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Soluble E-cadherin contributes to inflammation in acute lung injury via VEGF/VEGFR2 signaling

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Abstract

As a gatekeeper of the airway epithelial cells, E-cadherin is not only a critical component for the maintenance of epithelial integrity, but also engaged in pathological processes through the release of a soluble form (sE-cadherin). This study was aimed to investigate the role of sE-cadherin in ALI/ARDS. Serum samples from patients with ARDS and healthy volunteers were collected for the detection of sE-cadherin. An LPS-induced mouse model was induced to analyze the expression of sE-cadherin, and a neutralizing antibody against sE-cadherin (DECMA-1) was given to the LPS-exposed mice. The effects of recombinant sE-cadherin were tested both in vitro and in vivo, and VEGFR2 inhibition was used to explore a possible mechanism for sE-cadherin-induced pulmonary inflammation. We observed an increased level of sE-cadherin in ARDS patients as well as in LPS-exposed mice. In vivo treatment of DECMA-1 significantly attenuated LPS-induced inflammation. In vitro, exogenous sE-cadherin can dramatically upregulate the expression of VEGF in THP1-derived macrophages and human primary macrophages. In addition, intratracheal instillation of recombinant sE-cadherin leads to significant increased infiltration of neutrophils as well as overproduction of IL-6 and IL1β, which could be attenuated by inhibition of VEGF/VEGFR2 signaling. While blockade of the VEGF/VEGFR2 pathway inhibited pulmonary inflammatory responses in LPS-exposed mice. Taken together, our data demonstrated that sE-cadherin contributes to lung inflammation in ALI/ARDS, which is related to activation of the VEGF/VEGFR2 pathway.

Keywords Acute lung injury, Soluble E-cadherin, VEGF, VEGFR2

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Introduction

Acute lung injury (ALI), along with its more severe form acute respiratory distress syndrome (ARDS), is characterized by acute hypoxaemic respiratory failure and always associated with high mortality [1]. Over the past decades, considerable progresses have been made in understanding the epidemiology, pathogenesis and pathophysiology of ALI/ARDS, as well as relevant supportive care treatments. Yet effective pharmacological therapies for ALI/ARDS have not yet been available [2].

Multiple cells and mediators contribute to the pathogenesis of ALI/ARDS, among which the alveolar epithelia, endothelia and macrophages attract the most attention of researchers [3]. Yet the airway epithelial cells are rarely noticed. In fact, as the first line of defence against inhaled chemicals and pathogens, the airway epithelial cells also have a preeminent role in the development of ALI/ARDS. In 2004, Jansson AH et al. reported that rat trachea pretreated with LPS in situ significantly increased the influx of albumin from pulmonary circulation to the interstitium [4], indicating that activation of airway epithelial cells per se can induce lung edema. Later, with the aid of transgenic mice, Cheng DS and colleagues found that selective NFκB activation of the airway epithelium in vivo could lead to the production of copious amounts of inflammatory mediators, pulmonary edema, and neutrophil aggregation in the lung; while inhibition of airway epithelial NFκB prevented lung inflammation and injury induced by LPS delivered either to the airways or systemically [5], suggesting that the airway epithelial cells are crucial for the initiation of lung inflammation and injury in response to both local and systemic stimuli. Therefore, targeting the airway epithelial cells would be an effective therapeutic strategy for ALI/ARDS.

The airway epithelial cells display a highly coordinated and impermeable barrier along the trachea and bronchus through the assembly of tight junctions (TJs) composed of zonula occludens (ZO-1, ZO-2, ZO-3), occludin and claudins, as well as adherens junctions (AJs) composed of E-cadherin, β-catenin and etc [6]. As a gatekeeper of the airway epithelia, the transmembrane protein E-cadherin provides essential architecture and immunological functions in the airway [7, 8]. Currently, two forms of E-cadherin have been reported: a membrane-tethered form (full length) and a soluble form (cleaved form) [9]. Full-length E-cadherin (120 kDa) is anchored with other junctional proteins (including β-catenin) to maintain epithelial integrity. Soluble E-cadherin (sE-cadherin) is its cleaved product catalyzed by proteolytic enzymes such as α-secretase, a disintegrin and metalloproteases (ADAMs) and matrix metalloproteinases (MMPs). In contrast to the barrier function of full length E-cadherin, sE-cadherin acts in a totally different way. Free sE-cadherin can bind to the extracellular domain of full length E-cadherin to interfere the formation of adherens junction complex between adjacent epithelia [9]. Besides, a growing number of studies demonstrate that it's not only a critical contributor in tumor invasion and metastasis but also possesses potential proinflammatory capacities. Higher levels of sE-cadherin were detected in patients with chronic inflammatory diseases including COPD and arthritis, and were positively correlated with disease severity [10, 11]. In vitro, sE-caderin-positive exosomes could drive the activation of NFκB signaling cascade in endothelial cells, which can be inhibited by a neutralizing antibody against sE-cadherin [12], proposing a potential proinflammatory role for sE-cadherin. Likewise, in a recent study, we detected significantly increased levels of sE-cadherin in the airway both in patients with severe asthma and experimental murine models, and found that neutralizing sE-cadherin offered protection against allergen-induced mixed granulocytic airway inflammation in mice [13]. Previously, we've reported down-regulated expression of E-cadherin in the airway epithelia of LPS-induced ALI model [14], indicating the release of sE-cadherin. Therefore, in this study, we aimed to assess the expression pattern of sE-cadherin in this model and investigate its possible roles and mechanisms in ALI/ARDS.

Methods

Human samples

The use of human samples was approved by the Ethics Committee of Dongguan People's Hospital (KYKT2024-032). After obtaining written informed consent from participants or their legal surrogate, patients were recruited from the Department of Emergency Medicine and healthy volunteers were from the Department of Physical Examination of Dongguan People's Hospital during the period from 2023 to 2024. Serum samples from patients with ARDS were collected on the first day. ARDS was diagnosed when patients were admitted for respiratory support (day 1 from admission). Each patient with ARDS included in this study was diagnosed by the consensus of two independent experienced physicians based on the Berlin definition (18), and assessed fully for their clinical information, chest X-ray (CXR), echocardiography report, and PaO2/FiO2. Serum level of sE-cadherin was detected with a commercial ELISA kit (Elabscience Biotechnology Co., Ltd, Wuhan, China).

Reagents

Lipopolysaccharides (from Escherichia coli, O111: B4) was obtained from Sigma-Aldrich (Shanghai, China). ELISA kits for IL-6, IL-1β, TNF-α, HMGB1, MMP3, MMP7, MMP9 and sE-cadherin were purchased from Elabscience Biotechnology Co.,Ltd. (Wuhan, China).

Rabbit anti-VEGF, anti-VEGFR2 and phosoho-VEGFR2 (Tyr1175) antibodies were purchased from ABclonal Technology (Wuhan, China).

Animals

Six to eight week old specific pathogen-free (SPF) male BALB/c mice (20 ± 2 g) were purchased from Guangdong Medical Laboratory Animal Center. The mice were housed in a SPF condition with 12 h cycles of light and dark [temperature 23 ± 2℃, humidity range 40%~70%, 12 h light/dark cycle (lighting: 8:00–20:00)] and fed normally with sterile water and irradiated food ad libitum. All animal studies were conducted under the guidelines of the Committee of Guangzhou Medical University on the use and care of animals and approved by the Animal Subjects Committee of Guangzhou Medical University.

LPS-induced ALI model and treatments

LPS-induced ALI model was generated by intratracheal instillation of LPS (5 mg/kg; dissolved in 50 μL sterile saline) to the mice anesthetized by isoflurane inhalation. For isoflurane anesthesia, mice were placed in a small transparent box together with a cotton ball absorbent with isoflurane (0.5 ~ 1 mL). LPS was instilled when the skeletal muscles of their legs were fully paralysed (about 30 s in the box). Control mice received the same amount of sterile saline by the same procedure. In the first protocol, mice were enthanized at the 24^{th,} 48th, 72th and 120th hour (days 1, 2, 3 and 5) post LPS challenge to evaluate the time dependent responses induced by LPS.

In the second protocol, mice were randomly divided into four groups (8 ~ 10 mice per group): (1) saline-exposed and PBS-treated (control group); (2) LPS-exposed and PBS-treated (LPS group); (3) LPS-exposed and isotype IgG-treated (LPS + iso-IgG group); (4) LPS-exposed and DECMA-1-treated (LPS + DECMA-1 group). Anti-sE-cadherin monoclonal antibody [DECMA-1, Invitrogen (Catalog: 16-3249-82), 10 mg/kg per time] (Brouxhon et al., 2014), or the isotype control IgG [Invitrogen (Catalog: 16-4301-85), 10 mg/kg per time] was administered separately via the intraperitoneal (*i.p.*) route 1 h and 48 h after LPS challenge. Then the mice were sacrificed 72 h post LPS challenge for further analysis.

In the third protocol, mice were randomly divided into three groups (8 ~ 10 mice per group): (1) saline-exposed and vehicle-treated (control group); (2) LPS-exposed and vehicle-treated (LPS group); (3) LPS-exposed and ZM323881-treated (LPS + ZM group). ZM323881 (Selleck, Shanghai, China), a specific antagonist of VEGFR2, was given to mice through intraperitoneal injection 1 h, 24 h, and 48 h after LPS challenge, for a total of 3 times. ZM323881 was dissolved in PEG300 and diluted in PBS, and was given at the dose of 25 mg/kg per time. Control mice received the same volume of vehicle (25% PEG300 in PBS) by comparison.

Collection and analysis of bronchoalveolar lavage fluid

Mice were sacrificed by injection with an overdose of pentobarbital (100 mg/kg; *i.p.*). For bronchoalveolar lavage, mice were cannulated through the trachea and lavaged in situ by instilling and retrieving 0.8 mL of pre-warmed sterile saline into the lung. Then the recovered bronchoalveolar lavage fluids (BALF) were centrifuged at 500 rpm for 10 min at room temperature, and the pellet was immediately resuspended in 0.2 mL of sterile saline. Cell counts were then determined for each BALF sample, and differential cell counts were performed on cytospin preparations stained with haematoxylin and eosin (H&E). The total protein content of the BALF supernatants, an important indicator of alveolar-capillary permeability, was detected by the BCA kit (Thermo Fisher Scientific). The remaining supernatants were stored at -80℃ for further quantitative detection of IL1β, IL-6, TNF-α, sE-cadherin, HMGB1, MMP3, MMP7 and MMP9 according to the manufacturer's instructions (Elabscience Biotechnology Co.,Ltd., Wuhan, China).

Determination of pulmonary wet to dry (W/D) weight ratio

The W/D ratio of lung tissue is a critical indicator to assess the degree of pulmonary edema in ALI. To remove the excess blood in the intravascular pool, lungs were perfused with 5 mL of sterile saline via the pulmonary circulation, through catheterization of the right heart. Then, the lower right lung lobes of mice from each group were collected and weighed, which were recorded as wet weight. Afterward, they were placed into an 60 ℃ oven for 48 h for drying and then reweighed as dry weight. The ratio of wet weight to dry weigh (W/D) was then calculated for each mouse.

Histology

The left lung lobes were fixed in 4% neutral formalin, paraffin-embedded, cut in 4 μm sections, and stained with H&E for for blinded histopathological assessment. Lung injury was semi-quantified as previously described [14].

Cell culture and treatments

The BEAS-2B and A549 cell lines were purchased from ATCC and maintained in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). The third to fifteen passages of the cells were used in these experiments to ensure genetic stability of the culture. All cells were kept in a humidified incubator with 5% CO_2 at 37℃. Cells were cultured until 80% ~ 90% of confluence, and then starved for 10 h before treatment with recombinant human sE-cadherin.

THP-1 human monocytic cells (ATCC) were maintained in suspension at $2 \sim 8 \times 10^5$ cells/mL in RPMI-1640 supplemented with 10% heat-inactivated foetal bovine serum (Thermo Scientific) and 1% penicillin/streptomycin (Sigma-Aldrich). For experiments, 4×10^5 cells were plated in a 12-well plate and differentiated into macrophage-like cells by incubation with 100 nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) for 72 h. Differentiated cells were rested in PMA-free media for 24 h before experiments.

For human primary macrophages, peripheral blood mononuclear cells (PBMCs) were first isolated from healthy volunteers, following the protocol of Lymphoprep Density Gradient (Dakewe, Cat#7111011/7111012). Then 3×10^6 cells/well were seeded in 12-well plates in 1640 RPMI medium without supplements (37℃, 5% CO_2). Three hours later, supernatant was removed, and the cell monolayer was washed twice with PBS. Subsequently, cells were cultured in 1640 RPMI medium supplemented with 10% FBS, and 1% penicillin-streptomycin in the presence of 10 ng/mL human recombinant M-CSF (PeproTech), and incubated for 7 days at 37℃ in a humidified 5% CO_2 atmosphere to allow the differentiation of monocytes into macrophages.

Cytotoxicity assay

Cell viability was determined using the CCK-8 assay according to the manufacturer's instructions (Sigma). Briefly, 0.5×10^4 cells were seeded in 96-well plates and grown for 48 h before exposure to indicated treatments. Cells were then incubated with CCK-8 solution for 2 h at 37℃, then medium was aspirated to a microplate. The colorimetric analysis was measured at 450 nm with a microplate reader (Bio-Rad).

In vitro treatments of recombinant sE-cadherin

Cells were stimulated with 5 μg/mL or 10 μg/mL recombinant human sE-cadherin (R&D Systems, Catalog: 8505-EC) for 6–24 h.

Table 1 Demographic and clinical characteristics of enrolled subjects

	Healthy volunteers ($n=13$)	ARDS ($n=27$)
Demographics		
Age	48.08 ± 6.383	60.04 ± 19.35[§]
Gender (Female %)	61.54	22.22[☆]
Cause of ARDS		
Direct (%)	—	55.56
Indirect (%)	—	44.44
APACHE II score	—	18 (13.5, 22)
Oxygen index (PaO2/FiO2)	—	195.11 ± 66.43

[§]Age between groups was compared using Mann-Whitney test. $p=0.111$

[☆]Gender between groups was compared using Chi-squared test (Fisher's exact test). $p=0.0312$

Western blot

Lung homogenates or cell lysates were separated by 10% SDS-polyacrylamide gels and transferred onto PVDF membranes after mixed with 5×SDS loading buffer and boiled. Membranes were probed with antibodies against indicated antigens. After incubation with a IRDye® 800WC-conjugated secondary antibody (LI-COR Biosciences), immunoreactive bands were exposed to Odyssey® CLx Imager for image capture. Data analysis was done with Image J software.

Quantitative PCR

Fresh cells were harvested after exposure to recombinant sE-cadherin for 6–24 h. Total RNA was extracted with a RNAiso Plus kit (Takara, Guangzhou, China) and reverse-transcribed to complementary DNA using PrimeScriptTM RT reagent kit (Takara). Gene expression was quantified using SYBR Green Premix Ex Taq (Takara) by LightCycler 480 Fast Real-Time PCR System using the following amplification protocol: 95 °C/30 s followed by 40 cycles at 95 °C/5 s and 60 °C/30 s. The expression of each gene was normalized against the housekeeping gene gapdh in every PCR reaction. The primers used were listed in Supplementary Table S1.

In vivo treatments of recombinant sE-cadherin and VEGFR2 antagonist

Recombinant mouse sE-cadherin (R&D Systems, Catalog: 8875-EC) was diluted in PBS, and given to the mice via intratracheal instillation (5 μg/mouse, in 50 μL PBS). The VEGFR2 antagonist ZM323881 (0.1 μg/mouse in 50 μL PBS; Selleck, Shanghai, China) was diluted in PBS and intratracheally delivered into the lung. ZM323881 was administered in a single dose 30 min after sE-cadherin. 24 h after sE-cadherin treatments, lung tissue and BALF samples were collected for further detection.

Statistics

Analysis was done using GraphPad Prism Version 8 software (CA, USA). Data were presented as mean ± SD and the p-values between groups were calculated by using t test or one-way ANOVA with post hoc analysis (unless otherwise noted). $p \le 0.05$ was considered significant.

Results

Serum level of sE-cadherin was increased in patients with ARDS

The demographic and clinical characteristics of all subjects were shown in Table 1. A total of 27 ARDS patients and 13 healthy controls were enrolled, and their serum samples were collected for the detection of sE-cadherin.

As can be seen in Fig. 1A, the serum sE-cadherin level in ARDS patients was higher than that in healthy volunteers. In addition, we found that the increase in direct

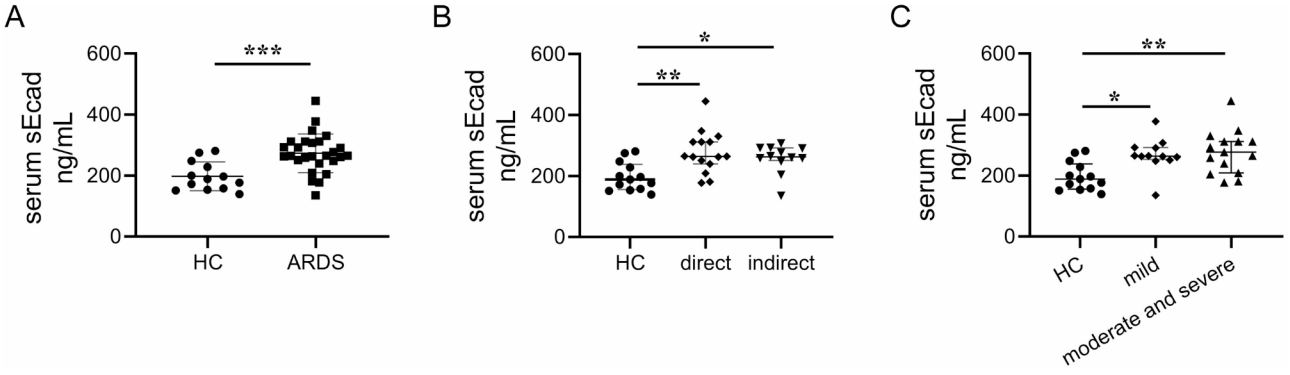

Fig. 1 The serum level of sE-cadherin was increased in ARDS patients. (**A**) Serum sE-cadherin levels were compared between healthy controls (HC, $n = 13$) and ARDS patients ($n = 27$). Data were expressed as mean ± SD and analysed using unpaired t test. (**B**) Serum sE-cadherin levels were compared among HC, patients with direct ARDS (caused by intrapulmonary factors) and patients indirect ARDS (caused by extrapulmonary or systemic factors). Data were expressed as median with IQR and analysed using Kruskal-Wallis test. (**C**) Serum sE-cadherin levels were compared among HC, mild ARDS ($200 < PaO2/FiO2 \leq 300$), as well as moderate and severe ARDS ($PaO2/FiO2 \leq 200$). Data were expressed as median with IQR and analysed using Kruskal-Wallis test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

ARDS patients (caused by intrapulmonary factors) was more significant than the increase in indirect ARDS patients (caused by extrapulmonary or systemic factors) when compared with healthy controls, though no statistical differences were observed between direct and indirect ARDS patients [278.0± 68.78 ng/mL vs. 257.5 ± 47.01 ng/mL] (Fig. 1, B). No significant differences of serum sE-cadherin level were detected between patients with moderate and severe ARDS ($PaO2/FiO2 \leq 200$) and patients with mild ARDS ($200 < PaO2/FiO2 \leq 300$) [277.5± 71.13 ng/mL vs. 268.1 ± 54.82 ng/mL] (Fig. 1, C). While no significant correlation between serum sE-cadherin level and APACHE II score was found ($p = 0.6169$, $R^2 = 0.0127$), nor between serum sE-cadherin level and oxygen index (PaO2/FiO2) ($p = 0.8868$, $R^2 = 0.0008$). These may be attributed to the limited number of samples in this study.

LPS-induced lung injury and inflammation was accompanied by time-dependent release of sE-cadherin in mice

Next we carried on to study the role of sE-cadherin in an animal model induced LPS. Previously, we've shown that lung inflammation rapidly increases within 3 days post LPS challenge [14]. In order to see if there is a peak of the response induced by LPS, pulmonary pathological changes as well as injury and inflammatory indicators were recorded every 24 h following LPS instillation. As can be seen in Fig. 2, LPS induced rapid weight loss within the first three days, and body weight of the mice reached the minimum at day 3 (Fig. 2, A). HE staining showed that alveolar and airway inflammation reached a peak at day 3 (Fig. 2, C and D), which agreed with the LPS-induced deaths within the first 3 days (Fig. 2, B). Analysis of BALF revealed that airway neutrophil counts rapidly increased from day 1 to day 3, and decreased on day 5 (Fig. 2, G). Similar changes were seen in BALF levels of IL1β, HMGB1 and sE-cadherin (Fig. 2, H, K and L), as well as W/D ratio of the lung and alveolar permeability as assessed by total BALF protein concentrations (Fig. 2, E and F). While peaks of BALF IL-6 and TNF-α levels were observed on day 1 and day 2 respectively (Fig. 2, I and J). Together, these data suggested that LPS induced time dependent responses in the lung and airway, with most severe injury and inflammation seen 3 days after LPS challenge. Therefore, in the following experiments, we chose this time point to evaluate the effect of neutralizing sE-cadherin.

Neutralizing sE-cadherin alleviated LPS-induced lung injury and inflammation

As the level of sE-cadherin was elevated within 24 h after LPS challenge and continued to raise till 72 h post LPS challenge, anti-sE-cadherin monoclonal antibody (DECMA-1) was given soon after LPS instillation, followed by a repeated dose at day 2. The antibody given at the dose of 10 mg/kg for twice neutralized more than half of the secreted sE-cadherin induced by LPS (LPS group vs. LPS + DECMA-1 group: 12.34 ± 3.732 vs. 3.381 ± 1.800 ng/mL) (Fig. 3, A). Accompanied by this were dramatically diminished airway and alveolar inflammation (Fig. 4, A and B), blunted neutrophil accumulation (Fig. 4, E), suppressed pulmonary edema (Fig. 4, C) and alveolar protein leakage (Fig. 4, D). BALF levels of HMGB1, VEGF, IL1β, IL-6, MMP3 and MMP9 were also significantly decreased (Fig. 3, B ~ E, G and I), while the LPS-induced TNF-α release was not affected by the neutralizing antibody (Fig. 3, F). The BALF level of MMP7 was not affected by either LPS or DECMA-1 (Fig. 3, H). These data demonstrated that blocking sE-cadherin can attenuate LPS-induced lung injury and inflammatory responses.

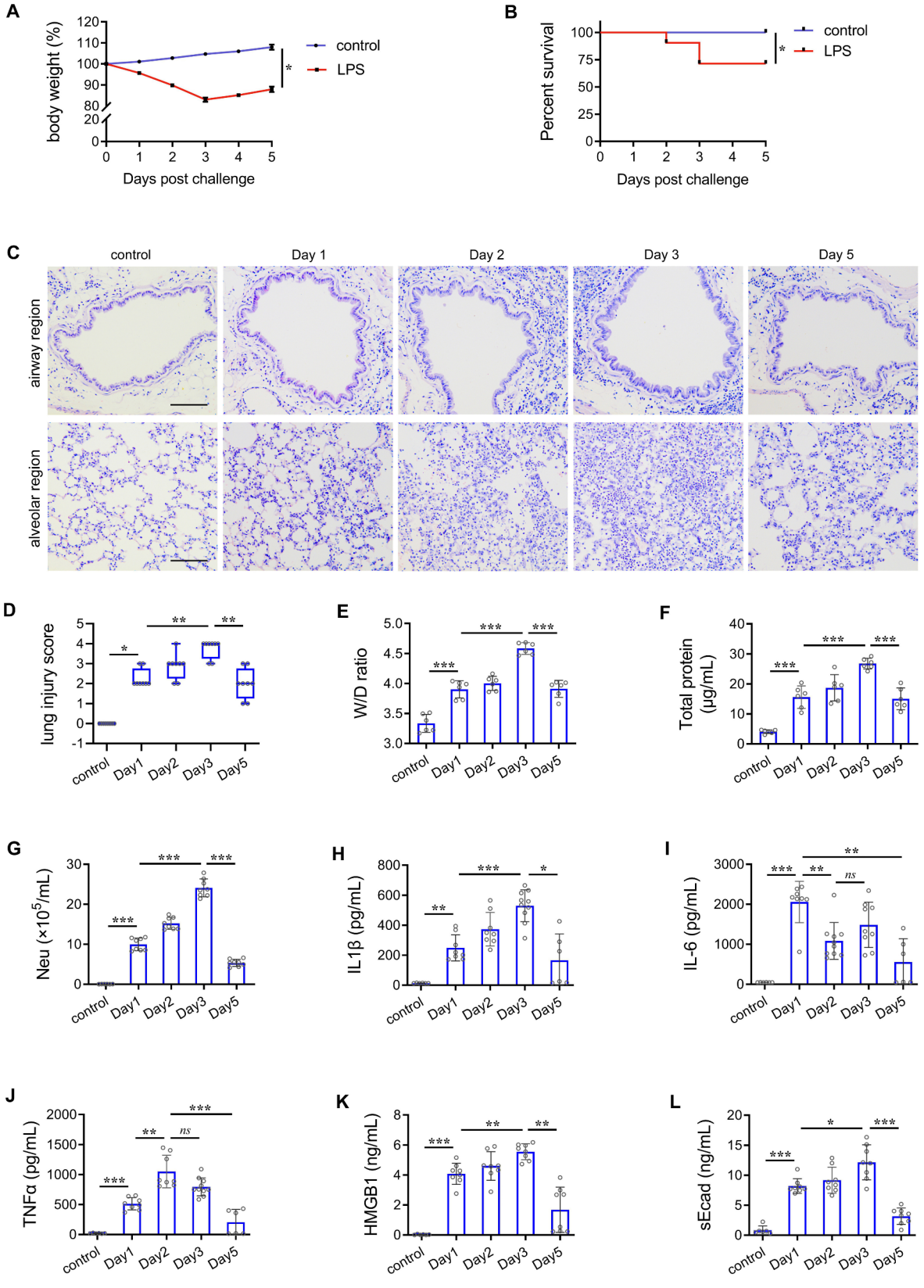

Fig. 2 (See legend on next page.)

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Fig. 2 LPS induced time-dependent lung injury and inflammation, as well as the release of sE-cadherin, with a peak seen 3 days post challenge. (**A and B**) Survival rates ($n = 20$) and body weight ($n = 8 \sim 9$) of the mice were recorded for five days immediately after LPS challenge. (**C**) Representative H&E stained lung sections at different time points after LPS challenge. Scale bar = 200 μm. (**D**) Lung injury score was semi-quantified. Data were expressed as median with IQR and differences between groups were compared using Mann-Whitney U test. (**E**) The pulmonary wet weight to dry weight (W/D) ratio was calculated to assess pulmonary edema. (**F ~ L**) Bronchoalveolar lavage fluids (BALF) were collected and analyzed.Total protein concentration in BALF was determined to assess alveolar-capillary permeability. Neutrophils were counted, and levels of sE-cadherin, HMGB1, IL1β, IL-6 and TNF-α were determined by ELISA. $n = 6 \sim 8$. Data were expressed as mean ± SD and analysed using One-way ANOVA unless otherwise indicated. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: none significance

Exogenous sE-cadherin increased the expression of VEGF in macrophages

Next we performed in vitro experiments to confirm the effects of sE-cadherin in cultured cell lines. The highest tolerated doses of recombinant sE-cadherin were determined by CCK-8 (supplementary Figure S1), and in the following experiments we used 10 μg/mL of sE-cadherin in BEAS-2B, 5 μg/mL in A549 and 10 μg/mL in THP-1 derived macrophages.

We first evaluated whether sE-cadherin had a direct proinflammatory capability. An array of inflammatory mediators including IL1β, IL-6, IL-8, TNF-α, VEGF-a (a major member of the VEGF family, VEGF hereafter), MMP3, MMP9, CSF3, CXCL1 and CXCL3 were detected after exposure to recombinant sE-cadherin for 6 h in both BEAS-2B and A549 cells. Yet none of their mRNA transcripts were up-regulated by recombinant sE-cadherin (supplementary Figure S2 and Figure S3). While in THP-1 derived macrophages, though no transcriptional changes of the proinflammatory mediators were seen within 6 h of sE-cadherin treatment (supplementary Figure S4), we found that the expression of VEGF was significantly up-regulated after 24 h exposure to recombinant sE-cadherin (Fig. 5, A), which was verified by western blot analysis (Fig. 5, C), while expression of the other inflammatory mediators still remained unchanged at the 24th hour (supplementary Figure S5). Similar results were seen when we repeated these experiments in primary human macrophages derived from peripheral blood mononuclear cells (PBMCs) (Fig. 5, B and C). Yet, in neither BEAS-2B or A549, the mRNA expression of VEGF was not altered after 24 h treatment of recombinant sE-cadherin (supplementary Figure S6).

VEGF/VEGFR2 is a critical proinflammatory signaling in LPS-induced acute lung injury

VEGF mainly acts through binding with its receptor VEGFR2[15], which contributes to neutrophil infiltration through phosphorylation at Y1175[16]. Though the proinflammatory role of VEGF in ALI/ARDS has been identified decades ago, here we carried on to ascertain this. We treated the LPS-challenged mice with a pharmacological inhibitor of VEGFR2, ZM323881. As shown in Fig. 6, treatment with ZM323881 significantly alleviated lung injury and edema (Fig. 6, A ~ C), neutrophil accumulation (Fig. 6, D), as well as alveolar protein leakage in the lung (Fig. 6, E), and reduced secretion of IL-6 and TNF-α (Fig. 6, G and H), though the LPS-induced increase of IL1β was not affected (Fig. 6, F). These data confirmed the proinflammatory role for VEGF/VEGFR2 in ALI/ARDS.

sE-cadherin induced neutrophilic lung inflammation partly through VEGF/VEGFR2

Having identified a pro-inflammatory role for sE-cadherin in vitro, we next sought to characterize its effects in vivo, by giving recombinant mouse sE-cadherin into the lungs of mice via intratracheal instillation. Consistent with the in vitro results, intratracheal recombinant sE-cadherin resulted in dramatically increased infiltration of total cells and neutrophils into the lungs after 24 h of treatment, accompanied by increased production of VEGF, IL-6, IL1β and TNF-α in BALF (Fig. 7, A ~ H). At the same time, we also detected a higher level of phosphorylated VEGFR2 (Y1175) in sE-cadherin-treated mice when compared with the vehicle control (Fig. 7, I and K), indicating activation of the VEGFR2 pathway. As VEGF mainly binds to VEGFR2 to initiate downstream angiogenic or proinflammatory functions [15], we treated the sE-cadherin-exposed mice with ZM323881, a small molecular antagonist of VEGFR2, to block the VEGF/VEGFR2 signaling. As expected, administration of ZM323881 significantly alleviated the neutrophil accumulation in the lung induced by sE-cadherin, and reduced the release of IL1β, IL-6, and TNF-α in BALF (Fig. 7), suggesting that the sE-cadherin-induced inflammatory response is partly dependent on VEGF/VEGFR2.

Discussion

ALI/ARDS is a devastating disorder with overwhelming intrapulmonary inflammation that can not be effectively prevented in most of the cases [17]. In this study, we found a significantly increased serum level of sE-cadherin in patients with ARDS, and demonstrated a pro-inflammatory role for sE-cadherin in LPS-induced lung injury and neutrophilic inflammation, partly through a VEGF/VEGFR2-dependent pathway.

ALI/ARDS is characterized by an uncontrolled inflammatory cascade that leads to disrupted pulmonary endothelial and epithelial barrier, increased vascular and alveolar permeability, as well as interstitial and alveolar pulmonary edema [18]. Most reported rodent models of

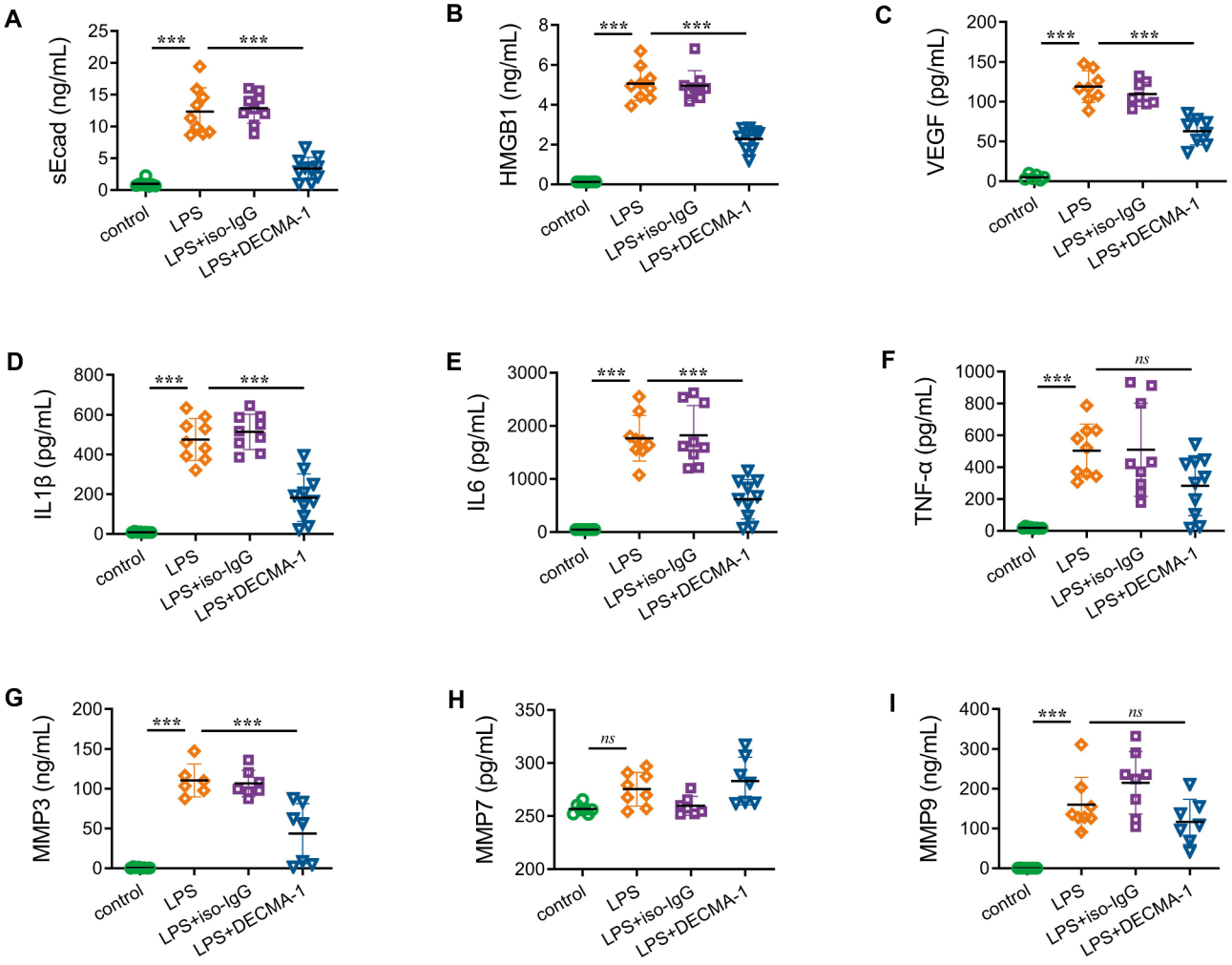

Fig. 3 Neutralizing sE-cadherin alleviated LPS-induced airway inflammation. (**A**) The level of sE-cadherin in BALF was quantified after treatment with the anti-sE-cadherin monoclonal antibody DECMA-1. (**B ~ I**) BALF levels of a list of proinflammatory mediators including HMGB1, VEGF, IL1β, IL-6, TNF-α, MMP3, MMP7 and MMP9 were detected. $n = 8 \sim 10$. Data were expressed as mean ± SD and analysed using One-way ANOVA. No significant differences were found between LPS group and LPS + iso-IgG group. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: none significance

ALI exhibited peaks of inflammation at the 24th hour following LPS challenge [19, 20]. Yet, clinically, this is not the case. Usually, pro-inflammatory mediators and neutrophil recruitment keep increasing rapidly from onset of the attack for days, leading to a vicious cycle that poses great challenges for the treatment of ALI/ARDS [21]. Previously, we've shown that lung injury and inflammation at the 72th hour after LPS challenge were far more severe than those at the 24th22. In order to find a peak of the response induced by LPS for future intervention studies, pulmonary injury and inflammatory indicators were assessed every 24 h following LPS instillation for a consecutive of five days. The results showed that mice exhibited the lightest body weight and the highest mortality rate at day 3, with the largest number of neutrophils in the lung, suggesting the peak of the disease. This agreed with the clinical finding that ALI/ARDS progresses rapidly with continuously growing production of TNF-α, IL1β, IL-6 and IL-8 within the first 3 days [22], and was in consistent with other rodent models of ALI [23, 24].

Apart from the inflammatory storm, epithelial injury is another cardinal feature of ALI/ARDS[2]. Of particular note, compromised integrity of the airway epithelial cells is not only a primary initiating factor but also an important determinant of the severity of ALI/ARDS [25]. Researchers have already demonstrated impaired airway epithelial barrier in patients with ARDS as well as murine models [26, 27]. In this study we also found that serum sE-cadherin, a marker of epithelial injury [28], was significantly increased in ARDS patients, indicating that airway epithelial injury may be a key step in the development of ALI/ARDS. Yet the direct consequences of injured epithelial barrier in ALI remains poorly characterized. Previously, we observed most significant epithelial E-cadherin down-regulation 72 h after LPS challenge

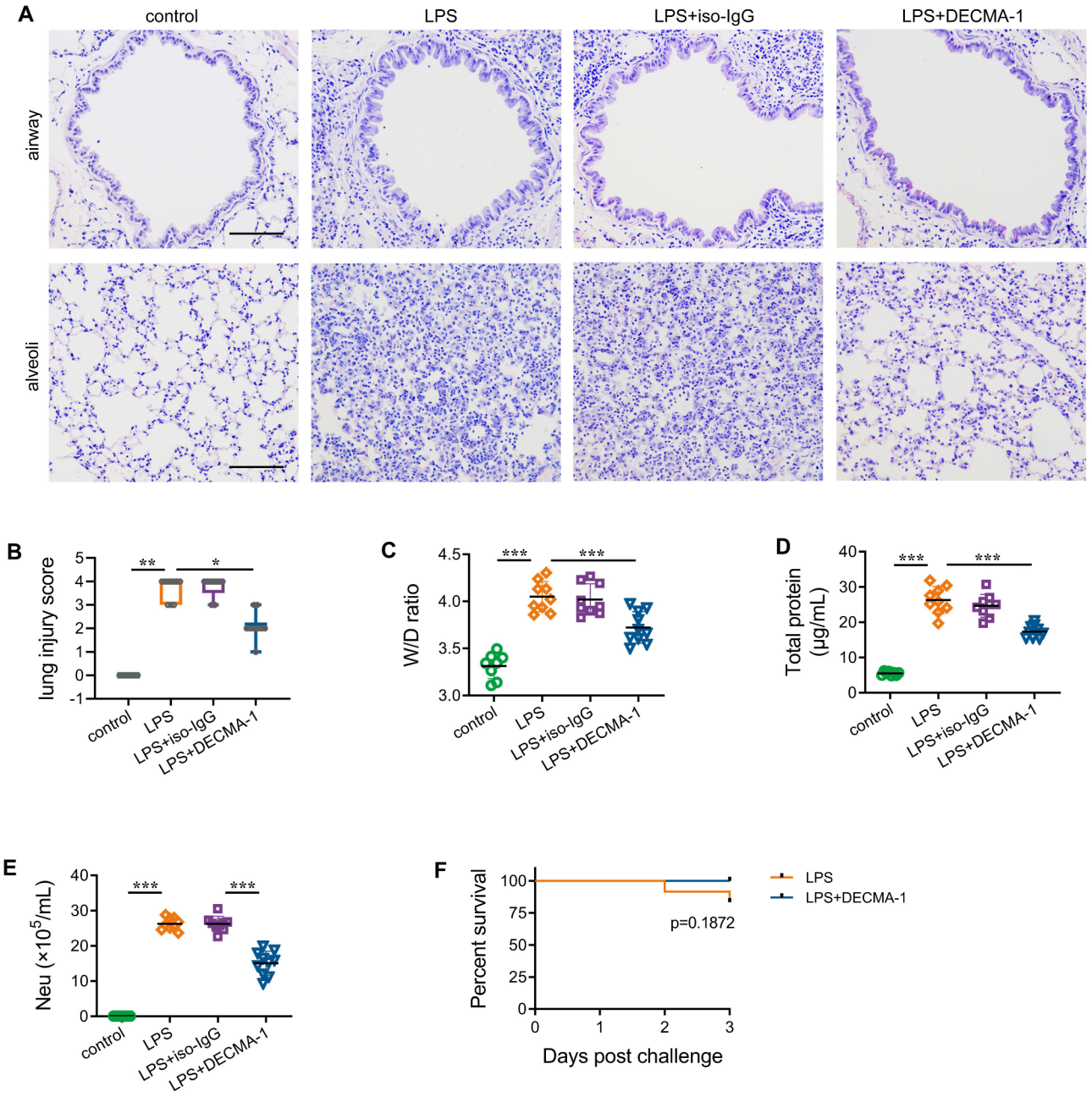

Fig. 4 Neutralizing sE-cadherin alleviated LPS-induced injury and inflammation in the lung. (**A and B**) Representative H&E stained stained lung sections of different treatment groups and semi-quantification of lung injury. Scale bar = 200 μm. Data were expressed as median with IQR and analysed using Kruskal-Wallis test. (**C**) The pulmonary wet weight to dry weight (W/D) ratio was calculated to assess pulmonary edema. (**D**) Total protein concentration in BALF was determined to assess alveolar-capillary permeability. (**E**) The number of neutrophils in BALF were analyzed after DECMA-1 treatment. $n = 8 \sim 10$. Data were expressed as mean ± SD and analysed using One-way ANOVA unless otherwise indicated. (**F**) Survival rates were compared between LPS group ($n = 12$) and LPS + DECMA-1 group ($n = 10$). No significant differences were found between LPS group and LPS + iso-IgG group. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

[14]. This was in good agreement with the considerable increases of sE-cadherin in BALF of LPS-exposed mice in the current study, whose changing curve coincided with those of pulmonary injury and neutrophilia. As a proteolytic product from the membrane-tethered full-length E-cadherin, sE-cadherin can be regarded as a marker of epithelial injury [28]. Intriguingly, the liberated sE-cadherin also displays a capacity to induce injury and inflammation. In vitro studies revealed that treatment with exogenous sE-cadherin would increase the release of TNF-α in KLRG1 + CD4 + T cells [29], while blocking sE-cadherin suppressed the activation of NFκB in

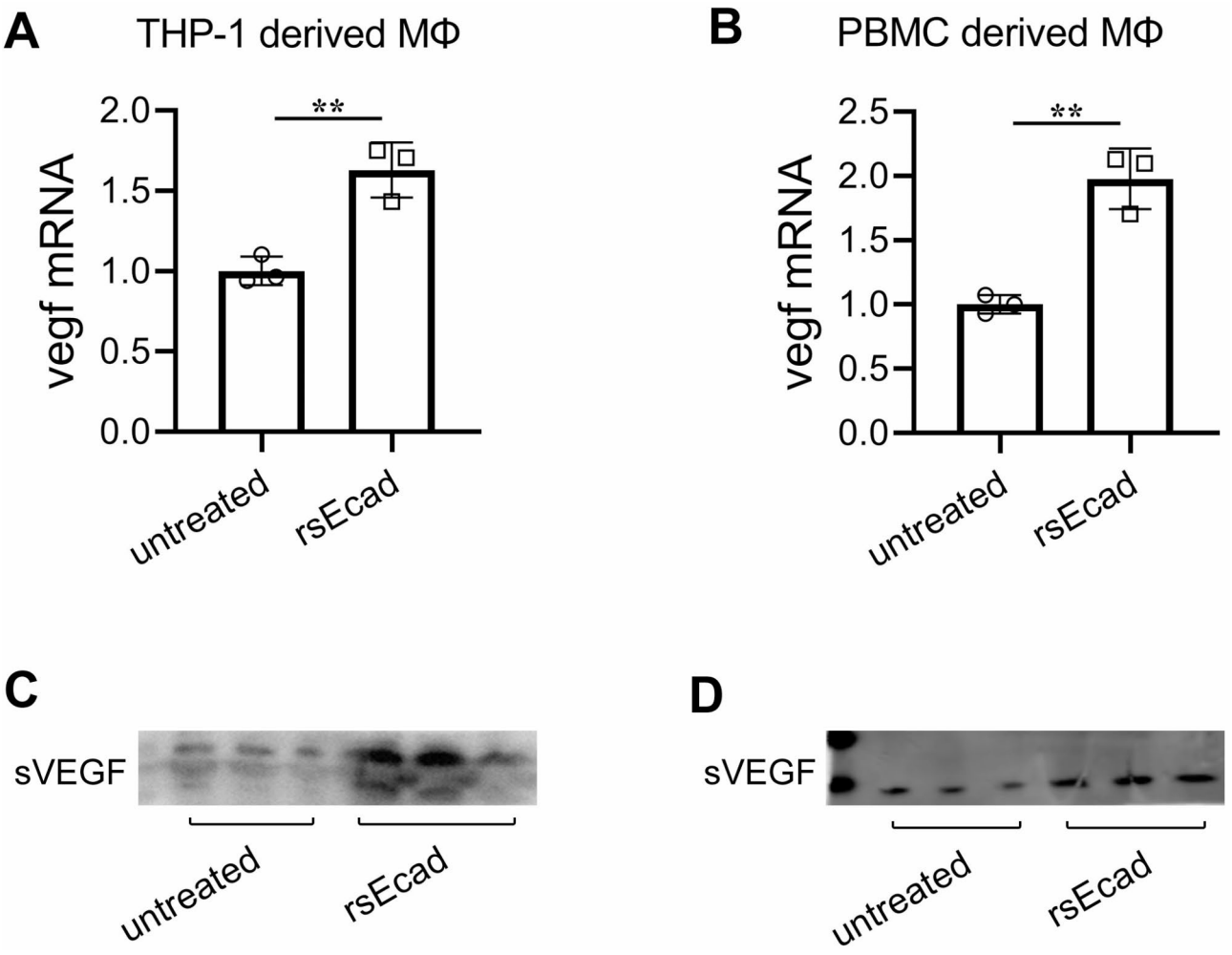

Fig. 5 Exogenous sE-cadherin increased the expression of VEGF in macrophages. THP-1 derived or PBMC derived macrophages (MΦ) were exposed to rsEcad (10 μg/mL) for 24 h. (**A and B**) Total RNA was extracted for qPCR quantification of the mRNA expression of VEGF. Its relative expression was normalized to the expression of gapdh. (**C ~ D**) Cell culture supernatants from THP-1 (**C**) or PBMC (**D**) derived MΦ were collected and subjected to western blot for analysis of secreted VEGF (sVEGF, 25KD). Data representative of at least three independent experiments ($n = 3$ wells/group) were expressed as mean ± SD and analysed using unpaired t test. **: $p < 0.01$

endothelial cells [13]. Therefore, in the present study, we used a sE-cadherin neutralizing antibody to investigate the possible role of sE-cadherin in ALI/ARDS. Though initially 1 mg/kg monoclonal antibody against sE-cadherin (DECMA-1) had no effects on LPS-induced lung injury in vivo (data not shown), treatment with 10 mg/kg DECMA-1 dramatically attenuated LPS-induced pulmonary edema, tissue injury, neutrophilia, as well as alveolar hyperpermeability, identifying the significance of E-cadherin and sE-cadherin in the development of ALI/ARDS. Despite the therapeutical effect of DECMA-1, we found that the LPS-induced secretion of TNF-α was not inhibited, which sounds in disagreement with the aforementioned in vitro studies [13, 29]. It should be noted that the LPS-induced peak of TNF-α release was at the second day, different from that of sE-cadherin and most other inflammatory indicators, indicating different pathways involved in the regulation of TNF-α expression. Though NFκB is the central signaling in inflammation, responsible for the upregulation of most inflammatory mediators including TNF-α, there are also NFκB-independent pathways involved in TNF-α expression. Likewise, Clarke CJ and colleagues also reported that IL-10 can suppress NFκB activity without affecting the expression of TNF-α [30]. These once again demonstrate the complex networks involved in sE-cadherin function, which calls for deeper investigations in the future.

There's substantial literature demonstrating that downregulation of E-cadherin in the bronchial epithelia would activate a series of signaling cascades that converge on an enhanced activity of the transcription factor NF-κB, leading to the production of excess amounts of proinflammatory mediators [31–33]. Release of sE-cadherin would induce similar responses [13]. Here we found that

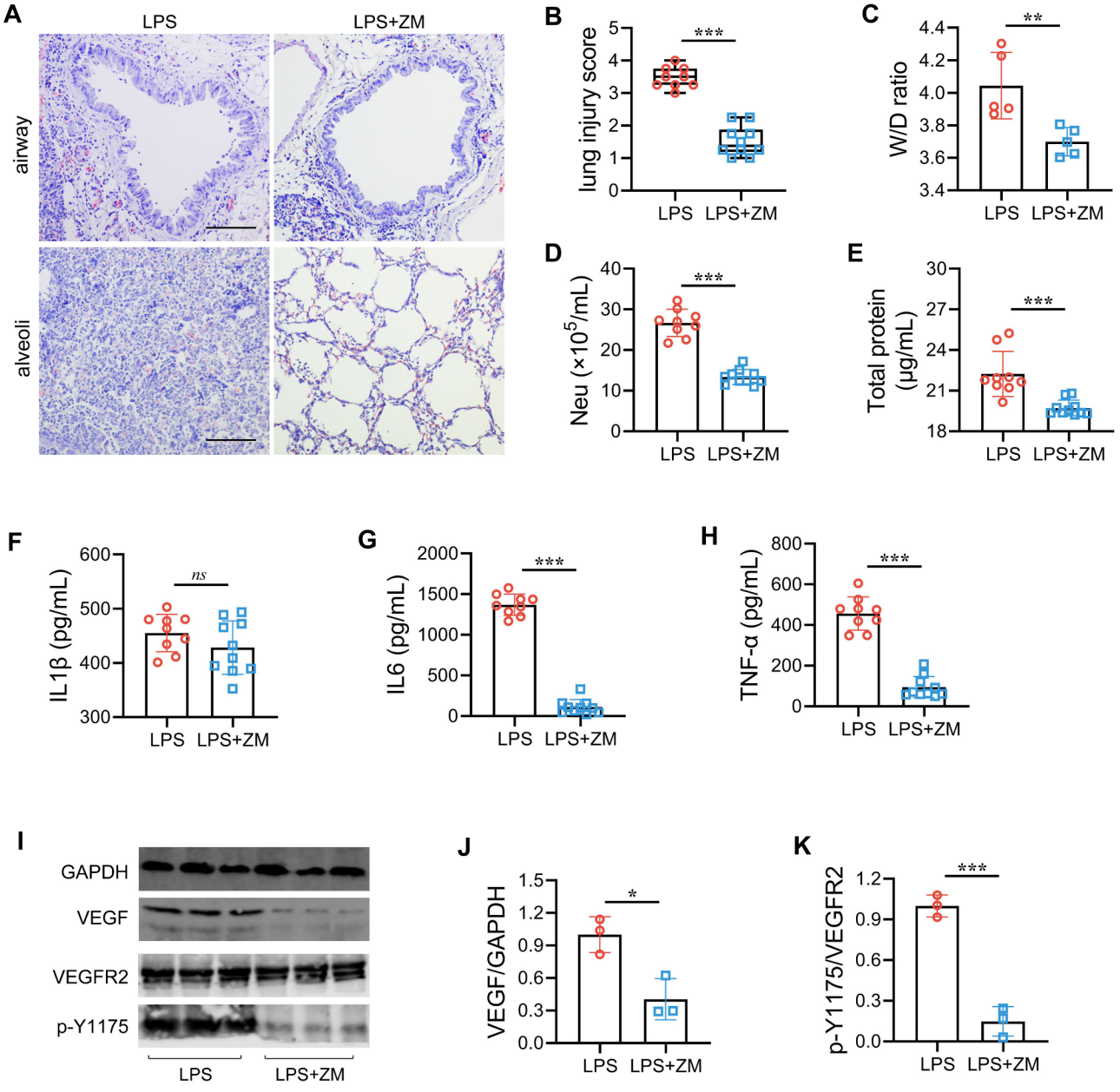

Fig. 6 VEGF/VEGFR2 contributes to LPS-induced lung injury and inflammation. A pharmacological antagonist of VEGFR2, ZM323881 (ZM, 25 mg/kg) was given intratracheally to mice after LPS challenge once daily for a consecutive of 3 days. (**A**) Representative HE-stained lung sections from different treatment groups showing inflammatory infiltration in the peribronchial (upper panel) and alveolar (lower panel) regions, as well as semi-quantification of lung injury (**B**). Scale bar = 200 μm. Data were expressed as median with IQR and analysed using Kruskal-Wallis test. (**C**) The pulmonary wet weight to dry weight (W/D) ratio was calculated to assess pulmonary edema. (**D**) The number of neutrophils in BALF were analyzed. (**E**) Total protein concentration in BALF was determined to assess alveolar-capillary permeability. (**F**~**H**) BALF levels of IL1β, IL-6, TNF-α were detected. $n = 8 \sim 10$. (**I**) Pulmonary expression of VEGF and phosphor-VEGFR2 (Y1175) was detected via western blot. Their relative expression was respectively normalized to GAPDH and VEGFR2. $n = 3$. Data were expressed as mean ± SD and analysed using One-way ANOVA unless otherwise indicated. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: none significance

IL-6, IL1β, MMP3 and MMP9 in BALF of LPS-exposed mice were all significantly down-regulated by treatment with DECMA-1. These led us to seek the direct effects of sE-cadherin on the expression of inflammatory mediators in vitro. The mRNA expression of a panel of canonical inflammatory mediators including IL1β, IL-6, IL-8, TNF-α, VEGF, MMP3, MMP9, CSF3, CXCL1 and CXCL3 were tested. Yet, none of them were found to be up-regulated by sE-cadherin within 24 h in BEAS-2B nor A549. It's worth noting that the functions of sE-cadherin are not confined to epithelial cells despite that they are the major sources of sE-cadherin. So we carried on to explore

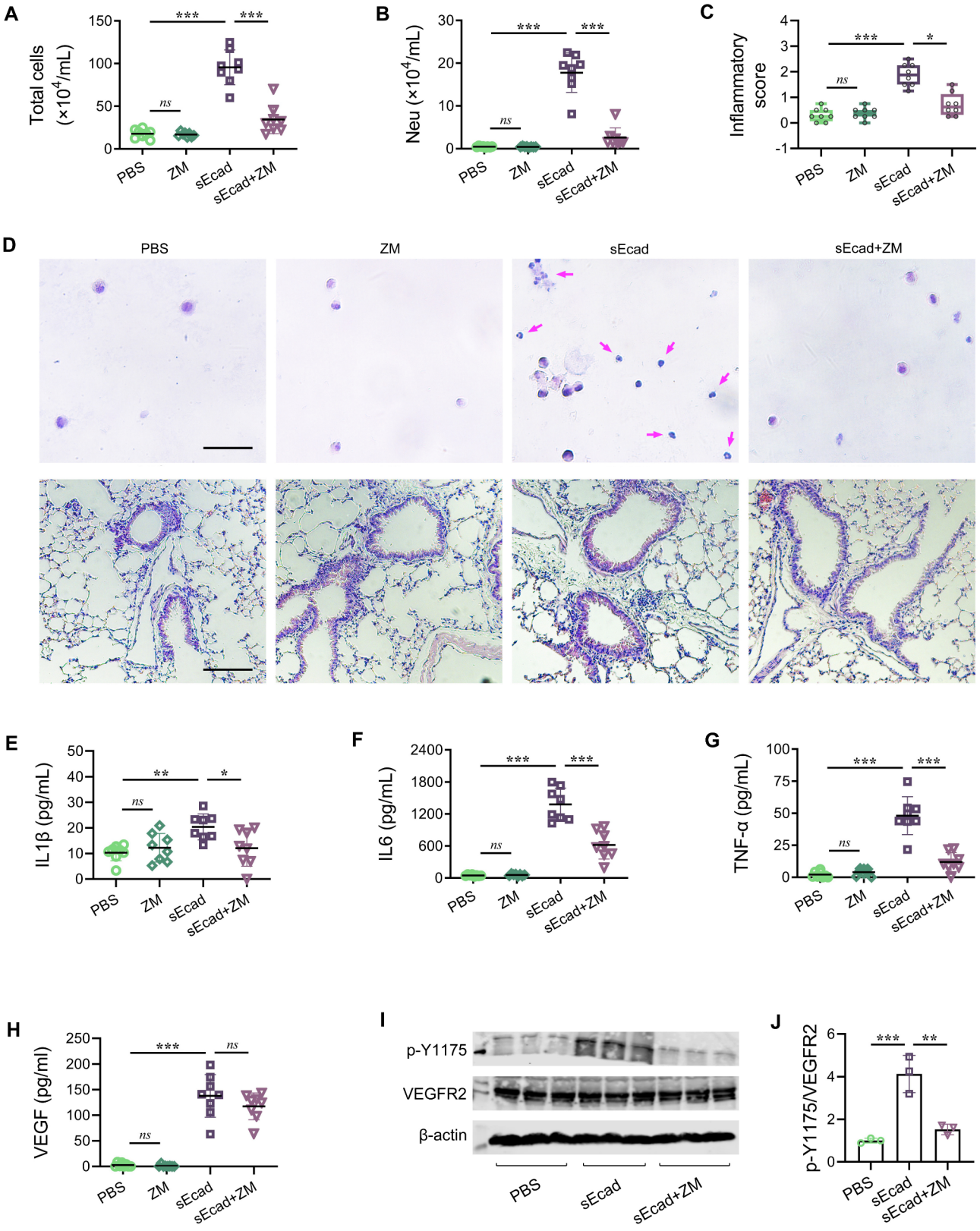

Fig. 7 (See legend on next page.)

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Fig. 7 sE-cadherin directly induced neutrophilic lung inflammation in a VEGF/VEGFR2 dependent pathway. Recombinant mouse sE-cadherin (5 μg/mouse) were given intratracheally to the mice, with or without the VEGFR2 antagonist ZM323881 (ZM, 0.1 μg/mouse). 24 h later, mice were sacrificed for analysis of lung inflammation. (**A and B**) The number of total leukocytes and neutrophils in BALF were determined in each group. (**C and D**) Semi-quantification of lung inflammation, as well as representative HE-stained cytospin samples from BALF (upper panel, Scale bar = 50 μm) and lung sections from different treatment groups (lower panel, Scale bar = 200 μm). Data were expressed as median with IQR and analysed using Kruskal-Wallis test. (**E ~ G**) The levels of IL1β, IL-6, TNF-α and VEGF in BALF were detected. $n = 8 \sim 10$. (**I and J**) Phosphor-VEGFR2 (Y1175) was detected via western blot analysis by using whole lung homogenates from mice treated with recombinant mouse E-cadherin. Its relative expression was normalized to total VEGFR2. $n = 3$. Data were expressed as mean ± SD and analysed using One-way ANOVA unless otherwise indicated. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: none significance

the potential effects of sE-cadherin in macrophages. And this time we discovered significantly increased production of VEGF. Subsequent in vivo experiments confirmed that intratracheal instillation of sE-cadherin could promote the release of VEGF in mice. Whether sE-cadherin up-regulates pro-inflammatory mediators in other cells is beyond the scope of this study. In brief, our data suggests that sE-cadherin could increase the expression of VEGF.

Originally isolated as an endothelial growth factor as well as a regulator of vascular permeability from cancer cells [34, 35], VEGF also acts as a powerful pro-inflammatory cytokine that is implicated in the pathogenesis of a multitude of inflammatory diseases [36–38], including ALI/ARDS. Elevated levels of VEGF were found in patients with ALI/ADRS as well as experimental rodent models [39, 40], and treatment with a neutralizing antibody significantly attenuated IR-induced lung injury and inflammation [41, 42], identifying a central role for VEGF in the pathogenesis of ALI/ARDS[43]. Though currently there's an apparent paradox of VEGF pertinent to ALI/ARDS, this suggests its double roles, namely, that VEGF facilitates epithelial repair following injury, but induces fluid and inflammatory flux across the exposed endothelium and epithelium if the alveolar-capillary barrier is functionally breached [43]. Without diminishing its repair function, which happens at the later proliferative and fibrotic phases of ALI/ARDS[2], here we mainly focused on its proinflammatory role in the early exudative phase. We detected up-regulated expression of VEGF in LPS-exposed mice, and found that inhibition of VEGF/VEGFR2 signaling dramatically alleviated the LPS-induced inflammatory responses in the lung. Downstream signaling of VEGF is mainly mediated by the receptor tyrosine kinase (RTK) VEGFR2, which directly binds to differential intracellular adaptor proteins upon phosphorylation, triggering a complex network of events that are crucial for its angiogenic, proliferative and proinflammatory functions [15, 44]. Several tyrosine phosphorylation sites of VEGFR2 have been discovered. Among those, phosphor-Y1175 and subsequent activation of the PLCγ-ERK axis are crucially engaged in driving neutrophil migration [16]. Of note, enhanced phosphorylation of VEGFR2 at Y1175 was detected in the lungs of mice after exposure to recombinant sE-cadherin for 24 h, an indication of activation of the VEGF/VEGFR2 signaling. Treatment with ZM323881, a specific VEGFR2 antagonist that inhibits tyrosine phosphorylation, significantly suppressed sE-cadherin-induced neutrophil infiltration into the lung and reduced the release of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α, implying that VEGF/VEGFR2 mediates neutrophil accumulation in the lung induced by sE-cadherin.

In summary, our data demonstrated that sE-cadherin contributes to lung inflammation in ALI/ARDS, which is related to activation of the VEGF/VEGFR2 pathway. Our study provides a link between barrier function and inflammatory response in the lung. Therefore, modulation of sE-cadherin may well have important clinical implications for the development of new therapeutic avenues toward ameliorating neutrophilic inflammatory responses in ALI/ARDS.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12964-025-02110-5.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Acknowledgements
Not applicable.

Author contributions
Tang H, Yao L, and Chen X designed this study. Yao L, Gan S and Chen Z performed most of the experiments. Li L, Fu C and Xie Z provided serum samples as well as clinical data from ARDS patients. Yao L and Tang H analyzed the results and wrote the manuscript. Gan S and Chen Z helped with the data analysis. Zhang H helped with the animal experiments. Yang C and Fu L helped with the in vitro experiments. Liu Y, Luo M, Kuang D and Hu G helped to collect clinical data of ARDS patients. Chen X, Yang L, Li L and Li S helped to revise the manuscript. All the authors agreed the final approval of the version to be published and ensured questions relating to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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

Declarations

Ethics approval and consent to participate
The use of human samples was approved by the Ethics Committee of Dongguan People's Hospital (KYKT2024-032). Informed consent was obtained from volunteers, patients or their families. All animal studies were conducted under the guidelines of the Committee of Guangzhou Medical University on the use and care of animals and approved by the Animal Subjects Committee of Guangzhou Medical University.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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