Original Article

Angiotensin-(1–7) alleviates acute lung injury by activating the Mas receptor in neutrophil

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Background: Acute lung injury (ALI) is a major cause of mortality and morbidity in the clinic. None of the current pharmacological interventions has achieved a detectable benefit. The renin-angiotensin system (RAS) is a complex humoral system essentially involved in the regulation of ALI. In the RAS family, angiotensin (Ang)-(1–7) was found to provide protection by counteracting the effects of Ang II in various cardiopulmonary disease models. The downstream receptor of Ang-(1–7) is the G protein-coupled receptor (GPCR) Mas. We hypothesize that the Ang-(1–7)-Mas pathway would protect patients from ALI.

Methods: To establish a 2-hit ALI model, the mice underwent intratracheal instillation of hydrochloric acid followed by ventilator-induced lung injury (VILI). ALI was evaluated based on lung edema, histology, myeloperoxidase activity, and proinflammatory cytokine production. The effects of the infusion or inhalation of Ang-(1–7) and Mas receptor blocker A779 were examined. The human neutrophils were isolated, and Mas receptor expression was examined. The neutrophil responses to platelet-activating factor (PAF) stimulation were tested by measuring the formation of reactive oxygen species (ROS), neutrophil adhesion, and chemotaxis. Next, in the mouse model, the neutrophils were depleted using an anti-ly6G antibody.

Results: The infusion or inhalation of Ang-(1–7) protected mice from ALI as evidenced by decreases in lung edema, the histological lung injury score, myeloperoxidase activity, and proinflammatory cytokine production. Such effects were largely blocked by the Mas receptor blocker A779. Mas receptor expression in the neutrophils was identified at both the messenger ribonucleic acid and protein levels. Ang-(1–7) prevented neutrophil responses to PAF stimulation, including the formation of ROS, neutrophil adhesion, and chemotaxis, while A779 alleviated these effects. The importance of neutrophils in ALI was further confirmed by neutrophil depletion using the anti-ly6G antibody; however, A779 partially reversed the protective role of neutrophil depletion in ALI, indicating the critical role of Ang-(1–7)-Mas signaling in other pulmonary cells.

Conclusions: Ang-(1–7)/Mas receptor attenuates the key features of ALI by regulating neutrophil activation. Our study provides new evidence of their role in the pathogenesis of ALI and may lead to the development of a promising therapeutic strategy.

Keywords: Acute lung injury (ALI); angiotensin-(1–7); Mas receptor; inflammation; neutrophil

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Introduction

Acute respiratory distress syndrome (ARDS) is a common clinic syndrome, characterized by the acute onset of hypoxemia and bilateral pulmonary edema due to excessive alveolocapillary permeability (1). ARDS has high mortality and mobility rates in the intensive care unit (2). Despite considerable advances in preventing ventilation-induced lung injury and fluid management (3,4), no specific pharmacotherapy has proven effective against ARDS.

It is widely acknowledged that the renin-angiotensin system (RAS) plays an indispensable role in regulating blood pressure and maintaining body fluid homeostasis. Recently, a growing area of research has highlighted the key role of RAS in the early pathophysiology of acute lung injury (ALI) (5).

Angiotensin (Ang)-(1–7) is the metabolite heptapeptide of Ang II by angiotensin-converting enzyme 2 (ACE2). Due to its antagonistic effect against Ang II, Ang-(1–7) has come to the fore in cardiovascular system research (6,7). Unlike the traditional Ang II receptor subtype 1 (AT1) and subtype 2 (AT2), Ang-(1–7) has a unique downstream messenger, Mas, which is a member of the G protein-coupled receptor (GPCR) family (8). The Mas receptor expression was reported to be upregulated by estrogen, contributing to sex differences in ALI (9).

The ACE2/Ang-(1–7)/Mas receptor axis counterbalances the deleterious actions of the ACE/ANG II/AT1 receptor axis. In experimental models, the Ang-(1–7)/Mas receptor axis appears to be beneficial in ALI (10,11). Ang-(1–7) has been shown to improve pulmonary function in terms of oxygenation and inflammatory cell recruitment in a 2-hit model of ARDS (10). However, the physiological and pathological roles of the Ang-(1–7)/Mas receptor axis in ARDS are not yet known.

Neutrophils have been implicated as harmful cells triggering acute inflammation in response to ALI (12). Depletion of neutrophils attenuated or reversed the development of ALI (13,14).

Based on previous findings, we hypothesized that Ang-(1–7) would protect form ARDS by inhibiting neutrophilic inflammation. To test our hypothesis, we examined the effect of Ang-(1–7) in an acid- and ventilation-induced ALI mouse model. Further, the expression of the Mas receptor was determined in human neutrophils, as was Ang-(1–7) mediated neutrophil activation, and alveolar transmigration with inflammatory stimuli. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6193/rc).

Highlight box

Key findings

- Ang-(1–7) protects against ALI partly through the interaction with the Mas receptor in neutrophils.

What is known and what is new?

- In the animal models of ALI, the administration of Ang-(1–7) provided some protective effects.
- Mas receptor is expressed in the neutrophils, and Ang-(1–7) works with the Mas receptor in the neutrophils.

What is the implication, and what should change now?

- Ang-(1–7) provides protection against ALI partly through the interaction with the Mas receptor in the neutrophils, which provides a new perspective for understanding the RAS role in the pathogenesis of ALI.

Methods

Animal studies

A protocol was prepared before the study without registration. *In vivo* experimental protocols were approved by the Ethics Committee of Fudan University, and all experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals (7th edition 1996)*. Wild-type C57BL/6 male mice (aged 8-weeks-old and weighing 25–30 g) were purchased from the Slac Laboratory Animal Center (Shanghai, China). The mice were anesthetized with intraperitoneal injections of sodium pentobarbital. The anaesthetized mice were placed in the supine position on a thermostatically controlled electric heated blanket to maintain their body temperature at 37.0–37.5 ℃. Following a tracheotomy and tracheal cannulation, the mice were ventilated (ALC-V8; Alcbio, Shanghai, China) with room air at 100 breaths/min, a positive end-

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expiratory pressure of 1 mmHg, and a tidal volume of 10 mL/kg body weight.

The mice were randomly assigned to the following experimental groups: the control group, which received no intervention, and the treatment groups, in which ALI was induced by intratracheal instillation of hydrochloric (HCl) acid (2 μL/g body weight; pH 1.5) followed by an increase in VT from 10 to 20 mL/kg body weight for 2 hours. In the treatment groups, an intravenous injection of Ang-(1–7) (6 nmol/kg) alone or with its antagonists d-Ala-Ang-(1–7) (A779; 12 nmol/kg) was initiated with ALI induction. To test the effects of the local administration, Ang-(1–7) (6 nmol/kg) alone or with A779 (12 nmol/kg) was intratracheally nebulized after which ALI was induced. After 2 hours, the mice were euthanized, the right main bronchus was ligated, and the right lung was excised and processed to determine the wet-to-dry lung weight ratio, myeloperoxidase (MPO) activity, and histology. The left lung was lavaged 3 times with aliquots of 0.6 mL phosphate buffered solution from which the bronchoalveolar lavage fluid (BALF) was recovered.

In the lipopolysaccharide (LPS)-induced ALI group, the mice were challenged with LPS (5 mg/kg, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) by intratracheal instillation. The neutrophils were depleted by an intraperitoneal injection of an anti-Ly6G antibody (200 μg, clone 1A8, BioXCell) 24 h before the ALI. The mice were sacrificed after 2 days, and the same procedure for sample collection was undertaken as that described above.

Assessment of lung injury

Lung edema was quantified by the wet-to-dry lung weight ratio (15). The MPO activity in the lung homogenates was determined by photometric assays (Sigma) and expressed as units per gram lung tissue (U/g) (15). The concentration of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10) in the BALF was quantified using enzyme-linked immunosorbent assay kits (Invitrogen™, Thermo Fisher, Shanghai, China). The histological evidence of lung injury was assessed from hematoxylin and eosin (H&E)-stained lung sections by a blinded investigator using an established histopathological score ranging from 0 to 2 (16).

Neutrophil isolation

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) with approval from the Ethics Committee of Zhongshan Hospital, Fudan University in Shanghai, China (No. B2021-715R). Human neutrophils were obtained from healthy male volunteers from Zhongshan Hospital, Fudan University. All healthy male volunteers signed an informed consent form. Peripheral venous blood samples were collected in heparinized tubes. Herman *et al.*'s (17) procedure for the isolation of neutrophils was adapted. Briefly, the blood was centrifuged at 323 g at 20 ℃ for 20 min to separate the blood sample into 2 distinct layers of platelet-rich plasma (PRP) and blood cells. The PRP was carefully transferred to a fresh 50-mL tube and centrifuged at 896 g for 20 min at 20 ℃ to pellet the platelets. Following the centrifugation of the PRP, the supernatant [platelet-poor plasma (PPP)] was transferred to a fresh 50-mL tube. The blood cells were transferred to a fresh 50-mL tube and 6 mL of the pre-warmed 6% dextran solution was added, and the solution was topped up to 50 mL with saline. Next, to mix the contents, the tube was gently inverted and left undisturbed at room temperature for 30 min. The pale upper leukocyte layer was then transferred to a clean 50-mL tube following centrifugation at 224 g at 20 ℃ for 6 min. After centrifugation was completed, the supernatant was removed and discarded, and the leukocyte pellet was gently resuspended in 2 mL of PPP. Next, the entire 2 mL of the resuspended leukocyte population was deposited on top of the upper phase of the gradient following centrifugation at 271 g for 11 min at 20 ℃ with no brake. The discontinuous plasma/Percoll gradient was as follows: Upper phase: 0.84 mL 90% Percoll + 1.16 mL of PPP; and lower phase: 1.02 mL of 90% Percoll + 0.98 mL of PPP. The granulocytes in the middle layer were then placed in a clean 50-mL tube and resuspended with 10 mL of PPP and Hank's Balanced Salt Solution (HBSS) following centrifugation at 504 g for 6 min at 20 ℃. After the centrifugation, the neutrophil pellet was collected.

Western blot

Mas protein expression in the isolated neutrophils was determined by western blotting as described previously (15). In brief, sample proteins (50 μg/slot) were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked, incubated with the primary antibodies of rabbit anti-Mas1 (Abcam, Cambridge, UK) and mouse anti-β-Actin (Sigma, St. Louis, MO, USA). The blots were then

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rinsed and incubated in IR-dye based secondary antibodies. The antibody signals were detected using the Odyssey scanner (LI-COR Biosciences, Lincoln, NE, USA).

Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA), and real-time polymerase chain reaction (RT-PCR) was performed in accordance with the manufacturer's instructions. Complementary deoxyribonucleic acid was synthesized using the Promega M-MLV Kit (Promega, Beijing, China). RT-PCR was performed with the Roche LightCycler 480 II (Roche, Mannheim, Germany) with SYBR Master Mixture (Takara, Dalian, China). The primer sequences were as follows:

Mas1: forward primer- GAAGAAGAGATTCAAGG AGTCC,

reverse primer- CTGTGACCGTATTACAATTGTC

GAPDH: forward primer- TGACTTCAACAGCGA CACCCA,

reverse primer- CACCCTGTTGCTGTAGCCAAA

All the reactions were run in triplicate. The expression level was normalized to the GAPDH control, and the relative expression values were determined against the control using the $2^{-\Delta\Delta CT}$ method.

Reactive oxygen species (ROS)

The generation of ROS in the neutrophils was measured using luminol-enhanced chemiluminescence (15). In brief, 3×10^5 neutrophils were suspended in 1 mL of HBSS. The cells were incubated for 30 minutes with saline, Ang-(1–7) (0.1 μmol/L), and Ang-(1–7) (0.1 μmol/L) combined with A779 (10 μmol/L), respectively, and then stimulated by 10 μmol/L of platelet-activating factor (PAF). ROS production was determined as the light released from the lumiphor luminol by a chemiluminometer (Autolumat LB953; Berthold Technologies, Bad Wildbad, Germany), and expressed as counts per second.

Neutrophil adhesion and chemotaxis

Neutrophil adhesion was measured as previously described (15). In brief, 3×10^4 human pulmonary microvascular endothelial cells (HPMECs; Cell Biologics, Inc., Chicago, IL) were grown to confluence in 96-well plates. Th isolated neutrophils were stained with Calcein-AM (Sigma, St. Louis, MO, USA), resuspended in HBSS (10^5/100 μL), and then incubated with saline, Ang-(1–7) (0.1 μmol/L), and Ang-(1–7) (0.1 μmol/L) combined with A779 (10 μmol/L), respectively, for 30 min. The neutrophils were then added to the HPMECs in the presence of 10 μmol/L of PAF. Fluorescence readings were obtained at excitation/emission wavelengths of 485/535nm with a fluorescence plate reader (Fluoroskan Ascent™; Thermo Scientific, Waltham, MA) before and after triple washing to remove any non-adherent neutrophils, and neutrophil adhesion was quantified as the ratio of the final/initial fluorescence.

The neutrophil chemotaxis experiments were performed as previously described (15). In brief, the HPMECs were seeded into 24-well transwells (8.0-μm pore-sized polycarbonate filters) and grown to confluence for 3–5 days. The lower chambers of the transwells were filled with 500 μL of serum-free endothelial cell media containing a vehicle (saline) or 10 μmol/L of PAF; 5×10^5 neutrophils pre-incubated with saline, Ang-(1–7) (0.1 μmol/L), and Ang-(1–7) (0.1 μmol/L) combined with A779 (10 μmol/L), respectively, for 30 min. suspended in 200 μL of serum-free medium were added to the upper chamber. The assembly was incubated at 37 ℃, with 5% carbon dioxide CO_2. After 3 hours, the migrated neutrophils were recovered from the lower chamber and counted using a hemocytometer.

MPO immunofluorescence staining

For the quantitative analysis of the infiltration of MPO positive cells (neutrophils) in the lung tissue, the embedded lung tissue sections were stained with an antibody for MPO (ab208670, Abcam). The MPO positive cells were counted and divided by the total number of cells.

Statistical analysis

All the values are expressed as the mean ± SEM. The quantitative variables were statistically analyzed using the Student's 2-tailed *t*-test for 2-group comparisons and a 1-way analysis of variance followed by Dunnett's test for multiple pairwise comparisons. A P value <0.05 was considered statistically significant. All the statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA).

Results

Ang-(1–7) protected ALI in the mouse model

The 2-hit model of instillation of HCl with over ventilation

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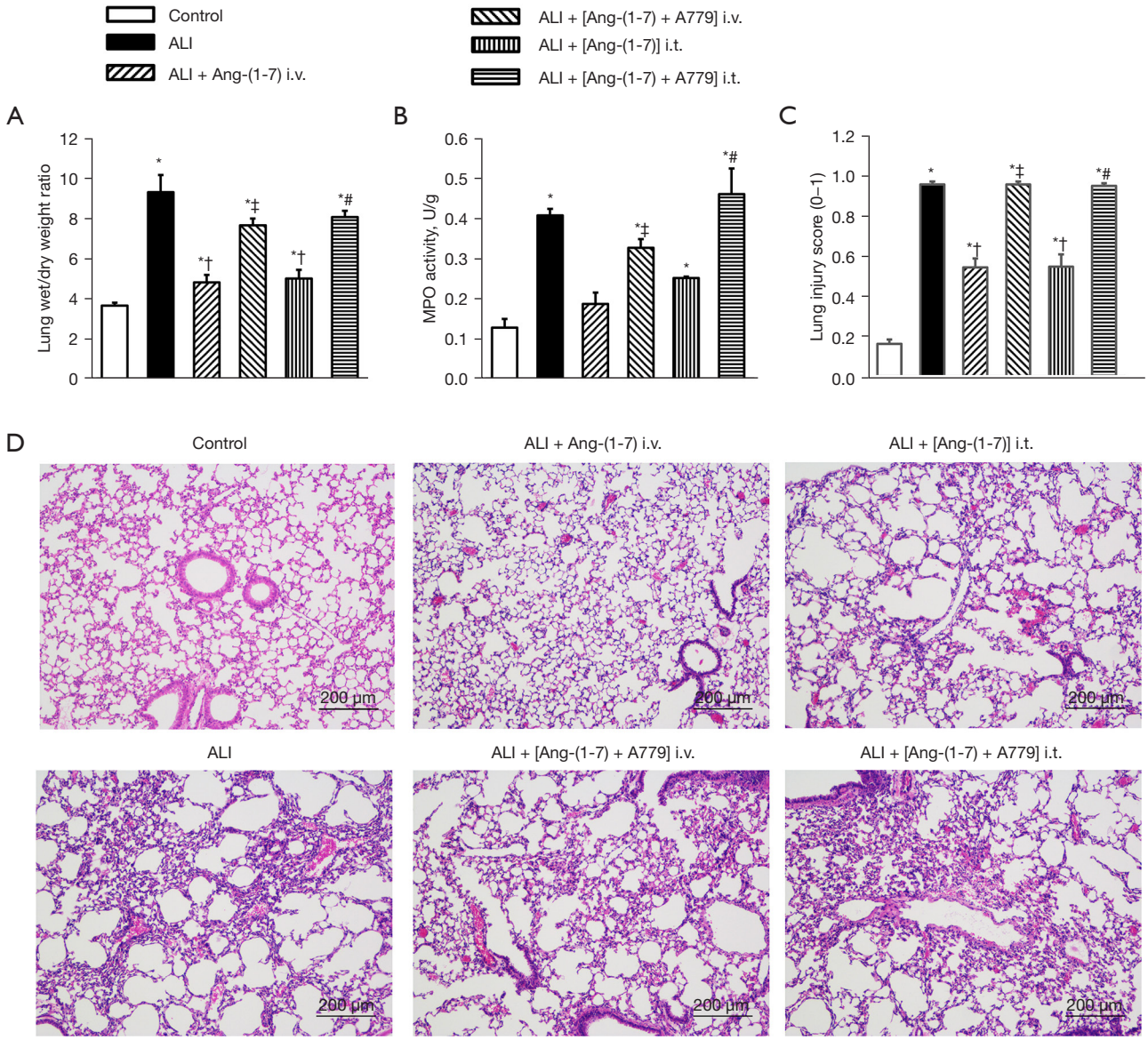

Figure 1 Effect of Ang-(1–7) and its receptor on lung pathological changes in acid- and ventilation-induced ALI mice. Group data showing the effects of Ang-(1–7) (6 nmol/kg) when administered either alone or in combination with the Ang-(1–7) receptor antagonist A779 (12 nmol/kg) concomitantly with the induction of acid- and ventilation-induced lung injury on the wet-to-dry lung weight ratio (A), lung MPO activity (B), and histological features of lung injury on a quantitative scale from 0 (no injury) to 1 (C). Histological features of lung injury are shown by representative histological micrographs by H&E staining (D). Data are mean ± SEM from n=5 mice per group. *, P<0.05 versus healthy control mice; †, P<0.05 versus HCl and over ventilation; ‡, P<0.05 versus HCl and over ventilation + Ang-(1–7) i.v.; #, P<0.05 versus HCl and over ventilation + Ang-(1–7) i.t.. ALI, acute lung injury; MPO, myeloperoxidase; SEM, standard error of mean; HCl, hydrochloric; i.v., intravenous injection; i.t., intratracheal instillation; H&E, hematoxylin and eosin.

caused the features characteristic of murine ALI. In the ALI group, the lung vascular barrier failure was evidenced by an increase in the lung wet-to-dry weight ratio (3.65±0.15 *vs.* 9.29±0.86), which was largely reversed following treatment with Ang-(1–7) by either intravenous infusion (4.81±0.37) or intratracheal nebulization (5.00±0.44). However, the effect of Ang-(1–7) was completely abrogated by the Mas receptor inhibitor A779 (7.65±0.33 and 8.05±0.32) (*Figure 1A*).

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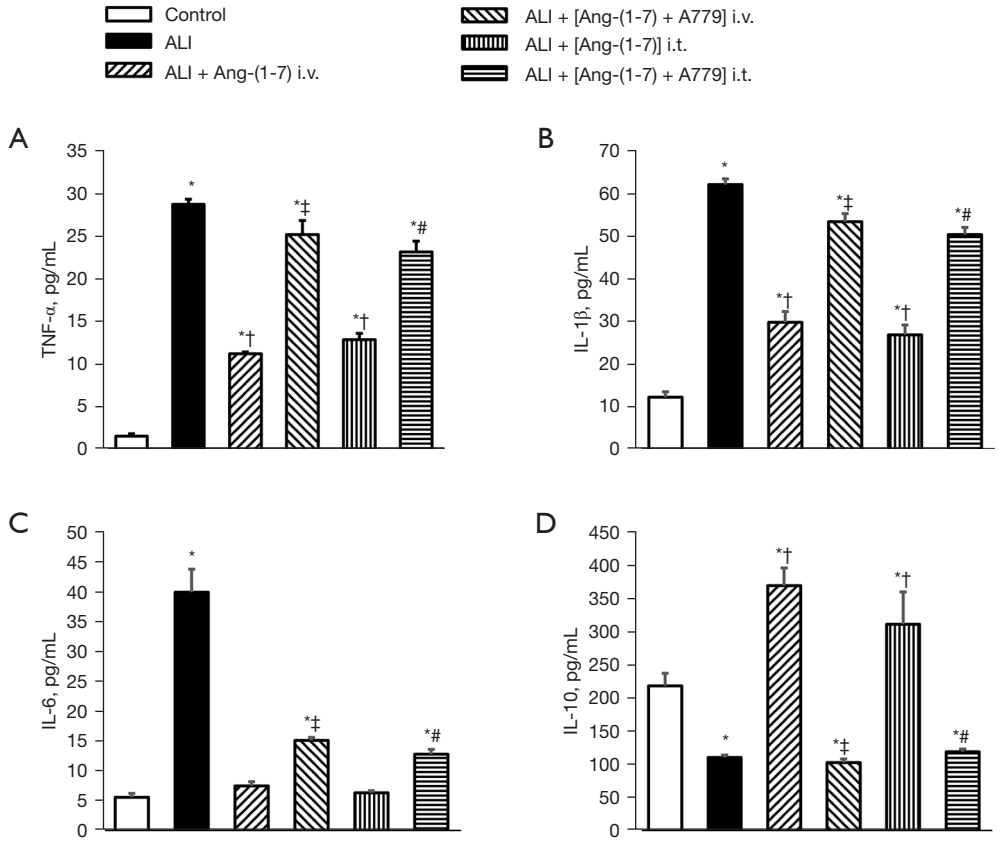

Figure 2 Effects of Ang-(1–7) and its receptor on the lung inflammatory cytokine response of ALI. Group data showing the effects of Ang-(1–7) (6 nmol/kg) when administered either alone or in combination with the Ang-(1–7) receptor antagonist A779 (12 nmol/kg) concomitantly with the induction of acid- and ventilation-induced lung injury on the concentration of TNF-α (A), IL-1β (B), IL-6 (C), and IL-10 (D). The data are expressed as the mean ± SEM from n=5 mice per group. *, P<0.05 versus healthy control mice; †, P<0.05 versus HCl and over ventilation; ‡, P<0.05 versus HCl and over ventilation + Ang-(1–7) i.v.; #, P<0.05 versus HCl and over ventilation + Ang-(1–7) i.t.. ALI, acute lung injury; TNF-α, tumor necrosis factor-α; IL, interleukin; SEM, standard error of mean; HCl, hydrochloric; i.v., intravenous injection; i.t., intratracheal instillation.

MPO activity is a hallmark of ALI and indicates the influx of inflammatory neutrophils. We examined the MPO activity in the lung homogenates. The 2-hit model significantly increased the MPO activity in the mice (0.13±0.02 *vs.* 0.41±0.02 U/g). However, treatment with Ang-(1–7) significantly attenuated the MPO activity (0.19±0.03 and 0.25±0.00 U/g), which was completely abrogated by the administration of A779 (0.33±0.02 and 0.46±0.06 U/g) (*Figure 1B*).

Lung injury was histologically evident in the H&E-stained lung sections, and quantitatively verified by blinded analyses based on lung injury scores (0.16±0.02 *vs.* 0.96±0.01). The administration of Ang-(1–7) significantly attenuated the histological damage (0.54±0.04 and 0.55±0.06). Conversely, the administration of A779 blocked the effect of Ang-(1–7) (0.96±0.01 *vs.* 0.95±0.01) (*Figure 1C*,*1D*).

ALI is associated with elevations in proinflammatory mediators. We measured the levels of TNF-α, IL-1β, and IL-6, and the anti-inflammatory cytokines as IL-10 in the BALF. The TNF-α, IL-1β, and IL-6, levels were significantly higher in the ALI group than the control group. The increase in the proinflammatory mediators was blocked following treatment with Ang-(1–7) but recovered following the administration of A779 (*Figure 2A-2C*). Conversely, the level of IL-10 was decreased in the ALI group. The Ang-(1–7) treatment increased the production of IL-10, while A779 blocked the effect of Ang-(1–7) on the IL-10 levels (*Figure 2D*).

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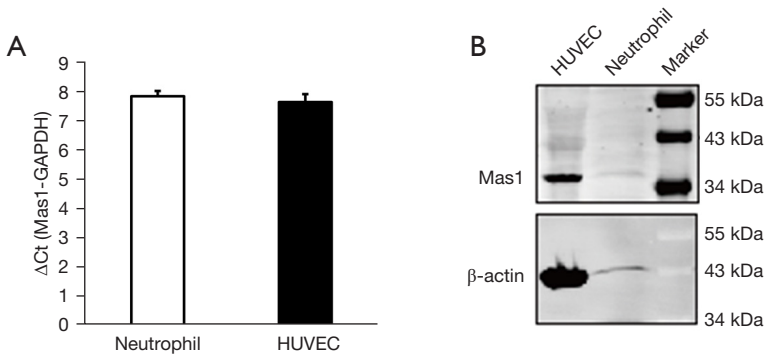

Figure 3 Mas receptor expression in human neutrophils. Mas receptor expression was detected in human neutrophils by quantitative RT-PCR (A), and western blot analyses (B). Human HUVECs were used as a control cell line. The data are presented as the mean ± SEM of 3 independent experiments. HUVEC, human umbilical vein endothelial cell; RT-PCR, real-time polymerase chain reaction; SEM, standard error of mean.

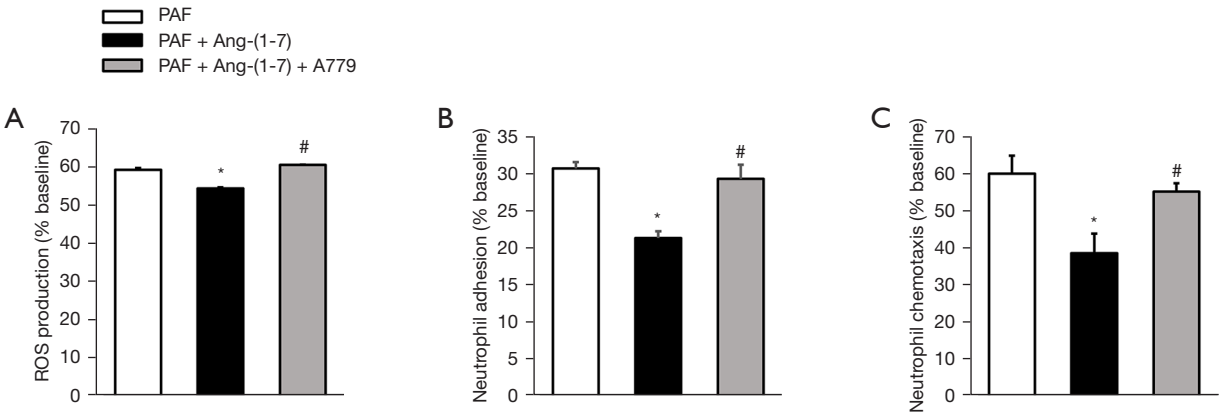

Figure 4 Ang-(1–7) attenuates respiratory burst, adhesion, and transmigration of neutrophils. Group data showing neutrophil ROS production (A), neutrophil adhesion to the HPMECs (B), and neutrophil chemotaxis across the monolayer of the HPMECs (C) when Ang-(1–7) (0.1 μmol/L) was administered either alone or in combination with the Ang-(1–7) receptor antagonist A779 (10 μmol/L) concomitantly in response to stimulation with PAF (10 mmol/L) in neutrophils. The data are presented as the mean ± SEM from n=3 per group. *, P<0.05 versus PAF control; #, P<0.05 versus PAF + Ang-(1–7). ROS, reactive oxygen species; HPMEC, human pulmonary microvascular endothelial cell; PAF, platelet-activating factor; SEM, standard error of mean.

Role of Ang-(1–7)/Mas in neutrophil function

The activation of the neutrophils and the interaction of the neutrophils with the endothelium represent substantial steps in the ALI. We examined the role of Ang-(1–7)/Mas in these processes. We first confirmed Mas receptor both protein and messenger RNA (mRNA) expression in the isolated human neutrophils (*Figure 3A,3B*). As Mas receptor expression was proven in the Human Umbilical Vein Endothelial Cell, it was used as the positive control.

Next, we tested the functional effects of Ang-(1–7)/Mas in the activation of the neutrophils. Stimulation with PAF caused a rapid, pronounced production of ROS (59.19%±0.45%), which is a key characteristic of respiratory bursts of neutrophils. However, in the group pretreated with Ang-(1–7), this response was largely attenuated (54.34%±0.25%). A779 reversed ROS production, which was inhibited by Ang-(1–7) (60.45%±0.12%) (*Figure 4A*).

We also investigated the effects of Ang-(1–7)/Mas on the neutrophil-endothelial interactions. After being triggered with PAF, 30.64%±0.83% of the neutrophils adhered to the endothelial monolayer. The number of adherent neutrophils significantly decreased due to pretreatment with Ang-(1–7)

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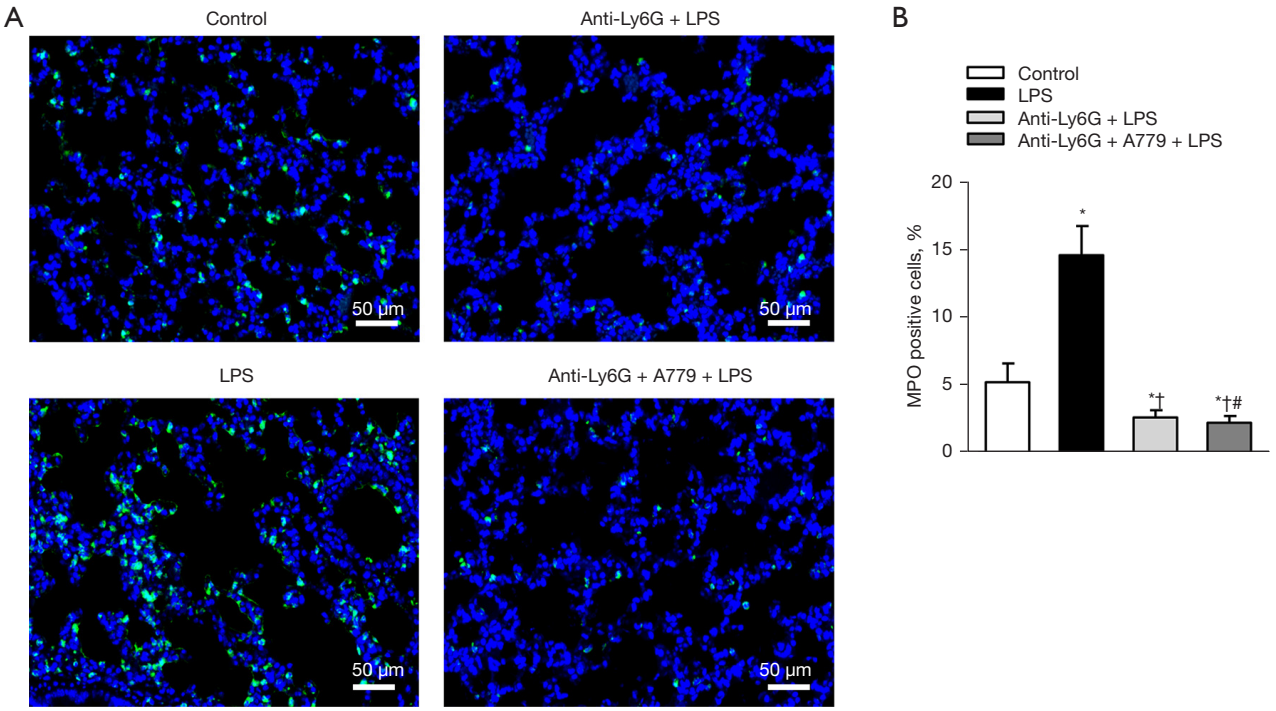

Figure 5 Neutrophil-depleting antibody anti-Ly6G reduces neutrophil recruitment in lungs. Anti-Ly6G antibody (clone 1A8, 200 μg) was administered intraperitoneally either alone or in combination with the Ang-(1–7) receptor antagonist A779 (12 nmol/kg) concomitantly with the induction of the LPS-induced ALI. Neutrophil depletion was confirmed by MPO immunofluorescence staining as shown by representative images in (A) and the percentage of MPO positive cells in (B). The data are presented as the mean ± SEM from n=5 mice per group. *, P<0.05 versus healthy control mice; †, P<0.05 versus LPS; #, P>0.05 versus *vs.* Anti-Ly6G + LPS. LPS, lipopolysaccharide; ALI, acute lung injury; MPO, myeloperoxidase; SEM, standard error of mean.

(21.26%±0.91%). Conversely, pretreatment with A779 resulted in an evident increase in the number of adherent neutrophils (29.24%±1.88%) (*Figure 4B*). Consistent with the results for Ang-(1–7)/Mas in relation to neutrophil adhesion to the endothelium, neutrophil transmigration across the endothelial monolayer was significantly reduced when the neutrophils were pretreated with Ang-(1–7) (59.83%±4.88% *vs.* 38.42%±5.30%); however, a concurrent pretreatment with Ang-(1–7) and A779 counteracted the effect of Ang-(1–7) (55.00%±2.27%) (*Figure 4C*).

Depletion of neutrophils attenuates ALI in the mouse model

To further examine the individual contribution of the neutrophils to ALI development, the neutrophils were depleted via the intraperitoneal administration of the anti-ly6G antibody 24 h before injury induction. The mice were then exposed to intratracheal LPS, and neutrophil recruitment and plasma leakage in the BALF were monitored.

Neutrophil depletion was first confirmed by MPO immunofluorescence staining, and the results showed that the anti-ly6G antibody significantly reduced neutrophil recruitment in the lungs (*Figure 5*). Neutrophil depletion abolished the BALF protein levels and the structural changes in the mice in response to LPS, which confirmed the importance of the neutrophils in ALI (*Figure 6A-6C*). Notably, the Ang-(1–7) receptor antagonist A779 partially reversed the protective role of neutrophil depletion in ALI (*Figure 6A-6C*), which shows the important role of Ang-(1–7)-Mas signaling in cells other than neutrophils.

Discussion

In the present study, we showed the critical role of Ang-(1–7) and its receptor Mas in the pathogenesis of ALI and in the activation of neutrophils. We developed a murine model of ALI that combined 2 injuries [i.e., the intratracheal instillation of HCl followed by ventilator-induced lung

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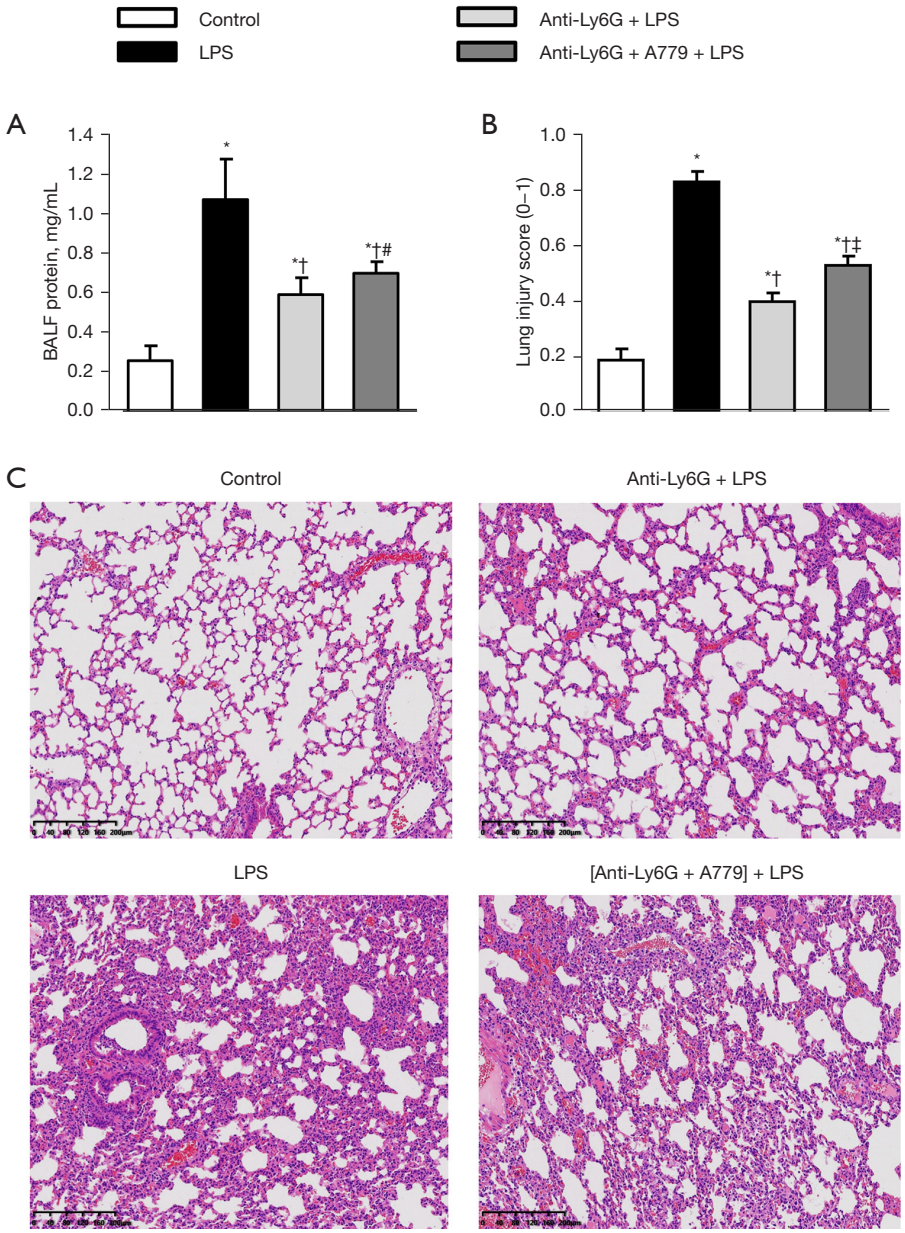

Figure 6 Depletion of neutrophils attenuates ALI in an animal model. Group data showing the effects of the anti-Ly6G antibody (clone 1A8, 200 µg, intraperitoneally) when administered either alone or in combination with the Ang-(1–7) receptor antagonist A779 (12 nmol/kg) concomitantly with the induction of the LPS-induced ALI on BALF protein concentration (A), and histological features of lung injury on a quantitative scale from 0 (no injury) to 1 (B). The histological features of lung injury are shown by representative histological micrographs by H&E staining (C). The data are presented as the mean ± SEM from n=5 mice per group. *, P<0.05 versus healthy control mice; †, P<0.05 versus LPS; #, P>0.05 versus LPS; ‡, P<0.05 versus anti-Ly6G + LPS. LPS, lipopolysaccharide; ALI, acute lung injury; BALF, bronchoalveolar lavage fluid; SEM, standard error of mean.

injury (VILI)]. Acid-aspiration mimics the clinical development of ARDS after the aspiration of the gastric contents (18). VILI is highly relevant in a wide variety of mechanical ventilation strategies in the care of critically ill patients (19). The 2-hit model of ALI addresses the limits of a simple injury that might only lead to a modest injury,

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and the fact that inducing ALI by a singular cause would fail to mimic its complex pathophysiological mechanisms.

The administration of exogenous Ang-(1–7) provided protection in the mouse models of ALI by attenuating the alveolo-capillary barrier failure, neutrophil invasion into the lungs, and inflammatory responses. The protective effect of Ang-(1–7) was essentially blocked by the Mas receptor blocker. In addition, we showed that Mas expression plays a critical role in circulating blood cells, as shown by Mas receptor expression in the human neutrophils. Further, we found that Ang-(1–7) was critical in neutrophil activation and function in response to PAF, as respiratory bursts, neutrophil adhesion, and migration were significantly reduced by exogenous Ang-(1–7). However, the protective effect of Ang-(1–7) was blocked by the Mas receptor blocker. Notably, we cannot exclude the effects of Ang-(1–7)-Mas signaling in cells other than neutrophils, as the Ang-(1–7) receptor antagonist A779 partially reversed the protective role of neutrophil depletion in ALI. The infiltration of other lymphocytes including T cells, Tregs and NK cells may also contribute to the process of ALI (20-22).

The RAS is a complex humoral system that is essentially involved in the regulation of blood pressure, electrolyte homeostasis, and water and sodium intake (23). Imai *et al.* found that ALI was more aggravated in ACE2-deficient mice than wild-type mice, and that the administration of recombinant human ACE2 attenuated ALI, and thus were the first to show the key role of the RAS in experimental models of ARDS (24). Over the past 2 decades, a number of animal and clinical studies have shown that the RAS plays an important role in the pathogenesis of ARDS (25-27). Ang-(1–7), as a metabolite of Ang II converted by ACE2, is the endogenous agonist for the Mas receptor. In animal models of ALI, the administration of Ang-(1–7) was shown to have some protective potential (e.g., it improved blood oxygen saturation, alleviated inflammation, and reduced fibrosis in the later stages of ARDS) (10,28,29). In our 2-hit mouse ALI model, the intravenous infusion of Ang-(1–7) attenuated the characteristic pathologic features of ALI; that is, acute lung edema, histological damage, and inflammatory responses. These results are in line with previous findings.

However, the underlying mechanisms by which Ang-(1–7) provides protection from ARDS are still not well understood. Cao *et al.* showed that the benefit of Ang-(1–7) administration in LPS-induced lung injury was related to the downregulation of AT1R expression (29). Erfinanda *et al.* revealed that the protection of ALI in females is partially attributable to endothelial barrier stabilization *via* the ACE2/Ang-(1–7)/Mas axis (9). Conversely, Ang-(1–7) has been shown to stimulate endothelial NO synthesis through the Mas receptor and increase NO production (30,31). Similarly, a previous study showed that Ang-(1–7) provided protection against ALI in different models partly by stimulating endothelial NO synthesis (11). To date, most research on Ang-(1–7) in ALI has focused on alveolar-capillary membrane permeability. However, the fundamental mechanisms that initiate and propagate the lung injury are complex. In addition to a defect in alveolocapillary permeability, dysregulated lung inflammation plays a crucial role in ALI (2).

Based on this notion, we examined the potential functional role of Ang-(1–7)/Mas in neutrophils that may be relevant to the pathogenesis of ALI. In the present study, we confirmed Mas receptor expression in human neutrophils at both the mRNA and protein levels. Next, we showed that the response of neutrophils to PAF is mediated by Ang-(1–7)/Mas. PAF has been shown to be an important mediator in ARDS (32), and it is a potent ether phospholipid mediator of inflammation that can activate neutrophil (33). Thus, it is ideally suited to examine the contributions of Ang-(1–7)/Mas to neutrophils in ALI.

We also demonstrated that ROS production in response to PAF, and neutrophil adhesion to and chemotaxis across endothelial cells were markedly attenuated by exogenous Ang-(1–7). Notably, this protective effect was achieved through the Mas receptor, and our subsequent finding showed that the benefits of the Ang-(1–7) treatment were effectively blocked by the Mas receptor blocker A779. These findings are in line with the *in vivo* results that MPO activity and the formation of inflammatory factors are regulated by Ang-(1–7)/Mas. The data showed that Ang-(1–7)/Mas provided protection against ALI by directly working on the neutrophils.

The Mas receptor is a member of the GPCR family. Neutrophils express a large number of GPCRs, including formyl-peptide receptors, classical chemoattractant receptors, and chemokine receptors (34,35). GPCRs are able to activate the chemotactic migration of neutrophils, and trigger ROS production and the exocytosis of intracellular granules and vesicles (36). In neutrophils, GPCRs transduce signals through the Gβγ heterodimer, activating 2 parallel pathways through PLCβ2/3 and PI3Kγ (37-39). The activation of PI3K and the subsequent production of PIP3 is one of the prominent pathways triggered by neutrophil GPCRs, which is in line with findings that the Ang-(1–7)/Mas axis activates PI3K/Akt/NO signaling in circulation and the kidneys

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(30,40,41). Thus, it may be that Ang-(1–7) mediates the activation of neutrophils through the Ang-(1–7)/Mas/PI3K/Akt/NO signaling pathway. Further research is required to investigate the underlying mechanisms.

Intratracheal nebulization increases drug delivery to the airway and alveoli, maximizing the exposure of the relevant sites. In addition, nebulization reduces the systemic effect of Ang-(1–7) on low blood pressure, as many patients with ARDS also suffer from vasopressor-dependent shock. Based on these notions, we tested the effect of intratracheal nebulization with Ang-(1–7). Remarkably, the protective effect of Ang-(1–7) was replicated in the mouse model of ALI with Ang-(1–7) intratracheal nebulization. To date, there is little data about Ang-(1–7) available for inhalation.

Among the members of the RAS, losartan and telmisartan had been reported with inhalation delivery in animal models (42,43). Recently, Magalhães *et.al*. reported that inhalation of Ang-(1–7) reversed inflammation and pulmonary remodeling in a mouse model of chronic asthma (44). Similar to our results, these findings show the suitability of delivering Ang-(1–7) via inhalation. However, a pharmacokinetic/pharmacodynamics study of Ang-(1–7) is unclear. Analyses of the drug discovery process, exposure, efficacy, and toxicity need to be conducted to confirm that inhalation results in localized or systemic effects. Nevertheless, our research showed the nebulized delivery of Ang-(1–7) attenuated ALI in an animal model.

Conclusions

The present study used a 2-hit mouse model of ALI by intratracheal instillation of HCl followed by over ventilation for the first time and demonstrated that exogenous Ang-(1–7) provided protection. The presence of Ang-(1–7) protects against ALI partly through its interaction with Mas receptor in neutrophils. Specifically, Ang-(1–7) abates the activation of neutrophils, reducing respiratory bursts, neutrophil adhesion, and migration. However, we cannot exclude the critical role of Ang-(1–7)-Mas signaling in other pulmonary cells. Our findings provide a new perspective for understanding the RAS role in the pathogenesis of ALI.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-6193/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6193/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. *In vivo* experimental protocols were approved by the Ethics Committee of Fudan University, and all experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals (7th edition 1996)*. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) with approval from the Ethics Committee of Zhongshan Hospital, Fudan University in Shanghai, China (No. B2021-715R). All healthy male volunteers signed an informed consent form.

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