–7) were dependent on the MasR. For this purpose, MasR-deficient mice were subjected to IAV infection and treated with Ang-(1–7) from the third to the tenth day post infection and followed for 21 days. As seen in Fig. 5, administration of Ang-(1–7) had no effect on weight loss (Fig. 5A) and lethality rates (Fig. 5B) after IAV infection in MasR$^{-/-}$ mice. These results reinforce that the actions of Ang-(1–7) in IAV infection is directly dependent on its effects on the MasR.

### 3.4. Treatment with Angiotensin-(1–7) in post-IAV pneumococcal infection is protective

A relevant factor that leads to high mortality of IAV infected patients is the occurrence of secondary bacterial pneumoniae, commonly due to *Streptococcus pneumoniae* [3]. Because of the protective effects of Ang-(1–7) in the model of viral pneumonia, we wondered if Ang-(1–7) given during the course of viral infection could benefit mice subsequently exposed to secondary bacterial infection. Mice were challenged with a sublethal dose of virus (500 PFU) and then infected with a sublethal dose of *S. pneumoniae* ($10^3$ CFU) at day fourteen after IAV infection. No virus was detected in the lungs of co-infected mice at day 14 post IAV infection and inflammatory parameters had virtually returned to background (data not shown). Ang-(1–7) treatment was given from the third to tenth day after IAV infection (Fig. 6A). As clearly seen in Fig. 6, a previous IAV infection significantly worsened the outcome of *S. pneumoniae* infection. Indeed, a mild infection, which did not result in lethality, resulted in 100 % death in the presence of a previous IAV infection (Fig. 6B). Treatment with Ang-(1–7) during the course of IAV infection resulted in decrease of lethality rates from 100 % to 46 % (Fig. 6B). In addition, Ang-(1–7) treatment decreased several parameters of inflammatory response, including the total leukocytes and mononuclear cells recruitment (Fig. 6C,D), neutrophilic infiltrate and MPO levels in the lungs seen after the sub sequential infections (Figs. 6E-F). Very importantly, whereas there was bacteremia in control mice, only 50 % of mice treated with Ang-(1–7) had detectable bacteria translocation to blood (Fig. 6G). There was not a significant change in number of bacteria in BALF (Fig. 6H). These data reveal that exogenous Ang-(1–7) treatment during influenza is beneficial for improving post influenza pneumococcal infection.

## 4. Discussion

Seasonal outbreaks of the influenza affect up to 5 million people worldwide annually, with approximately 500,000 deaths from pneumonia each year [37]. The severity of the disease is related to the inflammation intensity caused by Influenza virus [6] and secondary infections [38]. The intensity of inflammation must be balanced to guarantee viral clearance and avoid secondary infection while preventing lung injury and death. Therefore, it has been argued that certain strategies may improve inflammation resolution without disturbing the response of the host to infection [10]. The effects and relevance of Ang-(1–7) and its receptor Mas have not been explored in the context of IAV infection and post-IAV pneumococcal infection. In our study, we found that (i) the administration of Ang-(1–7) to WT mice caused major improvement of viral pneumonia. Indeed, the course of disease was less severe, as seen by decrease of infiltration of leukocytes in the lung (mainly neutrophil infiltrate in the alveoli), decreased tissue damage and lethality rates. There were lower viral titers in the lungs and expression of MasR was increased after administration of Ang-(1–7). (ii) On the other hand, the absence of MasR caused worsening of pneumonia during IAV infection, as characterized by increased viral load and death of all mice. (iii) The effects of Ang-(1–7) are dependent on its receptor, as Ang-(1–7) had no protective in MasR-deficient mice. (iv) Mechanistically, the beneficial effects of Ang-(1–7) were associated with decreased inflammation, increased neutrophil apoptosis and increased capacity of alveolar macrophages to interact and phagocytize apoptotic neutrophils. Finally, (v) exogenous administration of Ang-(1–7) during IAV prevented inflammation and death caused by a secondary pneumococcal infection.

Several pro-resolving molecules have been shown to decrease substantially inflammation and injury in different models of pulmonary disease, including fibrosis and asthma [16,18,39,40]. In addition, there is substantial evidence that pro-resolving molecules may have beneficial effects in the context of infection [10]. For example, a previous study showed that the treatment with Protectin D1 (PD1) attenuated IAV replication *via* RNA export machinery, decreasing lung injury and improving mice survival of severe influenza [41]. Another pro-resolving molecule, Annexin A1, limited IAV replication and attenuated lung damage and death induced by IAV [42]. The pro-resolving actions of Ang-(1–7) have only recently been described and further studies are necessary to dissect intracellular mechanisms of action of Ang-(1–7) [17,18,36]. The finding that Ang-(1–7) decreased lung injury and death induced by IAV is consistent with the anti-inflammatory and

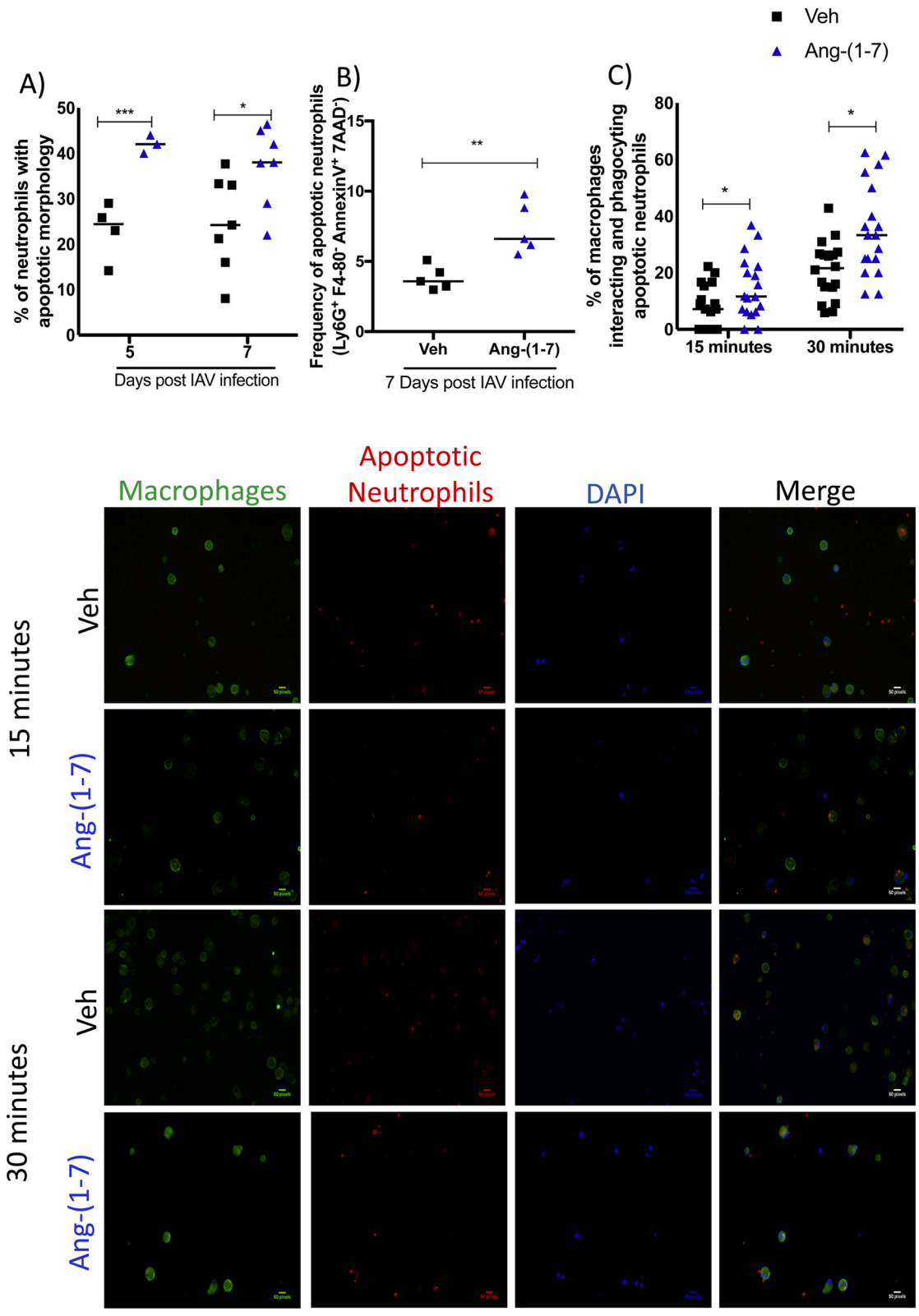


**Fig. 2. Angiotensin-(1-7) promotes neutrophil apoptosis, binding and efferocytosis**. WT mice were infected intranasally with $10^4$ PFU and treated twice a day, from day three to seven post-infection, Vehicle (92 μg/Kg of HPβCD) or Angiotensin-(1-7) [60 μg/Kg of Ang-(1-7) and 92 μg/Kg of HPβCD], twice a day by gavage. On days 5 and 7 post-IAV, mice were euthanized, and the frequency of apoptotic neutrophils was assessed by (A) microscopy (morphological criteria) and (B) flow cytometry (Annexin V + cells). Interaction (binding and efferocytosis) was analyzed *ex vivo* by incubating alveolar macrophages, treated with media (vehicle) or 300 nM of Ang-(1-7), with apoptotic neutrophils for 15 and 30 min. Immunofluorescence staining (C) was performed using antibodies for specific markers of macrophages (CD11c –FITC), neutrophils (Red Cell Tracker) and the nuclear staining was done with DAPI (blue). Magnification of 40 × . Graph shows the quantification of the interaction (binding and efferocytosis assay) between alveolar macrophages and apoptotic neutrophils. Data are presented as Median, n = 5-7 of three independent experiments. *, ** and *** for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, when indicated groups by Test-t.

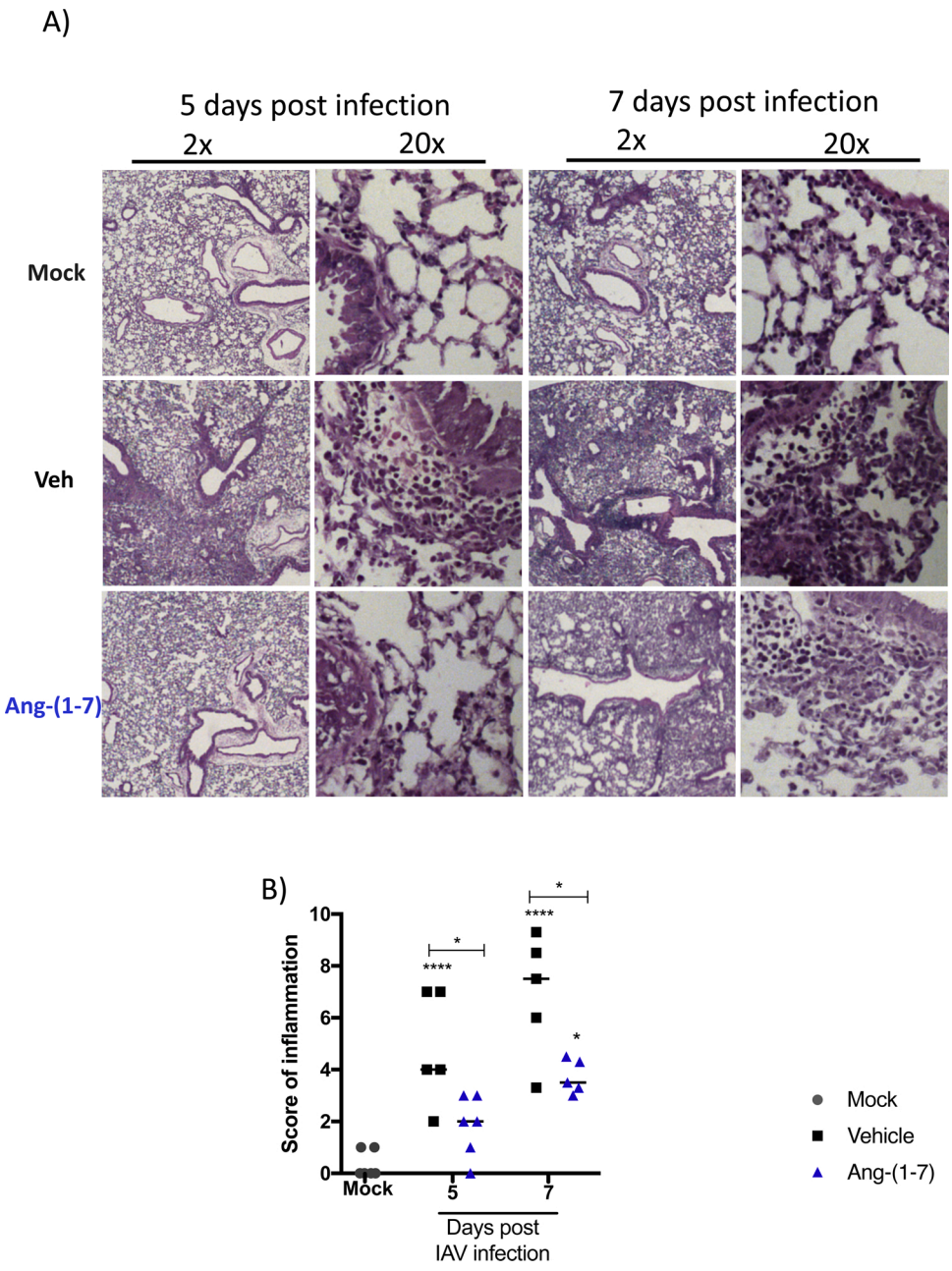


**Fig. 3. Angiotensin-(1-7) reduces the IAV-mediated lung injury.** (A) Representative lung slides of IAV infected-mice ($10^4$ PFU, i.n.) treated with Vehicle (92 μg/Kg of HPβCD) or Angiotensin-(1-7) [60 μg/Kg of Ang-(1-7) and 92 μg/Kg of HPβCD] (twice a day by gavage, from days 3-7) and uninfected controls (Mock). Images, from day 5 and 7 post IAV, were taken under 2× and 20x magnification. (B) Histopathological score (maximal of 18) evaluated airway, vascular, parenchymal inflammation, neutrophilic infiltration. The results are presented as Median, n = 5-6 mice per group, of three independent experiments. * and *** for $p < 0.05$ and $p < 0.001$, respectively, compared to Mock or indicated groups by one-way ANOVA.

pro-resolving actions of Ang-(1–7) in several systems [13–18,33,43,44] and agrees with the protective actions of other pro-resolving molecules in models of inflammation [10,38,39]. There was also partial reduction of viral load in Ang-(1–7)-treated animals. As the compound had no direct effect on viral replication, it is likely that the decrease in viral load is secondary to its immunomodulatory effects.

Previous studies have shown that ACE2 expression is decreased after IAV infection [45], which would lead to a drop in Ang-(1–7) levels. On the other hand, IAV infection promoted an increase in the expression of MasR. It is known that the MasR mediates the effects of Ang-(1–7) in various biological systems [21]. Experiments in MasR-deficient mice showed that these animals had worse disease after IAV infection. This is similar to findings in an arthritis model where absence of receptor was associated with a worse disease whereas administration of Ang-(1–7) was anti-inflammatory in mice [15]. In addition, the absence of the MasR increased macrophage recruitment, worsened the autoimmune encephalomyelitis model and induced atherosclerosis by affecting macrophage migration and infiltration [46]. Moreover, our results show that Ang-(1–7) was not protective in MasR-deficient mice, keeping with the relevance of this receptor for the effects of Ang-(1–7) in various biological systems.

Neutrophils appear to play a crucial role in the context of IAV infection. Indeed, the course of IAV infection is significantly worse in mice in which neutrophils had been depleted [28]. On the other hand, partial blockade of neutrophil accumulation and activation is known to prevent the pulmonary injury associate with IAV infection [22,23,47]. We have previously shown that modulation of the inflammatory response by blocking CXCR1/2 improves disease outcome during respiratory Influenza virus and pneumococcal infections, without compromising the ability of the murine host to deal with infection [23]. Administration of Ang-(1–7) significantly decreased neutrophil influx and this was associated with a decrease in the pulmonary levels of CXCL1, a chemokine that binds to CXCR2. Ang-(1–7) has been found to decrease CXCL1 and other neutrophil active chemokines in models of

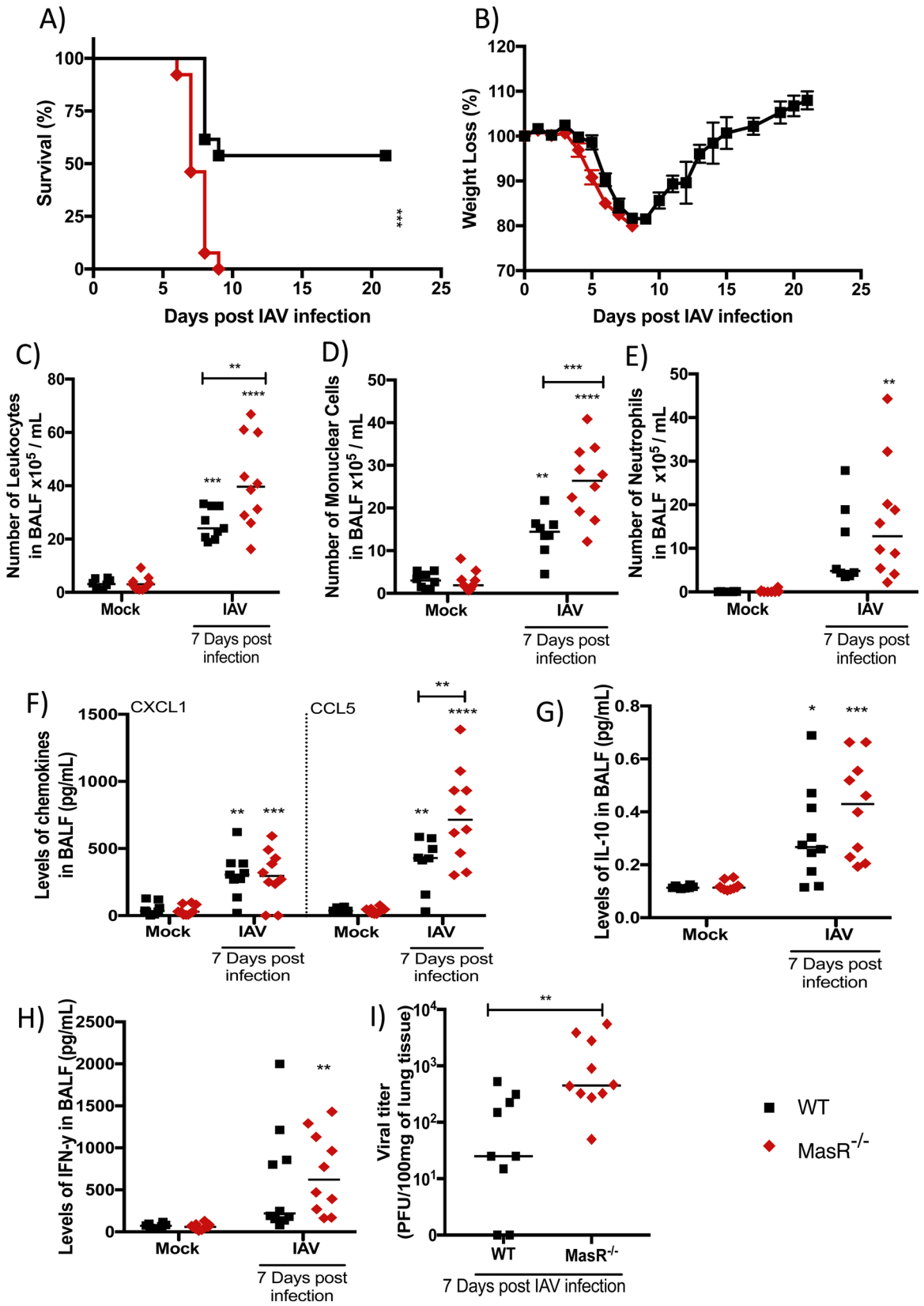


**Fig. 4. Receptor Mas is protective during influenza pneumonia.** Wild type (WT) and MasR knockout (MasR$^{-/-}$) mice were infected with IAV ($10^4$ PFU, i.n.) and evaluated daily for (A) lethality and (B) weight loss (*** for $p < 0.001$ by Log Rank Test – n = 13). On day 7 post-IAV, mice were euthanized for (C) total leukocytes, (D) mononuclear cells and (E) neutrophil counts in BALF. Uninfected mice (Mock) were used as controls. (F) Levels of CXCL1 and CCL5, (G) IL-10 and (H) IFN-γ were also evaluated in BALF by ELISA assay. (I) Viral loads were evaluated in the lungs from IAV-infected mice. Data are presented as Median, n = 5 mice per group of three independent experiments. ** and *** for $p < 0.01$ and $p < 0.001$, respectively, when compared to Mock or indicated groups by two-way ANOVA.

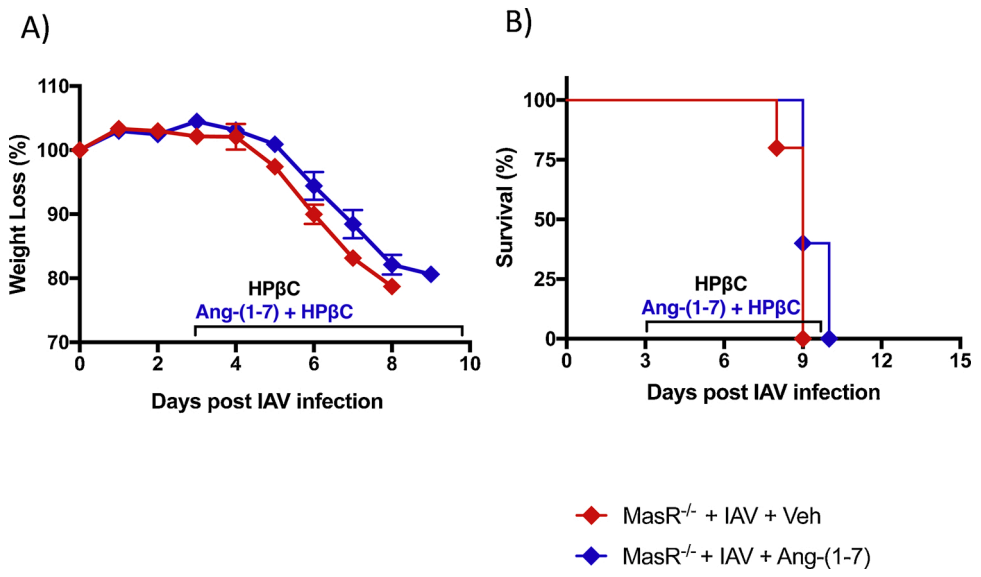


**Fig. 5. Angiotensin-(1-7) protective effects are MasR-dependent.** MasR-deficient mice (MasR$^{-/-}$) were infected with IAV ($10^4$ PFU, i.n) and treated twice a day, from day three to seven post-infection, with Vehicle (92 μg/Kg of HPβCD) or Angiotensin-(1-7) [60 μg/Kg of Ang-(1-7) and 92 μg/Kg of HPβCD], twice a day by gavage. (A) Weight loss and (B) lethality were evaluated daily.

arthritis [15] and renal reperfusion injury [13]. Therefore, the ability of Ang-(1–7) to decrease CXCL1 and consequent CXCL1 driven neutrophil influx may account for some of the beneficial effects observed. On the other hand, Ang-(1–7) did not alter neutrophilic elastase activity after infection, suggesting that it does not act in the formation of NETs, a finding that deserves further investigation. We have previously shown that in addition to inhibiting chemokine production [13,15] and directly inhibiting neutrophil influx [15], Ang-(1–7) is also capable of increasing neutrophil apoptosis *in vitro* and *in vivo* [17] and increasing binding and phagocytosis of apoptotic neutrophil by alveolar macrophages *ex vivo* as shown here. Similarly, treatment of mice with Ang-(1–7) enhanced apoptosis of neutrophils and their binding and efferocytosis by macrophages. These pro-resolution effects of Ang-(1–7) may clearly favor decrease of pulmonary injury with a delay in the production of IFN-γ and better clinical outcome after IAV infection. It is interesting to note that the decrease of neutrophilic inflammation and pulmonary injury were not associated with an increase in viral load in Ang-(1–7)-treated mice.

A secondary bacterial infection, especially by *S. pneumoniae*, is frequent after IAV infection and may account for significant amount of morbidity associated with the infection [48,49]. There are multiple mechanisms associated with enhanced severity of pneumococcal infection after IAV, including decreased beating of ciliary tracheal epithelial cells, epithelial cell death, decreased macrophage number and functionality and changes in microbiota [5,50,51]. In our model, treatment with Ang-(1–7) only during the course of viral pneumonia decreased by 50 % the death caused by post-IAV pneumococcal infection. The latter protective effects were associated with reduction of bacteria-induced neutrophil influx and lung injury. Although neutrophil numbers were reduced, there was better local control of infection and major decrease of spread of bacteria to the bloodstream. Of note, at the time of the second pneumococcal infection and based on the pharmacokinetics of Ang-(1–7) [25], it is highly unlikely that there is any relevant concentration of this peptide in mice. Therefore, the lower translocation of bacteria to blood appears to be due to the lesser overall inflammatory status of Ang-(1–7)-treated and infected mice and decreased breakdown of epithelial barrier integrity during IAV infection. Our results are in agreement with other findings that treatment with two other pro-resolution mediators, acetate and aspirin-triggered RvD1, in models of pneumococcal pneumonia after IAV infection [52,53]. Additionally, in another finding Ang-(1–7) rescued the phagocytic capacity by mice neutrophils in experimental Type 2 Diabetes Mellitus and these neutrophils phagocytized *Staphylococcus aureus*, a bacterium that also causes lung infection [54].

The inflammatory response plays a dual role during infection [6]. Whereas inflammatory responses are necessary to control proliferation and spread of the pathogen, misplaced or intense inflammatory responses may cause tissue injury and consequent aggravation of disease. Our results clearly show that treatment with the pro-resolving molecule Ang-(1–7) after the onset of pneumonia caused by IAV decreased pulmonary injury and death. Ang-(1–7) treatment also decreased the severity of pneumococcal pneumonia. Mechanisms associated with protection included decreased neutrophil accumulation and decreased neutrophil-associated injury. We suggest Ang-(1–7) may be a good candidate molecule to be developed as adjunct treatment of viral pneumonia.

## Authorship

E. M. Melo, J. P. Vago and M. M. Teixeira wrote the paper. E. M. Melo, J. L. Del Sarto and M. M. Teixeira designed the research. E. M. Melo, J. L. Del Sarto, J. P. Vago, A. P. F. Gonçalves, L. P. Tavares, F. Rago, M. G. Machado, I. Aranda-Pardos, B. V. S. Valiate, G. D. Cassali performed experiments and analyzes the data. V. Pinho and L. P. Sousa provided expertise. N. A-Gonzalez, M. J. Campagnole-Santos, M. Bader, R. A. S. Santos, A. V. Machado, S. Ludwig M. M. Teixeira provided essential tools and expertise.

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## Declaration of Competing Interest

The authors report no declarations of interest.

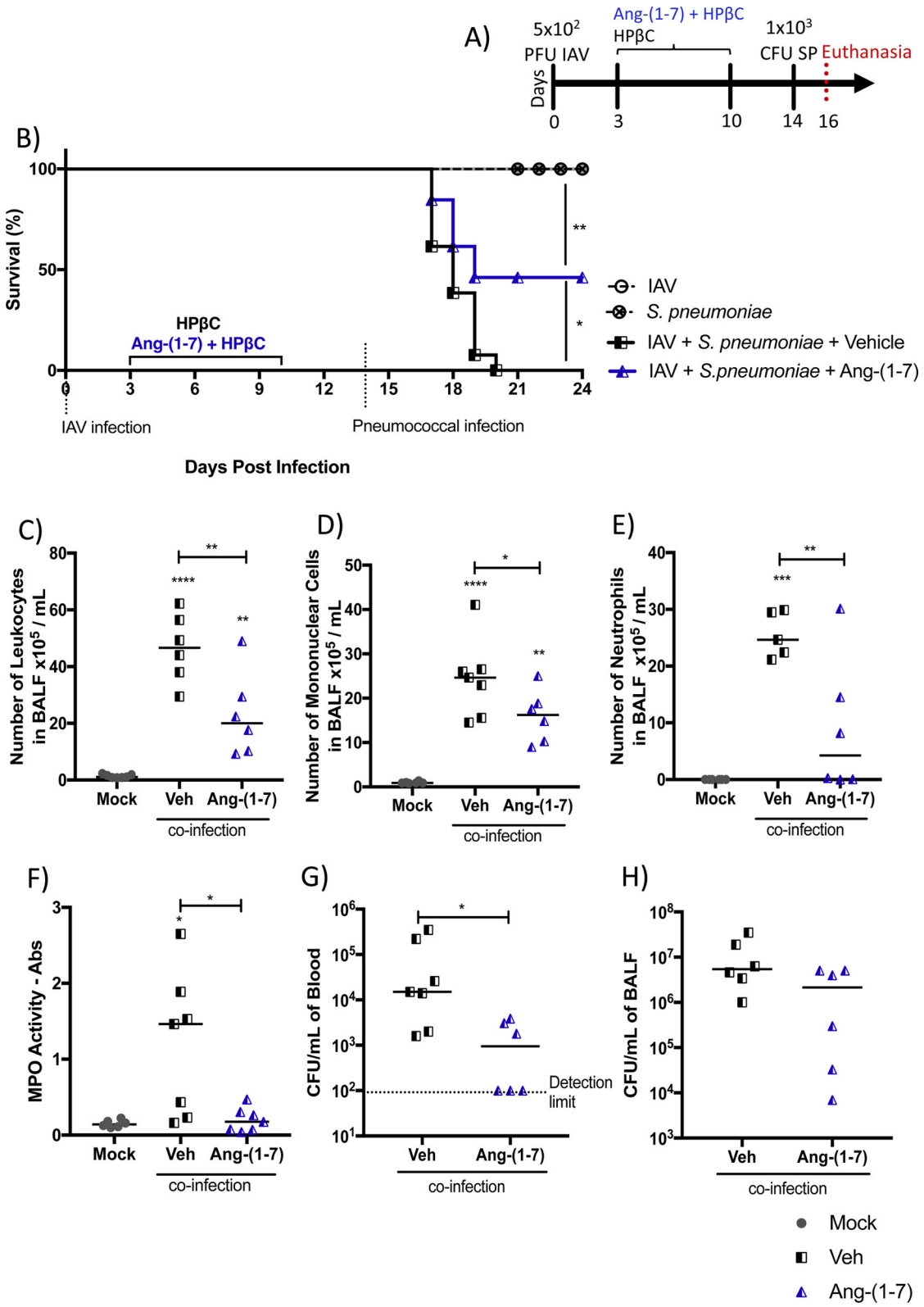


**Fig. 6. Angiotensin-(1-7) protects mice from post-IAV pneumococcal pneumonia.** (A) WT mice were infected with a non-lethal inoculum of IAV (500 PFU, i.n.) and treated twice a day, from day three to ten post-infection, with Vehicle (92 μg/Kg of HPβCD) or Angiotensin-(1-7) [60 μg/Kg of Ang-(1-7) and 92 μg/Kg of HPβCD], twice a day by gavage. On day 14 post-IAV, mice were secondarily infected with *Streptococcus pneumoniae* ($10^3$ CFU, i.n.). Single infected (IAV or *S. pneumoniae*) and uninfected mice were used as controls. (B) Survival was monitored daily for 24 days. IAV and *S. pneumoniae* single infected groups overlap in the frame of 100 % survival. Data are presented * for $p < 0.05$ and ** for $p < 0.01$ by Log Rank test, n = 8-9 mice per group of two independent-experiments. On day 2 post-bacterial challenge, mice were euthanized and BALF was harvested for (C) total leukocyte, (D) mononuclear cells and (E) neutrophil counts. (F) Neutrophil recruitment into the lung parenchyma was assessed by MPO analysis. (G—H) Bacteria burden was evaluated in blood and BALF of secondarily infected mice. Data are presented as Median, n = 6-7 of three independent experiments. *, ** and *** for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, when compared to Mock or indicated groups by one-way ANOVA.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2020.105292.

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