

The β 3/5 Integrin-MMP9 Axis Regulates Pulmonary Inflammatory Response and Endothelial Leakage in Acute Lung Injury

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Background: Acute lung injury (ALI) is a severe respiratory disease with high rates of morbidity and mortality. Many mediators regarding endogenous or exogenous are involved in the pathophysiology of ALI. Here, we have uncovered the involvement of integrins and matrix metalloproteinases, as critical determinants of excessive inflammation and endothelial permeability, in the regulation of ALI.

Methods: Inflammatory cytokines were measured by quantitative real-time PCR for mRNA levels and ELISA for secretion levels. Endothelial permeability assay was detected by the passage of rhodamine B isothiocyanate-dextran. Mice lung permeability was assayed by Evans blue albumin (EBA). Western blot was used for protein level measurements. The intracellular reactive oxygen species (ROS) were evaluated using a cell-permeable probe, DCFH-DA. Intratracheal injection of lipopolysaccharide (LPS) into mice was conducted to establish the lung injury model.

Results: Exogenous MMP-9 significantly aggravated the inflammatory response and permeability in mouse pulmonary microvascular endothelial cells (PMVECs) treated by LPS, whereas knockdown of MMP-9 exhibited the opposite phenotypes. Knockdown of integrin β 3 or β 5 in LPS-treated PMVECs significantly downregulated MMP-9 expression and decreased inflammatory response and permeability in the presence or absence of exogenous MMP-9. Additionally, the interaction of MMP-9 and integrin β 5 was impaired by a ROS scavenger, which further decreased the pro-inflammatory cytokines production and endothelial leakage in PMVECs subjected to co-treatment (LPS with exogenous MMP-9). In vivo studies, exogenous MMP-9 treatment or knockdown β 3 integrin significantly decreased survival in ALI mice. Notably, knockdown of β 5 integrin alone had no remarkable effect on survival, but which combined with anti-MMP-9 treatment significantly improved the survival by ameliorating excessive lung inflammation and permeability in ALI mice.

Conclusion: These findings support the β 3/5 integrin-MMP-9 axis as an endogenous signal that could play a pivotal role in regulating inflammatory response and alveolar-capillary permeability in ALI.

Keywords: acute lung injury, endothelial cells, integrin, MMP-9

Introduction

Acute lung injury (ALI) is a lethal pulmonary disease, characterized by increased vascular permeability, intrapulmonary retention of neutrophils, and synthesis of proinflammatory cytokines.¹ Lung endothelial cells (ECs) injury plays an important role in the dysfunction of alveolar-capillary barrier, which is a characteristic feature of ALI.^{2,3} ECs were activated in the setting of inflammation, and resulted in

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increasing pro-inflammatory cytokines release, recruitment of circulating leukocytes adhesion to ECs, and deteriorated ECs permeability.^{4,5} Lipopolysaccharide (LPS), also named endotoxin, has a pro-inflammatory effect and involves in the pathogenesis of Gram-negative bacterial infection, which stimulates innate immunity and triggers biochemical and cellular responses that lead to inflammation and toxicity.^{6,7} LPS is a primary pathogenic mediator of ECs activation, which activates the classic NF- κ B pathway, to induce the expression of several endogenous molecules, such as high-mobility group box 1 (HMGB1), integrins, and matrix metalloproteinases.^{8–10} This study was focused on integrins and matrix metalloproteinases in ECs and ALI.

Integrins are a family of cell surface glycoproteins, which include 24 receptors, each involving a single α subunit and a single β subunit. Integrins are expressed in all cell types and serve as a family of cell-cell and cell-extracellular matrix (ECM) binding transmembrane receptors capable of signal transduction.¹¹ Studies have indicated many integrin receptors play pivotal roles in the process of inflammatory responses.^{9,12} We and others have demonstrated critical roles for three integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$) in regulating lung inflammation and permeability.^{13–15} These results have identified these three integrins as potential therapeutic targets in ALI. Integrins are transmembrane adhesion receptors essential for cell communication with the environment and in particular with the ECM, which can be processed by several enzymes. Matrix metalloproteinases (MMPs) are one of the largest members involved in this process.

MMPs, a family of zinc-dependent extracellular enzymes secreted into the extracellular space and have important roles in many physiological and pathological processes, involving tissue repair, tumor invasion, and acute lung injury.^{16–18} Matrix metalloproteinase-9 (MMP-9 or gelatinase B) belongs to a family of MMPs that are released by inflammatory and noninflammatory cells, such as neutrophils and endothelial cells.^{19,20} Previous studies have focused on the role of MMP-9 in various LPS-induced acute inflammatory models, and MMP-9 can act as a regulator to promote the secretion of other cytokines by leukocytes.^{21,22} However, the precise mechanism for MMP-9 in the regulation of ECs and ALI remains unclear. In the current study, we performed to uncover the regulatory effect of $\beta_3/5$ integrin and MMP-9 on mouse pulmonary microvascular endothelial cells (PMVECs) in vitro, as

well as the LPS induced murine lung injury models in vivo.

Materials and Methods

Reagents

Antibodies against MMP-9 (ab228402), V5 (ab27671), Integrin β_3 (ab119992), ICAM-1 (ab222736), VCAM-1 (ab134047), TRAF-6 (ab33915) and β -actin (ab8227) were purchased from Abcam. Anti- Integrin β_5 (#3629), and NF- κ B proteins (NF- κ B Pathway Sampler Kit #9936) were purchased from Cell Signaling Technology. Pierce™ Protein A/G Plus Agarose was obtained from Invitrogen. LPS, rhodamine B isothiocyanate-dextran, and p-aminophenylmercuric acetate (APMA) were purchased from Sigma-Aldrich. Murine recombinant MMP-9 (R&D, 909-MM), MMP-9 ELISA kits (MMPT90), IL-6 ELISA kits (M6000B), TNF- α ELISA kits (DY410), CXCL-1 ELISA kits (MKC00B), and MPO ELISA kits (DY3667) were from R&D Systems. MMP-9 neutralizing monoclonal antibody (IM09L) was from Sigma-Aldrich. NF- κ B inhibitor, PDTC, and ROS scavenger, N-acetyl-L-cysteine (Nac), were obtained from MedChem Express (Shanghai, China). LipoJet™ reagent and GeneMute siRNA transfection reagent were purchased from SignaGen (Ijamsville, MD, USA).

Animals and ALI Models

Male wild-type mice (C57BL/6; 8–12 weeks) were housed and cared in the specific pathogen-free animal care facility at Shanghai Jiao Tong University following the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals as well as the guidelines of Shanghai Ruijin Hospital (approval number: SYXK 2018-0027). Mice in this study were randomly assigned to indicated groups by an investigator who was blinded to the group allocation. Acute lung injury (ALI) models were performed by intratracheal administration of LPS (2 mg per kg body weight) for 24h. Besides, C57/BL6 mice were intravenously administered of lentivirus vectors or lenti-Itgb3/5 (5×10^7 plaque-forming units per mouse) for 5 days prior to intratracheal administration of LPS.

Cell Culture and Transfection

Mouse pulmonary microvascular endothelial cells (PMVECs) and HEK-293T cells were obtained from Procell and cultured in DMEM with 10% fetal bovine serum. Penicillin (50 μ g/mL) and streptomycin (50 μ g/

mL) were added to cell cultures. PMVECs were authenticated and tested for mycoplasma contamination before conducting experiments. LipoJet™ reagent was used to overexpress *Itgb3/5* in PMVECs, and 24 or 48hr later, cells were treated with stimulators. The coding sequence of mouse *Itgb3* or *Itgb5* with C-terminal V5 epitope was synthesized and cloned into pCMV between HindIII and BamHI sites. For target genes knockdown, scramble siRNA, *MMP-9* siRNA, *Itgb3* siRNA, *Itgb5* siRNA were used to transfect PMVECs for 48 h by GeneMute siRNA transfection reagent. Cell lysates were collected in Lysis Buffer (150 mM NaCl, 50 mM Tris, 1.0 mM EDTA, 2 mM dithiothreitol, 0.025% sodium azide, and 1 mM phenylmethylsulfonyl fluoride), and prepared by brief sonication at 4°C.

Western Blot Analysis

The lysates of cells or lung tissues were extracted in ice-cold lysis buffer RIPA with Protease and Phosphatase Inhibitor Cocktail (Invitrogen). Lysates were prepared by brief sonication at 4°C. Insoluble cellular debris was precipitated via centrifugation at 12,000g for 15 min at 4°C. Lysate supernatant was normalized for protein concentrations, which were measured with use of a Bio-Rad Protein Assay Kit. Protein was eluted by dilution in denaturing loading buffer to a final 1X mixture and then incubated at 90°C for five minutes. Equal amounts of proteins were resolved by SDS-PAGE and then electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). These membranes were blocked with 5% skim milk for 1 h at room temperature and subsequently incubated with a primary antibody overnight at 4 °C. The membranes were then washed three times at 10 min intervals with PBST. After the addition of a secondary antibody for 1 h at room temperature, membranes were screened by the Odyssey Infrared Imaging System (LI-COR) and quantified through the Image J software.

RNA Extraction, Reverse Transcription PCR, Quantitative PCR

The total RNA was isolated from PMVECs cells using TRIzol reagent (Life Technologies, Grand Island, NY) and 1µg total RNA was reverse-transcribed to cDNA using the first-strand cDNA synthesis kit (Takara) according to standard protocols. The target genes were amplified and quantified in a LightCycler 480 Instrument II machine

(Roche Life Science) using SYBR Green PCR mixture (KAPA Biosystems). The data were normalized to GAPDH and analyzed with the $2^{-\Delta\Delta Cq}$ method. Primers for qPCR in this study are presented in [Supplementary Table 1](#).

Intracellular ROS Detection

A cell-permeable probe, DCFH-DA was used to measure intracellular ROS levels. PMVECs were seeded in 6-well plates and treated as indicated before incubation with DCFH-DA for 30 min at 37 °C. Cells were washed with PBS and then measured using flow cytometry.

Immunoprecipitation Assay

PMVECs were seeded in 10-cm dishes and treated with LPS with or without Nac before collection. Cell lysates were extracted in ice-cold IP buffer (0.25% Triton-X-100 in 1x PBS, pH 7.6, 0.025% sodium azide, and 1 mM phenylmethylsulfonyl fluoride) and prepared by brief sonication at 4°C. Lysate supernatant was collected by centrifugation at 12,000g for 15 min at 4°C and normalized for protein concentration. Supernatants were incubated with the indicated antibody for three hours at 25 °C. The immunoprecipitated protein was captured with 25 µL resuspended volume of Protein A/G PLUS-Agarose for 1 h before three rounds of washing with IP buffer (1 mL). Protein was eluted by dilution in denaturing loading buffer and incubation at 88°C for five minutes. Eluted samples were resolved by SDS-PAGE and subjected to immunoblotting.

Endothelial Permeability Assay

PMVECs monolayers were seeded in transwell filters (0.4-µm pore size; Corning) and treated as indicated prior to measurement by the passage of rhodamine B isothiocyanate-dextran (average MW ~70,000; Sigma) as described.²³ Briefly, the top well was placed with rhodamine-dextran at 20 mg/mL, and the fluorescence in the bottom well was screened by detecting 40 µL medium aliquots in a time course using a SpectraMax microplate reader at 544 nm excitation and 590 nm emission.

Alveolar-Capillary Permeability by Evans Blue Albumin (EBA)

Lung alveolar-capillary permeability was estimated using EBA based on our previous description.¹⁰ Mice were anesthetized and injected vena jugularis externa with

EBA (20 mg/kg). At 30 min after EBA injection, mice were killed, and lung tissue was harvested for EBA measurement.

NF- κ B Promotor Assay

Cignal NF- κ B Reporter luciferase plasmids were co-transfected with empty, *Itgb5*, control siRNA, or *Itgb5* siRNA for 24 to 48 hours before vehicle, LPS with or without rMMP-9 treatment for an additional 6 to 18 hours. Cells were then collected and assayed for firefly and Renilla luciferase activity. NF- κ B promoter activity was normalized by firefly and Renilla luciferase activity ratio.

H&E Staining and Lung Injury Scoring

Hematoxylin&eosin (H&E) staining and assessment of histological lung damage were performed as described previously.²⁴ Briefly, the left lungs from mice were dehydrated and embedded in paraffin. All lung fields were performed to H&E for the measurement of inflammatory cells infiltration and pulmonary morphology and determined at $\times 20$ magnification for each sample. Lung histology was scored by an expert clinical panel blinded to the group allocation.

Lentivirus Construction

To generate lentivirus encoding *Itgb3/5*, pLVX-*Itgb3/5* plasmid was co-transfected with Lenti-X HTX packaging plasmids (Clontech) into 293FT cells following the manufacturer's instructions. Seventy-two hours later, virus was collected and concentrated using Lenti-X concentrator.

Statistical Analysis

All data in this study were expressed as means \pm SEM of independent experiments. Group comparisons were performed using *t*-test or one-way analysis of variance (ANOVA) with Tukey's post hoc test. Survival curve was measured by Mantel-Cox test. $P < 0.05$ ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$) was regarded as statistically significant. All statistical analyses were carried out using the GraphPad Prism 8.0 program.

Results

MMP-9 Regulates Inflammation and Permeability in LPS-Induced PMVECs

As MMP-9 was regarded as an inflammatory mediator, we determined to explore its role in PMVECs treated with or without LPS. We found exogenous recombinant MMP-9

(rMMP-9) significantly enhanced the mRNA levels of *IL-6*, *CXCL-1*, *ICAM-1* and *VCAM-1* in PMVECs treated by LPS, whereas rMMP-9 treated alone did not notably affect these genes expression (Figure 1A–D). The protein levels also showed a similar pattern, with rMMP-9/LPS co-treatment increasing TRAF6, ICAM-1, and VCAM-1 protein amounts among treatment groups (Figure 1E). In addition, the NF- κ B signaling inhibitor, PDTC (pyrrolidinedithiocarbamate), drastically decreased MMP-9 protein amounts in LPS-treated PMVECs (Figure 1F).

To further explore whether MMP-9 would affect NF- κ B signaling, exogenous rMMP-9 was used to treat 293T cells and showed a significant increase in LPS (100 ng/mL) mediated NF- κ B promoter activity (Figure 1G), whereas *MMP-9* knockdown drastically reduced NF- κ B promoter activity (Figure 1H). The expression of endogenous MMP-9 in protein and mRNA levels was efficiently inhibited by siRNAs (Figure S1A and B). Based on the NF- κ B promoter activity assay, we found siRNA to *MMP-9* treated cells showed a significant decrease in LPS mediated increases in phospho-NF- κ B proteins, including p-IKK, p-P65, and p-I κ B α at different time points (Figure S1C). Exogenous rMMP-9 did not affect NF- κ B signaling, but in the presence of LPS, rMMP-9 significantly increased phospho-NF- κ B proteins in PMVECs (Figure S1D). Next, mouse pulmonary microvascular endothelial cells (PMVECs) were transfected with siRNA (or scrambled control) to *MMP-9* and exposed to LPS (1 μ g/mL) at indicated time points. siRNA to *MMP-9* treated cells showed a significant decrease in LPS mediated increases in IL-6 levels (Figure 1I). Exogenous rMMP-9, by itself, did not affect IL-6 secretion, but in the presence of LPS, rMMP-9 increased expression of IL-6 (Figure 1J). Further, the permeability assay was showed by detecting the traversing of rhodamine-dextran probes via PMVECs monolayers growing on 0.4- μ m filters. Similar to the above-mentioned results, *MMP-9* knockdown significantly reduced PMVECs leakage (Figure 1K), whereas treatment of rMMP-9 exhibited a significant increase in LPS mediated permeability (Figure 1L).

We next used PDTC to explore the role of MMP-9 in the inflammation and permeability of PMVECs. We found PDTC drastically diminished the effect of MMP-9 on promoting inflammatory cytokine production and rhodamine penetration in LPS-treated cells (Figure S1E and F). Collectively, these results suggest that MMP-9 modulates inflammatory response and permeability partially via NF- κ B signaling in PMVECs.

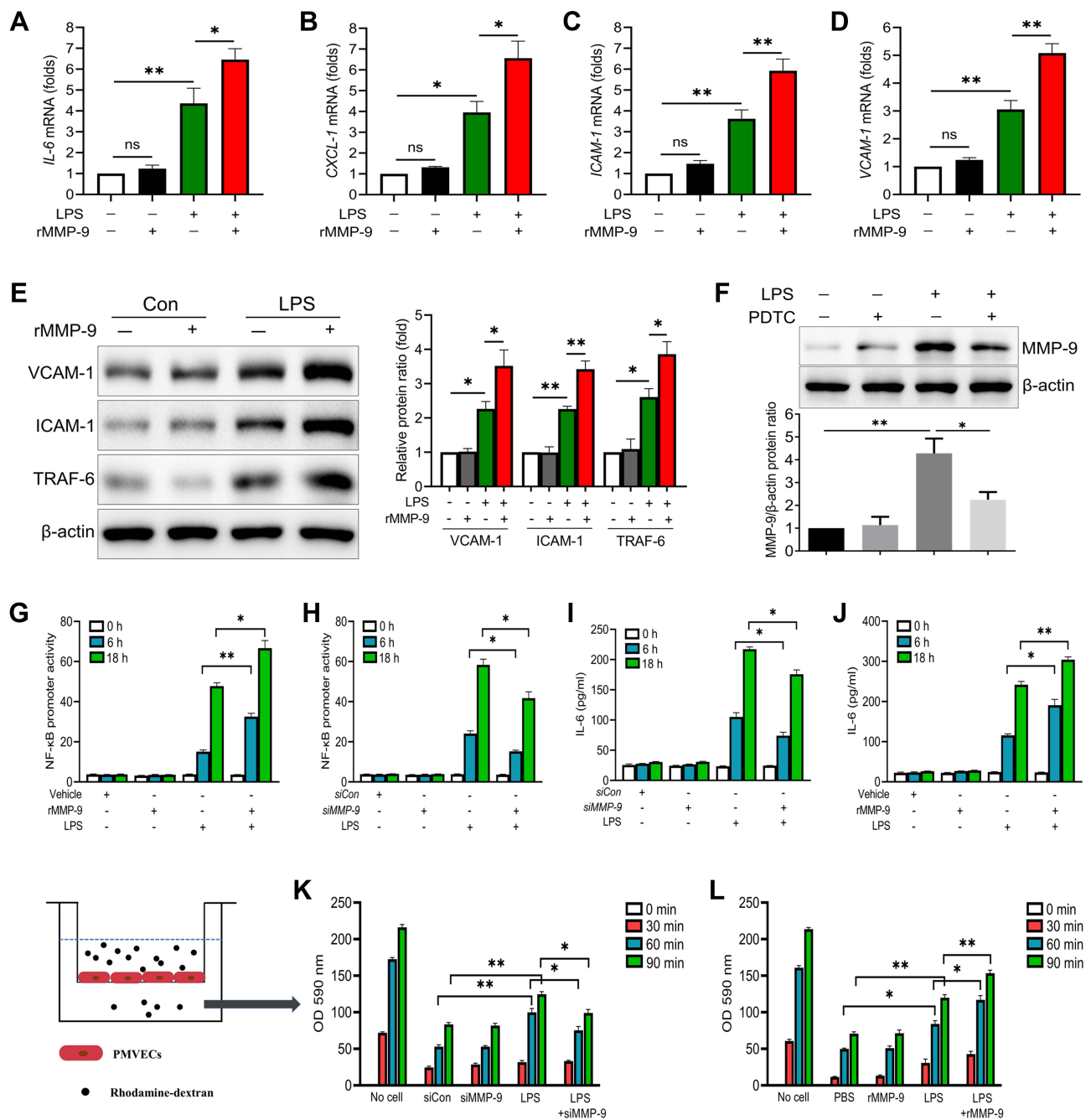


Figure 1 MMP-9 regulates inflammation and permeability in LPS-treated cells. (A–D) PMVECs were treated with LPS (1 μg/mL) with or without rMMP-9 (10 ng/mL) for 4 h. The mRNA levels of *IL-6*, *CXCL-1*, *ICAM-1*, and *VCAM-1* were detected. (E) The protein levels of VCAM-1, ICAM-1 and TRAF-6 were detected by Western blot analysis in PMVECs exposed to LPS with or without rMMP-9 treatment. (F) MMP-9 protein levels were detected by immunoblotting in PMVECs exposed to LPS with or without PDTC treatment. (G) 293T cells were transfected with Cignal NF-κB dual luciferase reporter plasmids for 48 h, and then cells were treated with LPS (1 μg/mL) with or without rMMP-9 (10 ng/mL) for an additional 6 h or 18 h. (H) 293T cells were co-transfected with NF-κB dual luciferase reporter plasmids along with control siRNA, or MMP-9 siRNA for 48 h, and then cells were treated with LPS (1 μg/mL) for an additional 6 h or 18 h. Cells were then collected and assayed for luciferase activity to evaluate NF-κB promoter activity. (I) PMVECs were transfected with control siRNA, or MMP-9 siRNA for 48 h, and then cells were treated with LPS (1 μg/mL) in a time course. Supernatant IL-6 secretion was measured by ELISA. (J) PMVECs were treated by APMA (APMA was used to active rMMP9 in vitro, and served as a control of active rMMP9) or active rMMP9 (10 ng/mL) 1 h before LPS challenged at indicated times, and then supernatant IL-6 was detected. (K and L) The permeability of treated PMVEC monolayers grown on 0.4 mm filters was determined by the appearance of rhodamine-dextran, which was added to the top well at the beginning of the experiment and detected the absorbance at 590 nm in the bottom well during a 90 min time course. **P*<0.05, ***P*<0.01. All the results are from at least three independent experiments; Data represent means ±SEM.

MMP-9 Potentiates Lung Injury and Lowers Survival in LPS-Induced ALI Mice

To determine whether MMP-9 contributed to the pathology of lung injury, we used a clinically relevant model for pneumonia and severe lung injury initiated by intratracheal administration of LPS. Mice were treated with vehicle (PBS), LPS (2mg/kg, i.t.), recombinant MMP-9 (rMMP-9, 50µg/kg, i.v.), or their combination, and we collected lung tissues and Bronchoalveolar lavage fluid (BALF) 18 h later. LPS increased BALF protein amounts, inflammatory cytokines IL-6 and TNF- α , cell counts, neutrophils, and the MPO levels. In contrast, rMMP-9 treatment alone had only a modest effect on the same parameters. However, co-treatment of rMMP-9 with LPS dramatically increased the amount of BALF protein, cell counts, inflammatory cytokines and MPO (Figure 2A–F) compared to LPS alone. The EBA permeability and lung histology also displayed a similar pattern, with rMMP-9/LPS co-treatment increasing EBA uptake and the extent of lung damage most dramatically among treatment groups (Figure 2G–I). Additionally, we used a lethal dose of LPS (20mg/kg) to detect survival. Mice were divided into three groups: Control group (rMMP-9, i.v.), ALI group (LPS, i.t.), and Co-treatment group (rMMP-9 with LPS), and seven days survival were recorded. Mice in ALI group had a significantly lower survival rate than in the control group, whereas co-treatment group further reduced the survival (Figure 2J).

Next, we determined to explore whether MMP-9 neutralizing antibody ameliorates lung injury in the LPS-induced mouse model. Mice were co-treated with LPS (2 mg/kg, i.t.) and either IgG (control) or a neutralizing antibody against MMP-9 (3 mg/kg, i.v.). We discovered that BALF cell counts, neutrophils, MPO levels, inflammatory cytokines IL-6 and TNF- α were all significantly reduced in mice treated with anti-MMP-9 compared to IgG-treated control mice (Figure S2A–E). However, blocking MMP-9 treatment had no significant effect on BALF protein amounts (Figure S2F), EBA uptake (Figure S2G), and the survival rate of ALI mice (Figure S2H). Taken together, these results suggest that MMP-9 aggravates LPS-induced lung injury.

Integrin β 3/5 Dampens MMP-9 Expression in LPS-Induced PMVECs

Many integrin receptors involve in the process of lung injury, and three (Integrin β 3/5/6) of these integrins are

related to vascular endothelial damage.²⁵ We determined whether Integrin β 3/5/6 would regulate any MMPs expressions in LPS-induced PMVECs and uncovered that *Integrin β 3/5* knockdown (*siItgb3/5*) significantly down-regulated *MMP-9* transcription in LPS treated PMVECs (Figure 3A–D). In line with the gene expression, MMP-9 protein levels were drastically decreased in PMVECs transfected with *siItgb3/5* (Figure 3E and F). Conversely, we over-expressed *Itgb3/5* in PMVECs and led to a dose-dependent increase of MMP-9 protein amounts (Figure 3G and H). MMP-9 secretion was also detected in cell supernatant by ELISA. Over-expression of *Itgb3/5* in PMVECs resulted in a significant increase in LPS mediated MMP-9 release (Figure 3I), whereas *Itgb3/5* knockdown drastically reduced MMP-9 secretion (Figure 3J).

Integrin β 3 Mitigates Permeability and Protects Survival in vitro and in vivo

Integrin β 3 has been verified in modulating the NF- κ B signaling pathway.²⁶ We first detected whether integrin β 3 would regulate NF- κ B signaling in LPS/MMP-9 co-treated PMVECs. Over-expressed integrin β 3 can give rise to a significant increase in phospho-NF- κ B proteins, including p-IKK, p-P65, and p-I κ B α (Figure 4A), whereas integrin β 3 knockdown significantly decreased phospho-NF- κ B proteins (Figure 4B) in PMVECs caused by LPS and rMMP-9 co-treatment at different times. We also observed increased IL-6 and CXCL-1 in the supernatant of LPS/MMP-9 co-treated PMVECs compared to LPS treated alone at 18h (Figure 4C and E). PMVECs were transfected with *siItgb3* and we found IL-6 and CXCL-1 secretions were drastically decreased in LPS/MMP-9 co-treatment compared to LPS treated alone (Figure 4D and F). The permeability assay revealed that over-expressed *itgb3* did not affect permeation improvement (Figure 4G), whereas *itgb3* knockdown enhances the rhodamine penetration (Figure 4H).

Next, we infected mice intravenously with empty lentivirus or lentivirus encoding *Itgb3* for 5 days and subsequently challenged them with LPS (2mg/kg, i.t.) with or without rMMP-9 (50 µg/kg, i.v.). Ectopic expression of *Itgb3* in the lungs was confirmed by immunohistochemistry staining (Figure S3A). *Itgb3* gene transfer significantly aggravated lung inflammation caused by LPS and rMMP-9 co-treatment as shown by increased BALF cell counts, neutrophils, and cytokine release (Figure S3B–D). However, there was no significant

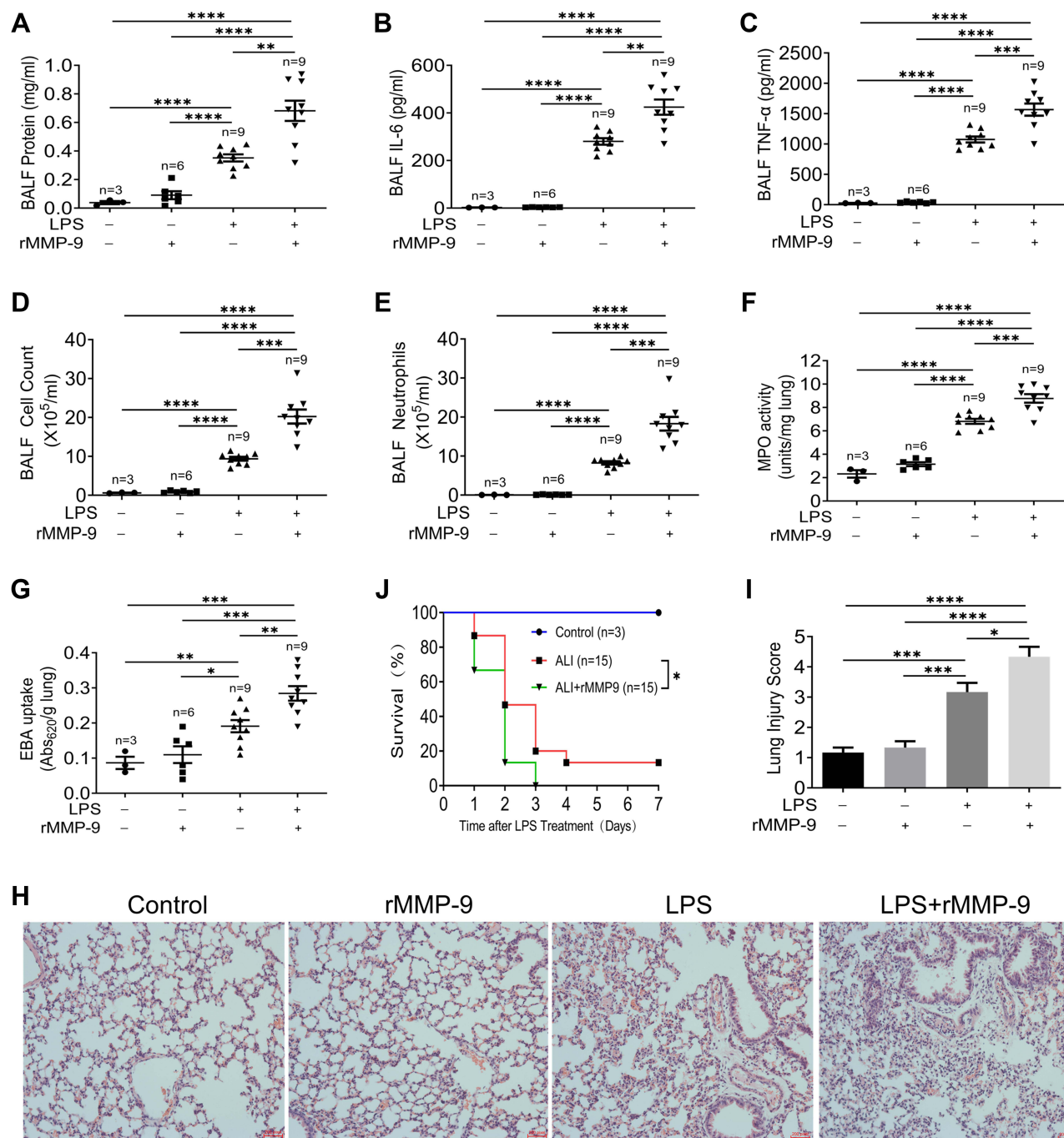


Figure 2 MMP-9 worsens LPS-induced lung injury in vivo. Mice were treated with LPS (2mg/kg, i.t.), recombinant MMP-9 (rMMP-9, 50 μ g/kg, i.v.), or their combination. 18 h later, bronchoalveolar lavage fluid (BALF) and lung tissues were collected. The protein concentration (A), IL-6 (B), TNF- α (C), cell counts (D), neutrophils (E) in Bronchoalveolar lavage fluid (BALF), and MPO activity (F) from the lung of mice were measured. (G) EBA uptake and (H) Hematoxylin and eosin (H&E)-stained lung sections ($\times 200$ magnification) with the injury score (I) were detected. Mice were treated by a lethal dose of LPS (20mg/kg, i.t.) with or without rMMP-9 (50 μ g/kg, i.v.), and then survival rates (J) were investigated. Data were analyzed by the Mantel-Cox test (survival). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Data represent means \pm SEM.

difference in the lung EBA permeability and BALF protein concentrations (Figure S3E and F). To determine the role of anti-integrin $\beta 3$ in LPS/MMP-9-induced lung injury, we injected lentivirus intravenously encoding control *shRNA* or *Itgb3* *shRNA* and then suffered from LPS

with or without rMMP-9. *Itgb3* knockdown significantly alleviated lung inflammation, but enhanced lung permeability as shown by decreased BALF cell counts, neutrophils, cytokine release (Figure S3G–I), and increased EBA uptake and BALF protein concentrations (Figure

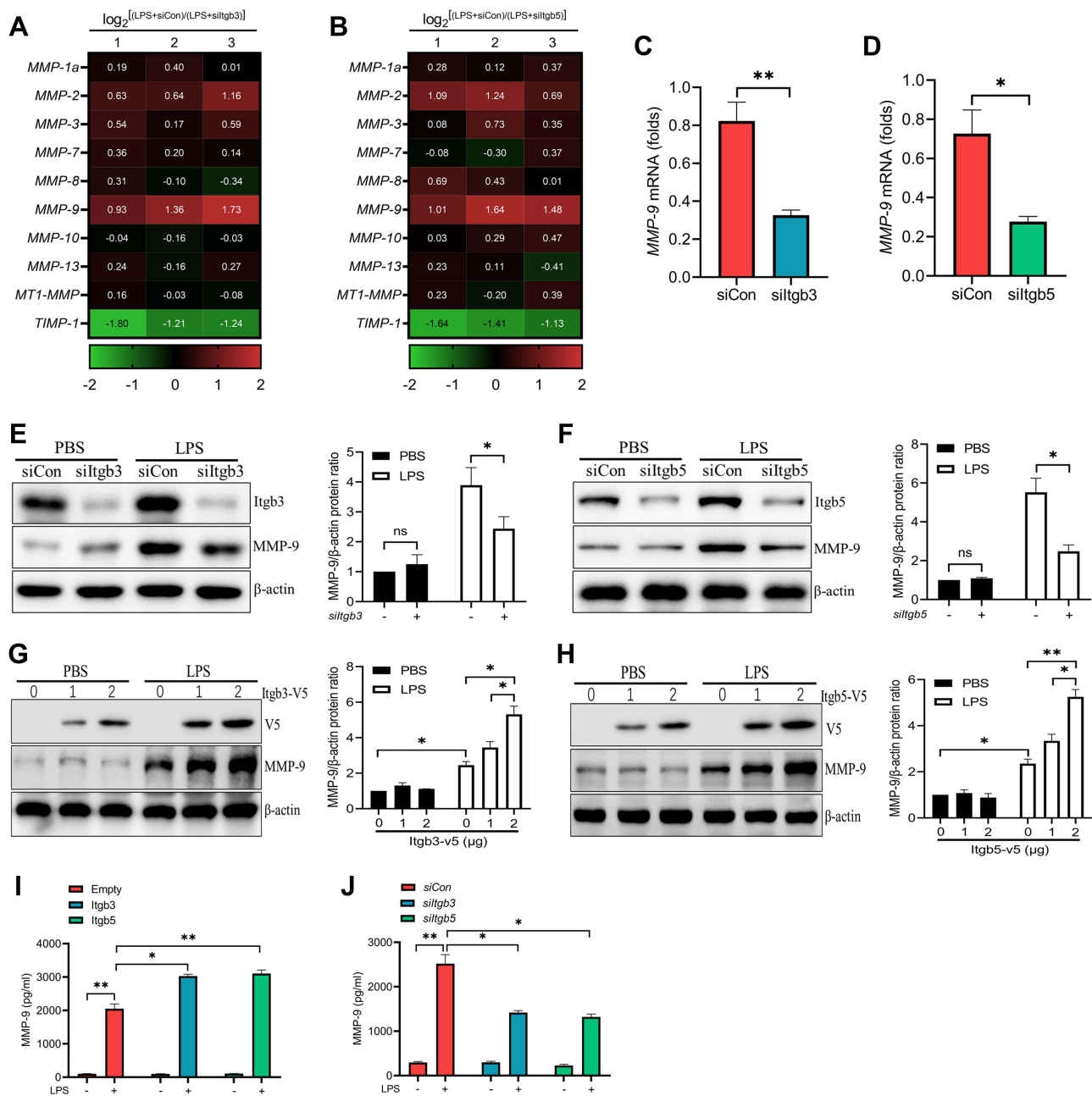


Figure 3 Integrin $\beta 3/5$ regulates MMP-9 expression in LPS-treated PMVECs. **(A and B)** Heat map of downregulated (green) and upregulated (red) mRNAs from PMVECs transfected with *Itgb3* siRNA (*siItgb3*) or *Itgb5* siRNA (*siItgb5*) for 48 h, and then subjected to 18 h of LPS (1 $\mu\text{g}/\text{mL}$) treatment. Numbers 1, 2, and 3 refer to three independent experimental analyses of $\log_2 \left[\frac{(\text{LPS}+\text{siCon})}{(\text{LPS}+\text{siItgb3/5})} \right]$. **(C and D)** MMP-9 gene expressions were measured in PMVECs transfected with *siItgb3/5* for 48 h prior to 18 h of LPS (1 $\mu\text{g}/\text{mL}$) treatment. **(E and F)** Western blot analysis of MMP-9 protein levels from PMVECs transfected with control siRNA (*siCon*) or *siItgb3/5* for 48 h prior to 18 h of PBS or LPS (1 $\mu\text{g}/\text{mL}$) treatment. **(G and H)** Western blot analysis of MMP-9 protein levels from PMVECs transfected with *Itgb3/5* plasmid in a dose course and then exposed to LPS (1 $\mu\text{g}/\text{mL}$). ELISA analysis of MMP-9 secretion from PMVECs transfected with *Itgb3/5* plasmid **(I)** or *siItgb3/5* **(J)** and then exposed to PBS or LPS (1 $\mu\text{g}/\text{mL}$). * $p < 0.05$, ** $p < 0.01$. All the results are from at least three independent experiments; Data represent means \pm SEM.

S3J and **K**). The silencing efficiency of *Itgb3* in lungs was detected by Western blot (**Figure S3L**). We also observed the survival in mice intravenously with lentivirus encoding *Itgb3* shRNA, and subsequently challenged them with LPS (2mg/kg, i.t.) with or without rMMP-9 (50 $\mu\text{g}/\text{kg}$, i.v.). *Itgb3* knockdown drastically

decreased the survival in LPS/MMP-9 co-treatment group compared to LPS treated group (**Figure S3M**). Taken together, these results suggest that inhibition of integrin $\beta 3$ could mitigate lung inflammation, but have the unwanted side effect of increasing lung endothelial permeability and lead to high mortality.

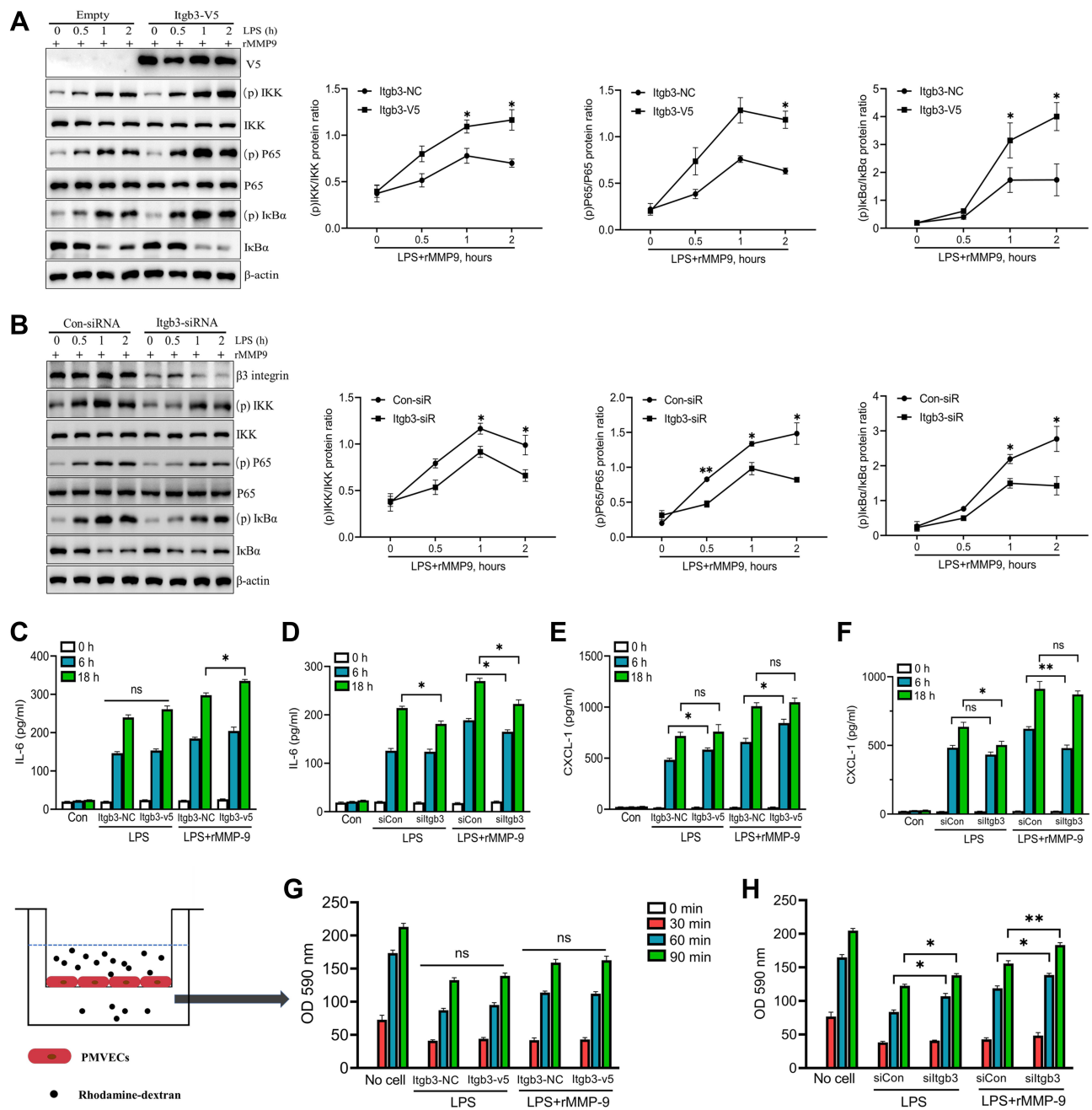


Figure 4 Integrin $\beta 3$ regulates inflammatory response and permeability in vitro. PMVECs were transfected with empty or *Itgb3* plasmid for 48 h, and then cells were exposed to rMMP-9 (10 ng/mL) for 1 h, followed by treatment with LPS (1 μ g/mL) at indicated times. (A) NF- κ B signaling proteins (p-IKK, IKK β , p-P65, P65, I κ B α , and p-I κ B α) were measured by Western blot. Supernatant IL-6 (C) and CXCL-1 (E) secretions were measured by ELISA. (G) The permeability of treated PMVEC monolayers grown on 0.4 mm filters was determined by the appearance of rhodamine-dextran. PMVECs were transfected with control siRNA, or *Itgb3* siRNA for 48 h, and then cells were exposed to rMMP-9 for 1 h, following by treatment with LPS at indicated times. (B) NF- κ B signaling proteins were measured by Western blot. Supernatant IL-6 (D) and CXCL-1 (F) secretions were measured by ELISA. (H) The permeability of treated PMVECs was determined by the appearance of rhodamine-dextran. * $P < 0.05$, ** $P < 0.01$. All the results are from at least three independent experiments; Data represent means \pm SEM.

$\beta 5$ Integrin/MMP-9 Axis Modulates Lung Injury in vitro and in vivo

We and others have uncovered that integrin $\beta 5$ contributes to NF- κ B-dependent pathological process.^{15,27} Thus, we explored whether integrin $\beta 5$ would regulate NF- κ B signaling in LPS/MMP-9 treated PMVECs. Over-expressed

integrin $\beta 5$ (*itgb5*) resulted in a significant increase in phospho-NF- κ B proteins, including p-IKK, p-P65, and p-I κ B α (Figure 5A), whereas integrin $\beta 5$ knockdown significantly decreased phospho-NF- κ B proteins (Figure 5B) in PMVECs caused by LPS and rMMP-9 co-treatment at different times. We also found *itgb5* overexpression

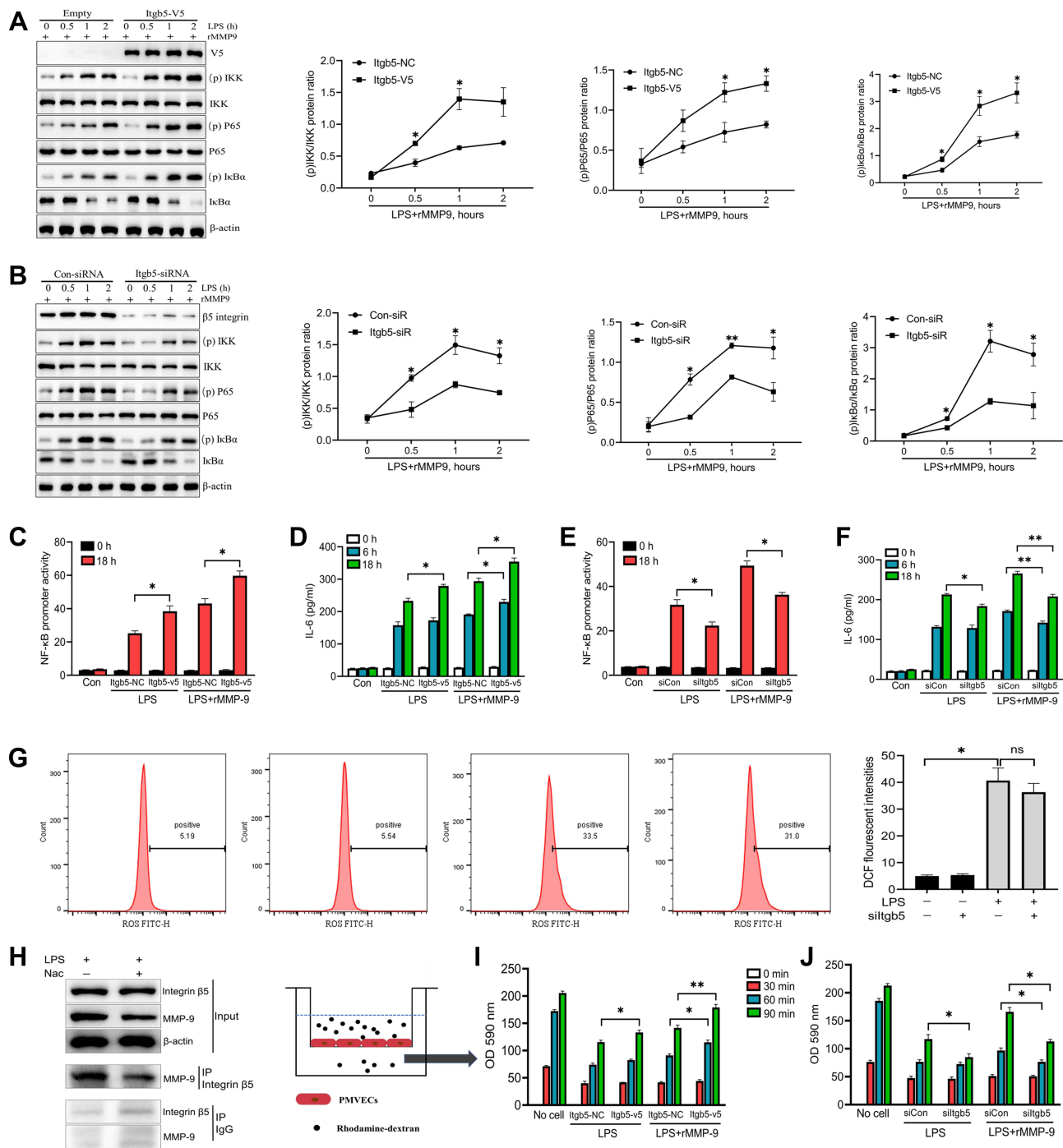


Figure 5 $\beta 5$ Integrin/MMP-9 axis modulates inflammatory response and permeability in vitro. PMVECs were transfected with empty or *Itgb5* plasmid, or control siRNA or *Itgb5* siRNA for 48 h, and then cells were exposed to rMMP-9 (10 ng/mL) for 1h, followed by treatment with LPS (1 μ g/mL) in a time course. (A and B) Immunoblot analysis of NF- κ B signaling protein abundances (p-IKK, IKK β , p-P65, P65, I κ B α , and p-I κ B α) was measured. (D and F) Supernatant IL-6 secretion was measured by ELISA. (I and J) The permeability of treated PMVECs was determined by the appearance of rhodamine-dextran. (C and E) 293T cells were co-transfected with NF- κ B dual luciferase reporter plasmids along with empty or *Itgb5* plasmid, or control siRNA or *Itgb5* siRNA for 48 h, and then cells were treated with LPS (1 μ g/mL) for an additional 6 h or 18 h. Cells were then collected and assayed for luciferase activity to evaluate NF- κ B promoter activity. (G) PMVECs were transfected with control siRNA or *Itgb5* siRNA for 48 h, and then cells were exposed to LPS for 18 h. The intercellular ROS levels were detected by flow cytometry. (H) PMVECs were treated with or without ROS scavenger, Nac (10 mM) for 1 h, prior to LPS (1 μ g/mL) administration for 18 h, and immunoblot analysis of PMVECs and immunoprecipitation (IP) of Integrin $\beta 5$. * P <0.05, ** P <0.01. All the results are from at least three independent experiments; Data represent means \pm SEM.

dramatically increased NF- κ B promoter activity (Figure 5C) and IL-6 secretion (Figure 5D). Conversely, *itgb5* knockdown significantly reduced NF- κ B promoter

activity (Figure 5E) and IL-6 production (Figure 5F) in LPS or LPS/MMP9 co-treatment models. Because vascular endothelial cell injury is associated with reactive

oxygen species (ROS) production, we next investigated whether integrin $\beta 5$ modulates ROS levels. We showed that LPS significantly enhanced the ROS production regardless of integrin $\beta 5$ expression in LPS-treated PMVECs (Figure 5G). Notably, the ROS scavenger was able to inhibit the interaction of MMP-9 and integrin $\beta 5$ (Figure 5H) and alleviate the secretion of the pro-inflammatory cytokine (Figure S4A and B) and endothelial leakage (Figure S4C) in PMVECs subjected to co-treatment (LPS with rMMP-9). Moreover, *itgb5* overexpression significantly increased the permeability in PMVECs as shown by enhanced rhodamine penetration (Figure 5I), and *Itgb5* knockdown exhibited the opposite phenotype (Figure 5J).

We next sought to investigate the role of $\beta 5$ integrin/MMP-9 axis during acute lung injury in vivo. Integrin $\beta 5$ and MMP-9 protein amounts were detected in lung homogenates after intratracheal LPS and found that both of them were increased compared to control mice (Figure 6A). Further, we measured *Itgb5* gene expression via RT-qPCR from BALF cells of ALI mice, and found a significant correlation to MMP-9 secretion from the same BALF (Figure 6B). We also detected enhanced MMP-9 in the BALF of LPS-treated mice compared to controls (Figure S5A). To explore the role of $\beta 5$ integrin in LPS/MMP-9 induced lung injury, we infected mice intravenously with empty lentivirus or lentivirus encoding *Itgb5* for 5 days prior to intratracheal LPS with or without intravenous rMMP-9 challenge. *Itgb5* gene transfer significantly aggravated lung inflammation and permeability caused by LPS/MMP-9 co-treatment as shown by increased EBA uptake, BALF protein concentrations, cell counts, neutrophils, lung histology (Figure 6C–F, K), and cytokines release (Figure S5B and C). Additionally, we administered lentivirus intravenously encoding control shRNA or *itgb5* shRNA and then exposed to LPS with or without MMP-9. *Itgb5* knockdown significantly alleviated lung inflammation and injury caused by LPS and rMMP-9 co-treatment as shown by decreased EBA uptake, BALF protein concentrations, cell counts, neutrophils, (Figure 6G–J), and cytokines release (Figure S5D and E). Intriguingly, histological analysis showed that *Itgb5* knockdown had no significant mitigating effect on the lung damage induced by LPS, but combined with anti-MMP9 treatment dramatically decreased the degree of lung injury (Figure S5F). Meanwhile, the survival rate revealed that *Itgb5* knockdown combined with anti-MMP9 treatment significantly increased the survival in

a lethal dose of LPS (20mg/kg) induced ALI mice, but not *Itgb5* knockdown treated alone (Figure 6L). Ectopic expression of *Itgb5* in the lungs was confirmed by immunohistochemistry staining (Figure S5G). The silencing efficiency of *Itgb5* in the lungs was detected by Western blot (Figure S5H). These results suggest that the $\beta 5$ integrin/MMP-9 axis modulates acute lung injury in vivo.

Discussion

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes, including gelatinases, collagenases, stromelysins, matrilysins, and membrane-type MMPs.²⁸ MMP-9 belongs to one of MMPs and has a critical role in the pathogenesis of inflammatory diseases with systemic or local destruction, such as sepsis, rheumatoid arthritis, and lung injury.^{29–31} Acute lung injury (ALI) is characterized by lesions of both lung endothelial and alveolar epithelial cells and finally leads to acute hypoxic respiratory insufficiency. Studies have reported the correlation between alveolar epithelial cells and MMP-9.^{32,33} Nonetheless, the role of MMP-9 is somewhat unclear in lung endothelial cells. MMP-9 can be secreted by many cell types, including neutrophils, macrophages, lung epithelial cells, and fibroblasts. Here, we showed pulmonary endothelial cells (ECs) also release MMP-9 in the setting of LPS stimulation. The activation of MMP-9 expression in ECs is concomitant with increases in pro-inflammatory cytokine production. This finding is in line with our previous study proposing that MMP-9 is released by immune cells and aggravates inflammatory response in macrophages exposed to LPS.¹⁰ MMP-9 degrades extracellular matrix (ECM), and leads to subsequent activation of major proangiogenic factors, including vascular endothelial growth factor (VEGF) and intercellular adhesion molecule 1 (ICAM-1).^{34,35} Excessive VEGF or ICAM-1 expression is associated with vascular endothelial cell injury. In this regard, we noted enhancement of endothelial permeability caused by rMMP-9 in a simple model of LPS-treated PMVECs, whereas rMMP-9 itself did not affect the permeability. Studies have found MMP-9 levels can be regulated by transcription factor NF- κ B in vascular smooth muscle cells and macrophages.^{36,37} We found PDTC, as a NF- κ B inhibitor, drastically reduced MMP-9 levels and abrogated the effect of rMMP-9 on promoting pro-inflammatory cytokine production and permeability in LPS-treated PMVECs.

MMP-9, as an important regulator in lung injury, has been reported. Nonetheless, its certain role in ALI is not

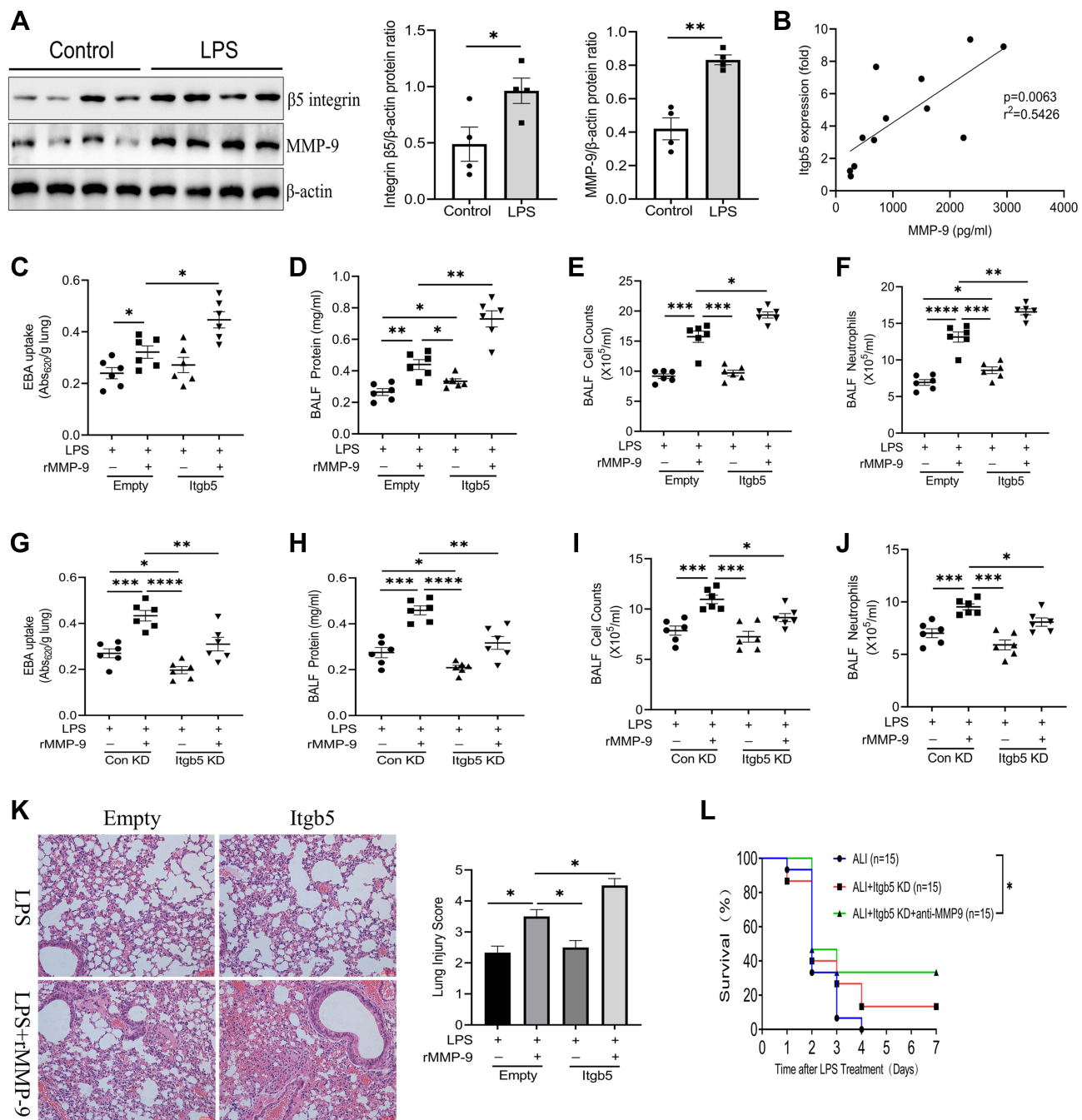


Figure 6 $\beta 5$ Integrin/MMP-9 axis regulates acute lung injury in vivo. **(A)** Immunoblot analysis of $\beta 5$ Integrin and MMP-9 from mice intratracheally treated with control or LPS for 18 h. Data and means \pm SEM of 4 mice per group. **(B)** Linear regression of *Itgb5* gene expression normalized to control mice vs BALF MMP-9 in ALI subjects ($n=12$). **(C)** EBA uptake, **(D)** protein concentrations, **(E)** total cell counts, and **(F)** neutrophils from BAL fluid of mice intratracheally treated with Lenti-Empty or Lenti-*Itgb5* and then treated with LPS and PBS or recombinant MMP-9, as indicated. Data and means \pm SEM pooled of 3 mice per group are from 2 independent experiments. **(G)** EBA uptake, **(H)** protein concentrations, **(I)** total cell counts, and **(J)** neutrophils from BAL fluid of mice intratracheally treated with Lenti-control shRNA or Lenti-*Itgb5* shRNA and then treated with LPS and PBS or recombinant MMP-9, as indicated. Data and means \pm SEM pooled of 3 mice per group are from 2 independent experiments. **(K)** Histological analysis of lung samples from mice treated as indicated. Images are representative of all independent experiments. **(L)** Survival rates of mice treated as indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

well established. Albaiceta et al and Yoon et al have indicated that MMP-9 has a protective effect on lung injury caused by ventilator or ozone.^{38,39} In contrast, Warner et al and Kim et al have found MMP-9 acts as

a negative regulator in the lung by which blocking MMP-9 can against lung injury.^{40,41} In this study, we found endogenous MMP-9 aggravates lung injury induced by LPS, and anti-MMP-9 inhibits excessive inflammatory response

in ALI mice. Relevant to these findings, many other studies have demonstrated that MMP-9 may be injurious in ALI caused by cardiopulmonary bypass, pancreatitis, and endotoxin.^{42–44} However, despite the protective effect of MMP-9 deletion on lung inflammatory injury, we did not see any benefit in regards to the lung edema from ALI mice. This may be a reflection of the fact that there was no significant improvement in the survival rate of ALI mice treated with MMP-9 neutralizing antibody in this model. MMP-9 expression can be regulated by transcription factors, such as NF- κ B and AP-1.^{37,45} Besides, studies have implicated the importance of integrins in regulating the expression of MMP-9.^{46,47} By using a model of LPS-treated PMVECs, we demonstrated that blocking integrin β 3 or integrin β 5 could reduce MMP-9 expression at the gene and protein levels.

Integrins are a family of transmembrane, heterodimeric receptors that act a pivotal part in cell adhesion to the ECM and initiate “outside-in” signal transduction events that regulate cell functions, including gene expression, cell migration, and invasion.^{48,49} Redondo-Muñoz et al verified α 4 β 1 integrin as a novel MMP-9 cell surface docking complex in chronic leukemic.⁵⁰ In the current study, we found extracellular MMP-9 also could bind to β 5 integrin receptor, and knockdown of β 5 integrin ameliorates the role of rMMP-9 in promoting inflammatory cytokine production and permeability in LPS-treated PMVECs. These findings could explain exogenous MMP9 aggravates the inflammatory response and permeability partially through β 5 integrin in PMVECs exposed to LPS. Integrin β 5 is a closely related receptor to ECM components by showing its interaction with MMP-9 in vitro. Additionally, we further found this interaction was dampened by a ROS scavenger, which also drastically decreased the pro-inflammatory cytokines secretion and endothelial permeability in PMVECs with co-treatment (LPS with rMMP-9) in this study. LPS induced excessive ROS production independent of β 5 integrin, whereas the excessive ROS promoted the interaction between β 5 integrin with MMP-9, suggesting the ROS seems to affect the role of β 5 integrin/MMP-9 axis in acute lung injury.

Studies have documented several integrins involve in the process of acute lung injury by using either specific blocking antibodies or gene knockout mice, and identified critical roles for two integrins (α _v β 3 and α _v β 5) in regulating alveolar endothelial permeability.^{25,51} Nonetheless, integrin β 3 appears to be a protective regulator in the

setting of lung injury, as we found blocking integrin β 3 aggravates lung edema and even decreased the survival in mice subjected to LPS with or without rMMP-9. Our findings are in line with the studies that noted the agonistic effects of β 3-blocking on induced permeability in vitro and lung edema in vivo,^{25,52} but the certain mechanism is not distinct and will need further clarification. Endothelial cells widely express the integrin α _v β 3, which has overlapping ligand specificity with α _v β 5. Su et al have confirmed that targeting integrin β 5 can potently inhibit lung vascular permeability and pulmonary endothelial barrier during acute lung injury.⁵¹ We reported that the increase in permeability was inhibited by transfection with *integrin β 5* siRNA in PMVECs exposed to LPS with or without rMMP-9. Our study showed the integrin β 3 and β 5 have completely opposite roles in cell leakage. We wonder whether they mediate different and antagonistic effects on the actin stress fibers and cortical actin, which are regarded as critical mediators in endothelial permeability. Further functional study will be conducted to prove the reciprocal phenotype.

We next characterized integrin β 5 as an injurious modulator in vivo by showing its gene transfer or knockdown also has a similar phenotype in cytokine release and albumin extravasation into the lung from ALI mice treated with or without exogenous MMP-9. Intriguingly, the survival of ALI mice was significantly increased by combined blocking integrin β 5 and MMP-9, whereas blocking integrin β 5, by itself, did not show any dramatic improvement in the survival of mice suffered from LPS. Based on our findings, combination therapy with drugs or biomaterials that specifically targeting integrin β 5 and MMP-9 could be a potential method to antagonize ALI.

Collectively, our data imply that MMP-9 functions as an inflammatory accelerator protein that promotes lung innate immune response and albumin extravasation during acute lung injury (ALI). Inhibition of the β 3 integrin on endothelial cells can potently aggravate ALI, whereas β 5 integrin has the opposite effect of decreasing susceptibility to ALI. Besides, we found that β 3/5 integrin negatively mediated the expression of MMP-9, which could interact with β 5 integrin involves in the regulation of endothelial function. This study demonstrated a novel insight into the mechanism by which the β 3/5 Integrin-MMP-9 axis plays a critical role in lung inflammatory response and permeability, and might be a potential therapeutic target in ALI (Figure 7).

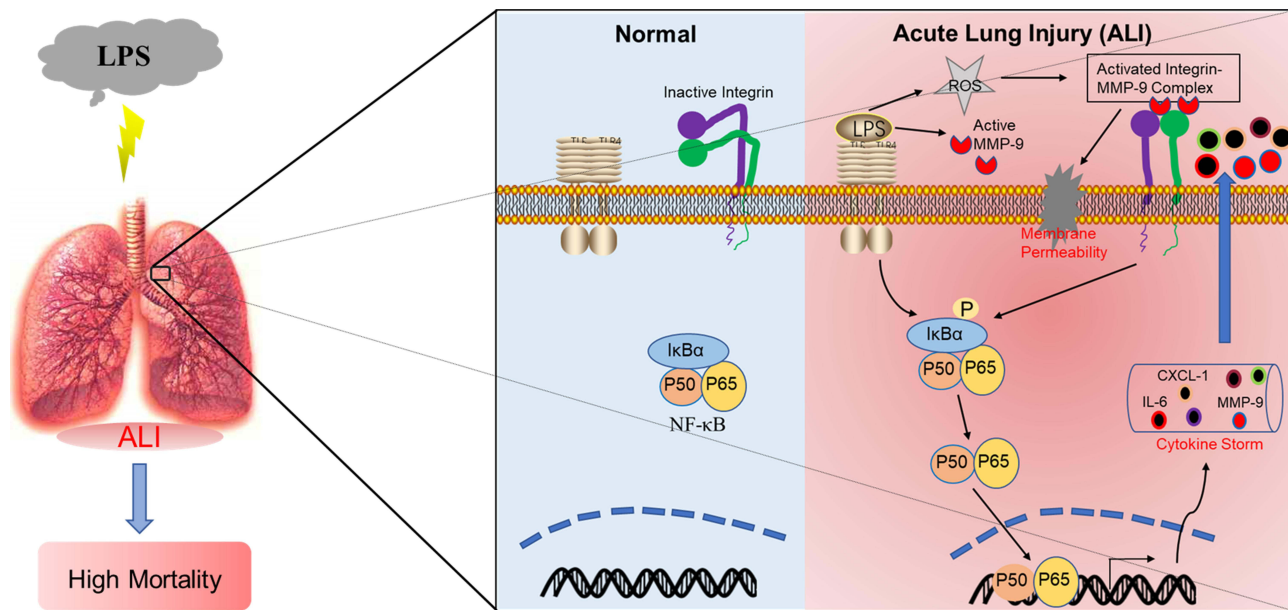


Figure 7 Scheme showing that $\beta 5$ integrin/MMP-9 axis-mediated acute lung injury by promoting pulmonary inflammatory disorders and endothelial permeability.

Funding

This study was supported by the National Natural Science Foundation of China (No. 81871101 to Luo Y, No. 81771138 to Lu H, No. 81900073 to Tong Y), and Shanghai Sailing Program (No.19YF1430700 to Tong Y).

Disclosure

The authors report no conflicts of interest in this work.

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