

Histone lactylation exacerbates acute lung injury in septic mice by promoting ferroptosis in pulmonary microvascular endothelial cells

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Abstract

Background: Circulating lactate is associated with poor prognosis in sepsis-induced acute lung injury (S-ALI). However, it remains unclear whether microvascular dysfunction, a hallmark of S-ALI, is related to circulating lactate levels and what the underlying mechanisms are. The aim of this study was to investigate the role and mechanisms of lactate in pulmonary microvascular dysfunction in S-ALI.

Methods: The effects of lactate on pulmonary microvascular function were assessed in a septic mouse model. Primary mouse pulmonary microvascular endothelial cells (MPMVECs) were isolated to evaluate the impact of lactate on MPMVEC permeability. Transcriptomic sequencing was employed to investigate the involvement of lactate in regulating MPMVEC ferroptosis, and the results were validated by *in vivo* and *in vitro* experiments. Histone lactylation was identified as a regulator of lipid peroxidation and iron homeostasis dysregulation in lactate-induced ferroptosis in MPMVECs. Gain- and loss-of-function approaches were used to assess the role of histone lactylation in regulating ferroptosis and pulmonary microvascular dysfunction. Correlations between serum lactate and ferroptosis levels and their associations with patient prognosis were investigated in patients with sepsis-associated acute respiratory distress syndrome (S-ARDS).

Results: The mouse serum lactate level reached a peak at 18 h after caecal ligation and puncture surgery. Elevated lactate levels during sepsis promoted ferroptosis in PMVECs, leading to increased pulmonary vascular permeability and exacerbation of ALI. Mechanistically, lactate increased the lactylation of histone H3 at K18 (H3K18la), which promoted ACSL4 transcription in MPMVECs, resulting in excessive lipid peroxidation. Additionally, elevated H3K18la promoted LC3 transcription and indirectly upregulated NCOA4 expression through the transcription factor GATA2, facilitating ferritinophagy. Serum lactate levels were significantly correlated with ferroptosis levels in S-ARDS patients, and both were associated with poor patient prognosis.

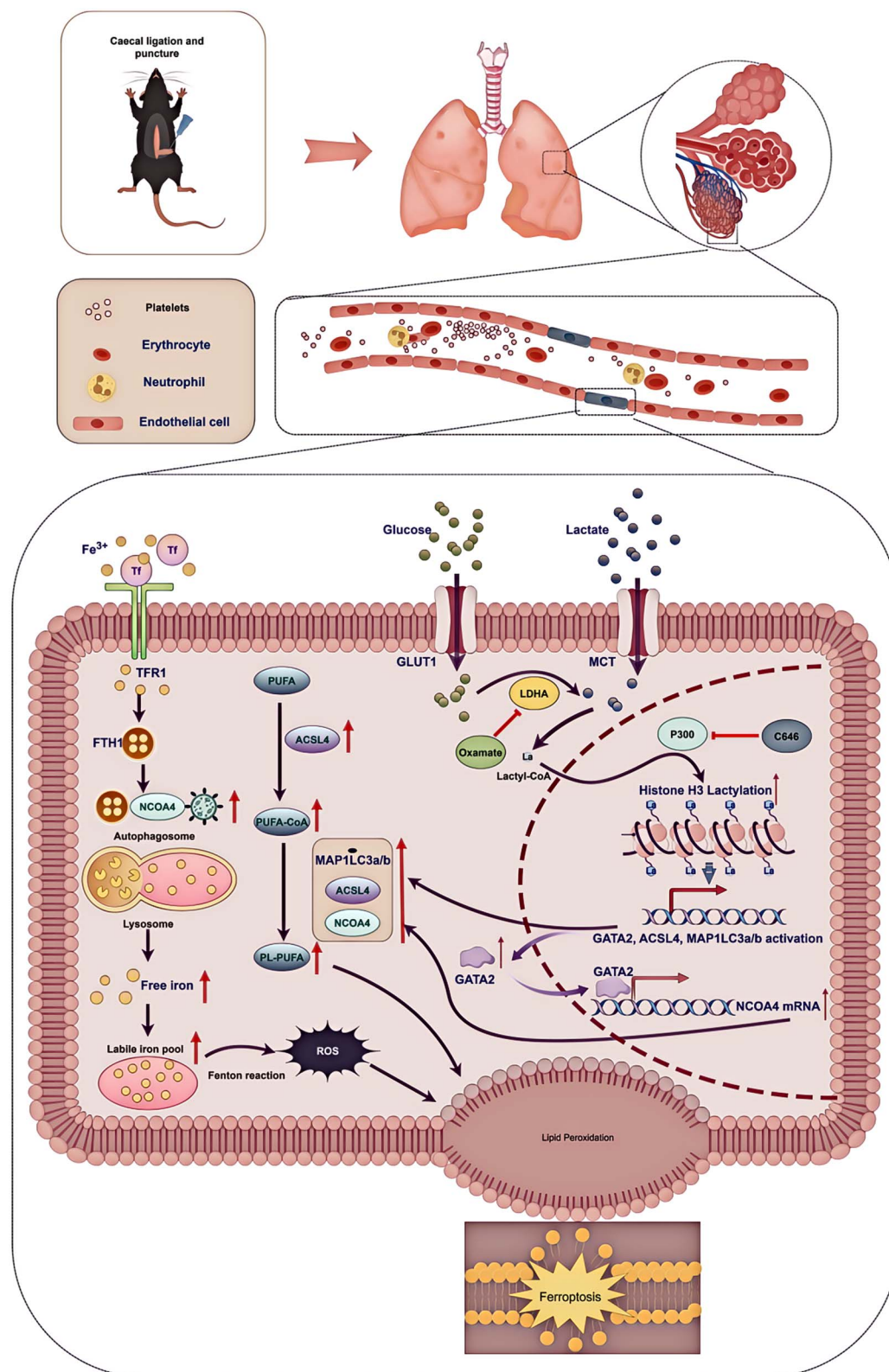
Conclusions: This study revealed a critical role for high lactate-derived histone lactylation in PMVEC ferroptosis and the progression of ALI during sepsis, providing new insights and potential therapeutic mechanisms.

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Graphical Abstract



Keywords: Sepsis-induced acute lung injury; Histone lactylation; Ferroptosis; Lipid peroxidation; Ferritinophagy

Highlights

- This study provides new insights and potential therapeutic mechanisms regarding the critical role of histone lactylation derived from high lactate levels in PMVEC ferroptosis and the development of ALI during sepsis.

- The present study emphasises that lactate promotes ACSL4 transcription by increasing lactylation of histone H3 at the K18 (H3K18la) site, leading to excessive lipid peroxidation.
- The present study also underlines that H3K18la promotes transcription of LC3 and indirectly up-regulates NCOA4 expression through the transcription factor GATA2, thereby promoting ferritinophagy.
- The study reveals that the transcription factor GATA2, a previously unrecognized regulator of ferroptosis, can upregulate NCOA4 transcription to promote ferritinophagy.

Background

Sepsis, a major cause of health loss worldwide, is an organ dysfunction associated with high mortality caused by dysregulated host responses to infection [1]. Microvascular dysfunction, a hallmark of sepsis, is closely associated with morbidity and mortality [2]. Acute lung injury (ALI), liver injury, and kidney injury are common complications of sepsis [3]. Among these organs, the lungs are the most commonly affected organ in sepsis-induced multiple organ dysfunction [4]. Microvascular dysfunction and the resulting interstitial oedema in terminal airways hinder gas exchange, which is a pathological hallmark of acute respiratory distress syndrome (ARDS) [5]. Additionally, pulmonary microvascular endothelial cell (PMVEC) injury increases vascular permeability and exacerbates inflammatory cell infiltration, further aggravating lung damage [6]. Therefore, therapies aimed at rescuing microvascular endothelial function are gaining increasing attention for patients with sepsis-associated acute lung injury (S-ALI).

Ferroptosis, a form of iron-dependent programmed cell death accompanied by high levels of lipid peroxidation, was first observed in 2012 by Dixon *et al.* in cancer cells with oncogenic Ras mutations [7]. Since then, ferroptosis has garnered widespread attention. As relevant research has progressed, accumulating evidence suggests that the dysregulation of ferroptosis is associated with various physiological and pathological conditions [8]. Numerous studies indicate a strong link between endothelial dysfunction and ferroptosis [9, 10]. Moreover, on the basis of the molecular mechanisms of ferroptosis, inhibiting endothelial cell ferroptosis represents a novel therapeutic strategy for protecting endothelial function [11]. Previous studies have shown that ferroptosis promotes sepsis-induced dysfunction in PMVECs, exacerbating ALI [12, 13]; in contrast, inhibiting ferroptosis in PMVECs has been shown to effectively alleviate S-ALI [10, 14]. Therefore, further research is warranted to elucidate the underlying mechanisms of PMVEC ferroptosis during sepsis and to identify potential therapeutic targets.

Studies have shown that metabolic dysregulation is a key factor in the pathogenesis of sepsis [15]. Alterations in metabolic pathways lead to hypermetabolism, increased energy consumption, and insulin resistance, and sepsis patients often exhibit hyperglycaemia, hyperlactatemia, and increased proteolysis [16]. In particular, during the hyperinflammatory phase of sepsis, the intense inflammatory response drives cells to rapidly obtain energy via glycolysis, a process that inevitably results in excessive lactate production and accumulation in the body [17]. Serum lactate levels are positively correlated with poor prognosis in sepsis patients [18, 19]. Lactate has traditionally been regarded as a clinical biomarker for assessing tissue perfusion and haemodynamic status [20]. With the advancement of scientific research, increasing attention has focused on the role of lactate as a signalling molecule in both physiological and pathological contexts, especially in diseases such as sepsis [21]. Endothelial cells are critical targets of lactate

accumulation in the hyperinflammatory microenvironment of sepsis. While immune cells and epithelial cells are major lactate producers, lactate passively diffuses or is transported via monocarboxylate transporters into endothelial cells, leading to intracellular lactate accumulation. This phenomenon has been well documented in sepsis-associated endothelial dysfunction [22, 23]. Extensive research has revealed a close link between energy metabolism and the dysregulation of lipid peroxidation and iron homeostasis in ferroptosis [24, 25]. However, the role of lactate in lipid peroxidation and iron homeostasis during ferroptosis remains unclear.

Recent studies have revealed that lactate can be converted into lactyl-CoA, which modifies protein lysine residues through lactylation catalysed by acetyltransferases [26]. Among these modifications, histone lactylation, an epigenetic modification mediated by the histone acetyltransferase p300/CBP, has attracted widespread attention because of its ability to directly stimulate gene transcription from chromatin [26]. Clinical studies have shown that histone H3 lysine 18 lactylation (H3K18la) is associated with the severity of sepsis [27]. Multiple studies have demonstrated that lactylation is linked to organ dysfunction during sepsis [28, 29]. However, it remains unclear whether lactylation is involved in sepsis-associated microvascular dysfunction.

In this study, we first investigated the role of histone lactylation in vascular injury in sepsis-associated acute lung injury (S-ALI). We observed that histone lactylation promotes lipid peroxidation and ferritinophagy, leading to ferroptosis in PMVECs. Mechanistically, lactate enhanced the p300-mediated enrichment of H3K18la at the promoters of acyl-coenzyme A (CoA) synthetase long-chain family member 4 (ACSL4) and microtubule-associated protein light chain 3 (LC3), increasing their transcription. This resulted in elevated polyunsaturated fatty acid content and increased ferritinophagy in PMVECs, causing excessive lipid peroxidation and iron overload, which triggered ferroptosis. Additionally, histone H3K18la promoted the transcription of GATA2, an upstream transcription factor of nuclear receptor coactivator 4 (NCOA4), leading to increased NCOA4 expression, increased ferritinophagy, and subsequent iron overload. The inhibition of lactylation alleviated endothelial damage, revealing a promising therapeutic approach for S-ALI. These findings establish a link between lactate and sepsis-associated microvascular dysfunction.

Methods

Animals

C57BL/6J mice were used in this study for all animal experiments. The mice were obtained from the Institute of Health and Medical Research, National Integrated Science Centre, Hefei. All experiments were approved by the Ethics Committee of the Institute of Health and Medicine, Hefei National Integrated Science Centre (approval number: IHM-AP-2020-004).

CLP-induced polymicrobial sepsis model

Male C57BL/6 mice (8–10 weeks old) were used in this study. Polymicrobial sepsis was induced by caecal ligation and puncture (CLP). After induction of anaesthesia with isoflurane, the animals were placed on a heating pad to maintain normothermia. A midline laparotomy was performed to exteriorize the caecum; ~1 cm of the distal cecum was ligated with a 4-0 silk suture and then perforated once with a 21-gauge needle. Gentle pressure was applied to extrude a small amount of faecal material before the caecum was returned to the peritoneal cavity. The abdominal wall was closed in layers, and postoperative care—including fluid resuscitation and analgesia—was provided. Sham-operated mice underwent identical surgical procedures except that the caecum was neither ligated nor punctured. At predefined time points, blood was obtained by cardiac puncture for serum lactate determination, and lung tissue was harvested for subsequent analyses.

Specific treatments

To study *in vivo* the relationship between serum lactate and iron death in the pulmonary microvascular endothelium, we injected lactate (pH 6.8, 0.5 g/kg body weight) intraperitoneally 6 h after CLP/SHAM operation. To inhibit lactate production, sodium oxalate (0.75 g/kg body weight) was injected intraperitoneally 3 h before CLP or SHAM operation. To inhibit iron death, Fer-1 (0.8 mg/kg body weight) was given by tail vein injection after CLP.

To silence P300 *in vivo*, AAV6 vectors carrying P300-shRNA were constructed and packaged using AAV6 vectors constructed and packaged by GenePharma (Shanghai, China). AAV6-NC and AAV6-shP300 were then injected via tail vein into C57BL/6J mice (6 weeks of age) (2.5×10^{11} vg per mouse). Four weeks later, P300-knockdown mice were subjected to CLP surgery. Then, lung tissues were collected for analysis. siRNA sequences were as follows:

sh P300: 5'-ATGACTTACCAGATGAATTAA-3'.

Mouse microcomputed tomography

Thoracic imaging and irradiation were performed on a Hiscan XM micro-CT system (Suzhou Hiscan Information Technology Co., Ltd, China). The X-ray source was operated at 60 kV and 134 μ A, and images were captured at an isotropic voxel size of 50 μ m. A full 360° rotation was completed in 0.5° increments, with a 50-ms exposure per step. Raw data were reconstructed using Hiscan Reconstruct software (v3.0; Suzhou Hiscan Information Technology), and volumetric analyses were carried out with Hiscan Analyzer software (v3.0; Suzhou Hiscan Information Technology).

Transmission electron microscopy analysis

Lung tissue and cell pellets were processed for ultrastructural analysis by standard electron microscopy (EM) protocols. Specimens were immersed in 2.5% glutaraldehyde (1 mL) for 2 h at room temperature and then kept at 4°C overnight. After three buffer washes, they were post-fixed in 1% osmium tetroxide for 1 h, rinsed again, and stained en bloc with 2% uranyl acetate for 30 min. Following graded ethanol dehydration, samples were embedded in epoxy resin, cut into 70-nm sections with an ultramicrotome, and examined on a Talos L120C G2 transmission electron microscope (Thermo Scientific, USA).

Pulmonary vascular permeability assessment

Evans Blue Dye (EBD) extravasation: Thirty minutes before euthanasia, mice received an intravenous bolus of 1% EBD. Lungs were then excised, blotted dry, weighed, and homogenized in formamide (4°C, 24 h) to elute the dye. After centrifugation, the absorbance of the supernatant was read at 620 nm and referenced against a standard curve to obtain the tissue EBD concentration.

Lung permeability index: Immediately after bronchoalveolar lavage, plasma was collected by cardiac puncture. Total protein in both bronchoalveolar lavage fluid (BALF) and plasma was quantified with a BCA kit (Beyotime Biotechnology, P0012) according to the manufacturer's protocol. The lung permeability index was expressed as the ratio of BALF protein to plasma protein.

BALF cell counting and classification

Bronchoalveolar lavage fluid was obtained by gently instilling and withdrawing 1 mL of sterile saline through a tracheal cannula. Total leukocyte counts were enumerated in a haemocytometer, and cytospin preparations were stained with a modified Giemsa reagent (Beyotime Biotechnology, China) to allow differential cell classification.

ATP measurement

Tissue ATP content was quantified with an ATP Bioluminescence Assay Kit (Nanjing Jiancheng Bioengineering Institute, A095-1-1). Lung samples were homogenized on ice in the kit's lysis buffer, clarified by centrifugation (12 000 $\times$ g, 4°C, 10 min), and the supernatant was immediately assayed. Luciferin-luciferase luminescence was read on a luminometer, and ATP concentration was interpolated from a concurrent standard curve supplied by the manufacturer.

H&E staining and evaluation of lung injury severity

Mouse lung specimens were fixed in 4% paraformaldehyde, dehydrated through graded ethanol, cleared in xylene, and embedded in paraffin. Serial 5- μ m sections were cut, mounted on glass slides, and stained with haematoxylin and eosin (H&E). Slides were examined under an Olympus light microscope by an investigator blinded to group allocation. Lung injury was scored according to four parameters—oedema, haemorrhage, neutrophil infiltration, and hyaline-membrane formation—each graded from 0 (absent) to 4 (severe), and the sum of the individual scores was taken as the total lung injury score.

DAB-enhanced Perls' Prussian blue staining

Paraffin-embedded tissue sections (5 μ m thick) were subjected to DAB-enhanced Perls' Prussian blue staining. After deparaffinization and water washing, the sections were immersed in Perls staining solution (2.5% potassium ferricyanide, 2.5% HCl, 1:1 ratio) for 30 min and then rinsed with distilled water. Sections were treated with 3% H₂O₂ methanol solution for 30 min, and then rinsed with 0.01 mol/L PBS three times for 5 min each. Colouring was achieved by applying DAB chromogenic solution (0.025% DAB, 0.003% H₂O₂) for 10 min, and nuclei were lightly stained with nuclear solid red for 10 min. After rinsing in tap water, the sections were dehydrated, made transparent, and sealed with neutral gum.

Labile iron pool measurement

Iron levels in mouse lung tissue as well as in collected cells were determined using an iron assay kit (ab83366; Abcam) according to the manufacturer's protocol.

Isolation and culture of primary MPMVECs

MPMVECs were isolated and cultured from mice according to our team's previous method. Fresh lung tissues were isolated from male SPF C57BL/6 mice and immediately immersed in ice-cold Dulbecco's modified Eagle's medium (DMEM) under sterile conditions. After excision of the visceral pleura of the lung tissue, the lung tissue at the edge of the lung lobes was taken and chopped into 1–2-mm² pieces. Subsequently, the chopped lung tissues were evenly dispersed into cell culture flasks with DMEM supplemented with 20% foetal bovine serum, 1% endothelial cell-derived growth factor, and 1% penicillin–streptomycin, respectively. After incubation for 60 h in a 37°C, 5% CO₂ humidified incubator, the lung tissues were discarded and the MPMVECs continued to be incubated in the incubator until the cells reached 80%–90% fusion. The purity of MPMVECs was tested by flow cytometry with anti-CD31.

Specific treatments

Primary MPMVECs were cultured at 37°C in a 5% carbon dioxide humidified incubator using complete growth medium that also had 1% vascular growth factor, and the medium was changed every 2 days. To mimic the *in vitro* inflammatory environment, MPMVECs were stimulated with 12 µg/mL *Escherichia coli* LPS for 12 h followed by 10 mM lactate or PBS for 7 h. In separate experiments, MPMVECs were pretreated with OXA (dissolved in PBS, 20 mM) for 3 h, Ferrostatin-1 (Fer-1, dissolved in PBS, 2 µM) for 6 h, P300 inhibitor (C646, dissolved in PBS, 5 nM) for 3 h, autophagy inhibitor (3-MA, dissolved in DMSO, 5 mM) for 6 h, proteasome inhibitor (MG132, dissolved in DMSO, 10 µM) for 6 h, and pan-caspase inhibitor (Z-VAD dissolved in DMSO, 20 µM) for 6 h.

Western blot analysis

Protein lysates were prepared by standard methods. Equal amounts of protein were resolved on 8%–15% SDS-polyacrylamide gels and electro-transferred to 0.45-µm PVDF membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk for 2 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies. Following three washes with TBST, horseradish peroxidase-conjugated secondary antibodies were applied for 1 h at room temperature. Reactive bands were visualized by enhanced chemiluminescence and quantified by densitometry using ImageJ software.

Antibodies and chemical reagents

The antibodies and chemical reagents used are listed as follows: anti-ACSL4 (Abcam, ab205199), anti-FTH1 (Abcam, ab183781), anti-GPX4 (Abcam, ab125066), anti-IRP2 (Abcam, ab181153), anti-TFR (Abcam, ab84036), anti-NCOA4 (Abcam, ab314553), anti-TF (Proteintech, 17435-1-AP), anti-FPN1 (Proteintech, 26601-1-AP), anti-GATA2 (Proteintech, 11103-1-AP), anti-LC3A/B (Abmart, TA5402), anti-HK3 (Proteintech, 67803-1-Ig), anti-HK2 (Proteintech,

22029-1-AP), anti-PFKFB3 (Proteintech, 13763-1-AP), anti-PKM2 (Proteintech, 15822-1-AP), anti-PKM2 (Proteintech, 15822-1-AP), anti-LDHA (Cell Signaling, 2012S), anti-VE-cadherin (Abmart, TA6265), anti-ZO-1 (Proteintech, 21773-1-AP), anti-IgG (Proteintech, 30000-0-AP), anti-histone-H3 (Proteintech, 17168-1-AP), anti-histone-H4 (Abcam, ab177840), anti-GAPDH (Proteintech, 60004-1-Ig), anti-LAMP1 (Proteintech, 67300-1-Ig), anti-SQSTM1 (Proteintech, 18420-1-AP), anti-TBP (Proteintech, 66166-1-Ig), anti-TBP (Proteintech, 66166-1-Ig), anti-acetyllysine (PTM Bio, PTM-105RM), anti-L-lactyl lysine (PTM Bio, PTM-1401RM), anti-L-lactyl-histone H3 (Lys18) (PTM Bio, PTM-1427RM), anti-acetyl-histone H3 (Lys18) (PTM Bio, PTM-114RM), anti-L-lactyl-histone H3 (Lys23) (PTM Bio, PTM-1413RM), anti-L-lactyl-histone H3 (Lys27) (PTM Bio, PTM-1428), CoraLite488-conjugated goat anti-mouse IgG (Proteintech, SA00013-1), CoraLite488-conjugated goat anti-rabbit IgG(H+L) (Proteintech, SA00013-2), CoraLite594-conjugated goat anti-rabbit IgG(H+L) (Proteintech, SA00013-4), CoraLite594-conjugated goat anti-mouse IgG(H+L) (Proteintech, SA00013-3), HRP-conjugated Affinipure goat anti-rabbit IgG(H+L) (Proteintech, SA00001-2), HRP-conjugated Affinipure goat anti-mouse IgG(H+L) (Proteintech, SA00001-1), 3-MA (Sigma, M9281), MG-132 (Sigma, 474787), Z-VAD (Sigma, 627610), L-(+)-lactic acid (Sigma, L6402), sodium oxamate (Sigma, O2751), *E. coli*-LPS (Sigma, L2630), C646 (Sigma, 382113), endothelial cell-derived growth factor (ScienCell, 1052), Fer-1 (MCE, HY-100579), and Z-VAD-FMK (MCE, HY-16658B).

RNA extraction and real-time quantitative PCR

Total RNA was extracted from tissues or cells with the HiPure Total RNA Mini Kit (Magen, R4111) and quantified on a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA). Contaminating genomic DNA was removed, and 1 µg RNA was reverse transcribed into cDNA using the HiScript IV RT SuperMix for quantitative PCR (qPCR) (+gDNA wiper) (Vazyme Biotech Co., R423). qPCR was carried out on a Roche LightCycler 480 II system (Roche, Switzerland) with ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Q311). Gene expression levels were normalized to the signals of GAPDH expression. The primer sequences are listed in Table S1.

In vitro permeability assay

Endothelial barrier integrity was quantified by measuring trans-endothelial electrical resistance (TEER) with an electric cell-substrate impedance sensing system (Millipore Millicell ERS-2, USA). Mouse pulmonary microvascular endothelial cells (MPMVECs) were seeded onto 0.4-µm Transwell inserts (Corning, USA) and grown to confluence in complete medium. Baseline TEER was recorded 1 h before any intervention; subsequent readings were taken at defined intervals to monitor treatment-induced changes in MPMVEC permeability.

Cell viability assay

Cell viability was evaluated with the Cell Counting Kit-8 (CCK-8) assay (APE×BIO, K1018). MPMVECs were seeded in 96-well plates and treated as indicated. After treatment, 10 µL of CCK-8 reagent was added to each well, followed by incubation for 2 h at 37°C in the dark. Absorbance was measured at 450 nm using a microplate reader (PerkinElmer, USA).

Determination of lipid peroxidation

Malondialdehyde (MDA) levels in serum samples, lung tissue, and cells were measured using the Lipid Peroxidation MDA Assay Kit (S0131; Beyotime) according to the manufacturer's instructions.

Appropriately treated cells were harvested with trypsin according to the manufacturer's instructions and incubated in PBS containing BODIPY 581/591C11 (S0043S, Beyotime) for 30 min in a water bath at 37°C. The cells were then washed with PBS and resuspended for analysis by flow cytometry (Beckman Coulter). Analyses were performed using FlowJo software.

Reactive oxygen species assay

According to the manufacturer's instructions, MPMVECs were treated with 10 μ M 2',7'-dichlorodihydrofluorescein (DCFH-DA) (S0033S; Beyotime). The fluorescence intensity of 2',7'-dichlorofluorescein was evaluated by fully automated live cell imager (Zeiss).

Determination of content of GSH

GSH was assayed in mouse lung tissue and MPMVECs according to the manufacturer's instructions (Beyotime Biotechnology, S0053).

Liquid chromatography–mass spectrometry for the analysis of untargeted lipidomics

Two hundred microlitres of water was added to the collected cells and vortexed for 5 s. Then, 240 μ L of pre-cooled methanol was added and the mixture was vortexed for 30 s. Then, 800 μ L of MTBE was added and the mixture was placed in a thermostat for 20 min of sonication time. The mixture was sonicated at 4°C for 20 min and then allowed to stand at room temperature for 30 min. The solution was centrifuged at 14 000 $\times$ g for 15 min at 10°C to obtain the upper organic solvent layer. The upper organic solvent layer was obtained and dried under nitrogen.

Reverse phase chromatography was selected for LC separation using CSH C18 column (1.7 μ m, 2.1 mm $\times$ 100 mm; Waters). The lipid extracts were re-dissolved in 200 μ L 90% isopropanol/acetonitrile, centrifuged at 14 000 $\times$ g for 15 min, and finally 3 μ L of sample was injected. Solvent A was acetonitrile–water (6:4, v/v) with 0.1% formic acid and 0.1 mM ammonium formate, and solvent B was acetonitrile–isopropanol (1:9, v/v) with 0.1% formic acid and 0.1 mM ammonium formate. The initial mobile phase was 30% solvent B at a flow rate of 300 μ L/min. It was held for 2 min, and then linearly increased to 100% solvent B in 23 min, followed by equilibrating at 5% solvent B for 10 min. Mass spectra were acquired by Q-Exactive Plus in positive and negative mode, respectively. ESI parameters were optimized and preset for all measurements as follows: source temperature, 300°C; capillary temperature, 350°C; ion spray voltage, 3000 V; S-Lens RF Level, 50%; and scan range of the instruments, m/z 200–1800. Lipid metabolomics analyses were performed by Personalbio (Nanjing, China).

RNA-seq and analysis

Total RNA was extracted from the cells according to the manufacturer's instructions. rRNA was then digested for reverse transcription and rRNA was degraded. A cDNA

library was created and sequenced using the DNBSEQ platform. DESeq was used to identify differentially expressed genes, with q -value <0.05 and $|\log_2\text{FC}| > 1$. Sequencing and bioinformatics analysis were performed by Huada Gene Company (Wuhan, China).

Immunoprecipitation

Immunoprecipitation was conducted following the protocol provided by the manufacturer for the Pierce Classic Magnetic IP/Co-IP Kit (Thermo Fisher, 88804). Cell lysates were incubated with anti-NCOA4 (Abcam, ab314553) overnight at 4°C to form immune complexes. Subsequently, Pierce Protein A/G Magnetic Beads were mixed with the antigen sample and antibody mixture at room temperature for 1 h. Binding was followed by elution of bound proteins for subsequent analysis.

Immunofluorescence staining and confocal microscopy

Tissue sections: Paraffin-embedded lungs were cut into 4- μ m sections, deparaffinized, and subjected to antigen retrieval by autoclaving in Tris-EDTA buffer (pH 8.0; Servicebio, G1206) for 2.5 min. After blocking with 3% BSA in PBS (Beyotime, ST2249) for 30 min at 37°C, sections were incubated overnight at 4°C with primary antibody. Following three PBS washes, slides were treated with Alexa-Fluor-conjugated secondary antibody (1:200) for 2 h at 37°C in the dark. Nuclei were counterstained with DAPI (Beyotime, C1005). Images were captured on a Zeiss LSM-880 confocal microscope and analysed with ImageJ and ZEN 2.0 (blue edition).

Cells: MPMVECs grown on 24-well chamber slides were fixed with 4% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 (Beyotime, P0096) for 5 min, and blocked with 3% BSA in PBS for 30 min at 37°C. Primary antibody was applied overnight at 4°C. After three PBS washes, cells were incubated with fluorescently labelled secondary antibody (1:200) for 2 h at 37°C in the dark, followed by DAPI counterstaining. Imaging and analysis were performed as described for tissue sections.

CUT&Tag

CUT&Tag libraries were prepared with the NovoNGS CUT&Tag 4.0 High-Sensitivity Kit for Illumina (NovoProtein, N259-YH01) following the manufacturer's instructions. In brief, lactate-treated MPMVECs were immobilized on concanavalin A-coated magnetic beads and permeabilized. Cells were first incubated with an anti-H3K18la primary antibody (PTM-1427RM; PTM Bio), followed by a secondary antibody, before simultaneous binding of the pAG-Tn5 transposome. Transposase activation and tagmentation were performed on-bead, after which DNA fragments were released, amplified, and purified to generate sequencing libraries. Post-amplification cleanup was carried out with NovoNGS DNA Clean Beads. Libraries were sequenced on an Illumina PE150 platform to a depth of ~ 20 G per sample. Sequencing and primary analysis were performed by APEXIO Technology LLC (Shanghai, China), and peaks were visualized with IGV v2.14.1.

Chromatin immunoprecipitation

Cells were cross-linked with 4% formaldehyde for 10 min at room temperature, then quenched with 125 mM glycine for 5 min. After two PBS washes, pellets were lysed in 1%

SDS, 5 mM EDTA, 50 mM Tris-HCl (pH 8.1) plus protease inhibitors for 10 min on ice. Chromatin was sheared to ~300 bp by sonication (60 s total, 10-s pulses, 10-s rests, 4°C). Lysates were pre-cleared and incubated overnight at 4°C with specific primary antibody or control IgG. Protein A/G magnetic beads (Beyotime, P2080S) were added and rotated for 60 min at 4°C. Beads were washed sequentially, bound complexes eluted, and cross-links reversed by heating at 65°C for 4 h. DNA was purified and analysed by ChIP-PCR or ChIP-qPCR. Primer sequences are listed in Table S2.

Dual-luciferase reporter gene assay

A dual-luciferase reporter gene system based on pGL3-basic vector was used. The luciferase vector carrying a fragment of the NCOA4 promoter region as well as a reporter plasmid for firefly luciferase, an internal reference plasmid, and an over-expression plasmid for GATA2 was constructed by APE×BIO (Shanghai, China). Cells were co-transfected with Lipofectamine 3000 (Thermo Fisher, L3000015) with the luciferase vector. Forty-eight hours after transfection, luciferase activity was measured using a dual-luciferase reporter gene assay system (Beyotime, RG042).

RNA interference

The siRNAs used in this study were synthesized by Shanghai GenePharma Company (Shanghai, China). The MPMVECs were transfected by using CALNPTM RNAi *in vitro* (D-Nano Therapeutics, DN001) according to the manufacturer's instructions. The corresponding siRNAs used were as follows:

siRNA-ACSL4 (sense 5'-GGCUGUAGGAUUGGAUACUTT-3', antisense 5'-AGUAUCCAAUCCUACAGCCTT-3')

siRNA-NCOA4 (sense 5'-CCCUACAAGAAGAACGUAATT-3', antisense 5'-UUACGUUCUUCUUGUAGGGTT-3').

Plasmids and transfection

All plasmids were obtained from Shanghai GenePharma (Shanghai, China) and delivered into target cells with Lipofectamine 3000 (Thermo Fisher, L3000015) according to the manufacturer's protocol. Culture supernatants were harvested 24 h post-transfection, while cell lysates were prepared at 72 h.

Lentivirus infection

Site-directed mutagenesis of H3c1-K18R was performed by PCR using mutagenic primers synthesized by GenePharma. Full-length H3c1WT (NC_000079.7) and the H3c1-K18R mutant were cloned into the lentiviral expression vector LV5 (EF-1α/GFP&Puro). Lentiviral particles were produced and used to transduce target cells following the manufacturer's protocol. Transduced cells were selected with puromycin (MedChemExpress, HY-B1743A) to establish stable lines.

Study population and sample collection

This study was ethically approved by the Research Ethics Committee of the Anhui Chest Hospital (No. KJ2023-40). All patients were informed of the research content and signed an informed consent before participating in the study. We recruited 50 patients diagnosed with sepsis-associated acute respiratory distress syndrome (S-ARDS) and 20 healthy controls at Anhui Chest Hospital from December 2023 to May

2024. Sepsis was diagnosed per the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis 3.0) guidelines, which define sepsis as a Sequential Organ Failure Assessment (SOFA) score ≥ 2 with documented or suspected infection. Additionally, ARDS patients were identified based on the ARDS Berlin Definition or the American-European Consensus Conference Diagnostic Criteria. The exclusion criteria were as follows: (1) patients aged <18 years; (2) patients with liver disease (including viral and autoimmune hepatitis, and drug-induced liver injury); (3) patients with chronic kidney disease; (4) patients with blood coagulation dysfunction; (5) patients with malignancies of any kind; (6) patients with cardiogenic factor induced ARDS; (7) patients who were on immune suppressors within 3 months before the study commenced; (8) pregnant women and lactating females; and (9) patients who were admitted for <24 h or with incomplete clinical information. Blood samples and medical histories were collected on the day of ICU admission for S-ARDS patients, and their outcomes were tracked. Clinical data collected for both S-ARDS patients and healthy controls included oxygenation index, duration of invasive mechanical ventilation, SOFA score, and Acute Physiology and Chronic Health Evaluation II (APACHE II) score. All patients provided written informed consent prior to participation. Serum samples from all participants were analysed to measure levels of lactate (Elabscience, E-BC-K044-S), MDA, long-chain acyl-CoA synthetase 4 (ACSL4) (AiFang biological, Changsha, China), and ferritin (AiFang biological, Changsha, China) following the instructions of manufacturers.

Statistical analysis

All analyses were performed with GraphPad Prism 8.3. Comparisons between two groups used an unpaired Student's *t*-test; three or more groups were analysed by one- or two-way ANOVA as appropriate. Survival curves were generated with the Kaplan–Meier method and compared by log-rank test. Data are presented as mean $\pm$ standard deviation (SD). Investigators were blinded to group allocation during experiments and data acquisition. Statistical significance was set at $p < 0.05$ and is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns denotes not significant.

Clinical data were first examined for normality with the Shapiro–Wilk test. Continuous variables that followed a normal distribution are presented as mean $\pm$ SD and were compared by one-way ANOVA; non-normally distributed data are given as median (interquartile range) and were analysed with the Kruskal–Wallis test. Categorical variables are reported as frequencies (percentages) and were compared using χ^2 or rank-sum tests as appropriate. Correlation and linear regression analyses were performed with Spearman's method for nonparametric data and Pearson's test for parametric data. All computations were carried out in SPSS 25 (IBM Corp., Armonk, NY) and R 3.6.2.

Results

Lactate exacerbates acute lung injury and increases pulmonary vascular permeability in CLP mice

Clinical studies have shown that elevated serum lactate levels are an independent risk factor for mortality in sepsis patients [30, 31]. In sepsis patients, serum lactate levels are positively correlated with respiratory drive and effort and

negatively correlated with dynamic lung compliance [32]. To better understand the association between lactate and ALI during sepsis, we established a CLP sepsis mouse model. Pathological analyses of lung tissues at different time points post-CLP revealed that lung injury increased with increasing duration of CLP (Figure S1a, b, see online supplementary material). A previous study confirmed that during sepsis, cells tend to rapidly obtain energy via glycolysis, producing the Warburg-like effect observed in tumour cells, resulting in the inevitable production and accumulation of lactate [15, 21, 33]. Our study revealed similar results. Compared with that in the SHAM group, the expression of key glycolytic enzymes in lung tissues of the CLP group, including LDHA, PFKFB3, PKM2, HK3, and PDK1, was significantly higher (Figure 1a), whereas ATP levels were lower (Figure 1b). The serum lactate levels in the CLP mice were significantly greater than those in the SHAM mice, increasing with increasing duration of CLP, peaking at 18 h postinjury and then declining (Figure 1c). We also observed a positive correlation between serum lactate levels and lung injury scores in mice (Figure S1c, see online supplementary material). To further understand whether lactate exacerbates ALI during sepsis, as shown in Figure S1d, we administered saline or oxamate (OXA; 0.75 g/kg body weight) intraperitoneally 3 h before SHAM or CLP surgery, followed by intraperitoneal lactate injection (0.5 g/kg body weight) after surgery. The mice were divided into six groups on the basis of treatment. The degree of ALI was assessed using lung CT, histopathology, and total cell and neutrophil counts in bronchoalveolar lavage fluid (BALF). As shown in Figure 1d–i, compared with the SHAM group, the lactate and CLP groups had higher serum lactate levels, worse lung injury, and higher total cell and neutrophil counts in BALF. The addition of lactate further worsened lung injury in CLP mice, increasing the total cell count in BALF. OXA treatment significantly reduced the serum lactate level in CLP mice, alleviated lung injury, and decreased the total cell and neutrophil counts in BALF. Previous studies have shown that inhibiting lactate production increases survival rates in CLP mice, whereas increased lactate levels reduce survival rates [29, 34]. Our study revealed consistent results, with lactate supplementation decreasing and OXA pretreatment increasing the 7-day survival rate of septic mice (Figure 1j).

Endothelial cells constitute the first line of defence in the pathological process of sepsis-induced lung injury. Endothelial cell damage leads to increased pulmonary vascular leakage and acute pulmonary oedema [35, 36]. In this study, transmission electron microscopy revealed structural damage to the vascular endothelial barrier in CLP mice, and lactate administration further exacerbated this damage, while OXA treatment reversed these effects (Figure S1e, see online supplementary material). To assess pulmonary vascular permeability comprehensively, we measured Evans blue dye (EBD) leakage into lung tissue, calculated lung permeability indices, and analysed the expression of the vascular junction proteins VE-cadherin and ZO-1 in lung tissue. We observed a significant increase in EBD leakage and the lung permeability index in CLP mouse lung tissues, with a marked reduction in vascular junction protein expression. Lactate supplementation further increased EBD leakage and the lung permeability index, whereas OXA treatment alleviated EBD leakage and increased the lung vascular permeability index in CLP mice and rescued the low expression of vascular junction

proteins. Under additional lactate exposure, SHAM mice also presented increased EBD leakage, a greater lung permeability index, and reduced expression of vascular junction proteins (Figure 1k–o). Moreover, we analysed the correlations between serum lactate levels and the above indicators. As expected, the serum lactate level was positively correlated with EBD leakage and the lung permeability index and negatively correlated with the expression of vascular proteins (Figure S1f–j, see online supplementary material). These data indicate that increased lactate levels in sepsis plays a crucial role in S-ALI and pulmonary vascular permeability.

Lactate exacerbates pulmonary vascular hyperpermeability by inducing ferroptosis in MPMVECs

We previously confirmed that pulmonary microvascular endothelial injury and dysfunction are key factors leading to imbalanced pulmonary vascular permeability [37, 38]. However, no studies have evaluated the effects of lactate on MPMVEC injury and function. Considering the correlation between increased lactate levels in sepsis patients and pulmonary vascular permeability, we hypothesized that high lactate levels disrupt the stability of pulmonary microvascular endothelial barrier structure and function. To test this hypothesis, we isolated and cultured MPMVECs *in vitro*. We treated the cells with increasing concentrations and durations of lactate. As shown in Figure 2a, after 7 h of treatment with 10 mM lactate, cell viability significantly decreased, and cell death markedly increased (Figure S2a, see online supplementary material); moreover, vascular junction protein levels significantly decreased (Figure 2b). The reduction in the TEER of the MPMVEC monolayer confirmed that lactate exposure impaired MPMVEC permeability (Figure 2c). Moreover, the apoptosis inhibitor Z-VAD-FMK (pan-caspase inhibitor) did not reverse lactate-induced cell death, indicating that lactate-induced cell death is nonapoptotic (Figure S2b, see online supplementary material). Lactate treatment leads to an acidic cellular environment, and studies have reported that acidic conditions do not promote endothelial permeability [22]. Consistent with this, we found that under acidic conditions equivalent to lactate conditions (pH = 6.8), there was no significant change in the TEER value for MPMVECs (Figure S2c, see online supplementary material), indicating that lactate itself, not the pH change, caused alterations in MPMVEC permeability. In a lipopolysaccharide (LPS)-induced sepsis cell model, lactate treatment reduced the expression of vascular junction proteins and TEER levels in the LPS group, whereas OXA pretreatment attenuated these effects (Figure 2d–e, Figure S2d, see online supplementary material). To elucidate the mechanism by which lactate mediates changes in MPMVEC permeability, we performed transcriptome sequencing of MPMVECs stimulated with lactate, followed by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. The results revealed that the main enriched pathways among the differentially expressed genes were related to cell junction disassembly, the collagen-containing extracellular matrix, and ferroptosis (Figure 2f). Previous studies have shown that pulmonary endothelial cell ferroptosis disrupts the barrier function of blood vessels, leading to increased permeability [10, 39]. Therefore, we speculated that lactate induces ferroptosis in pulmonary microvascular endothelial cells, leading to vascular barrier

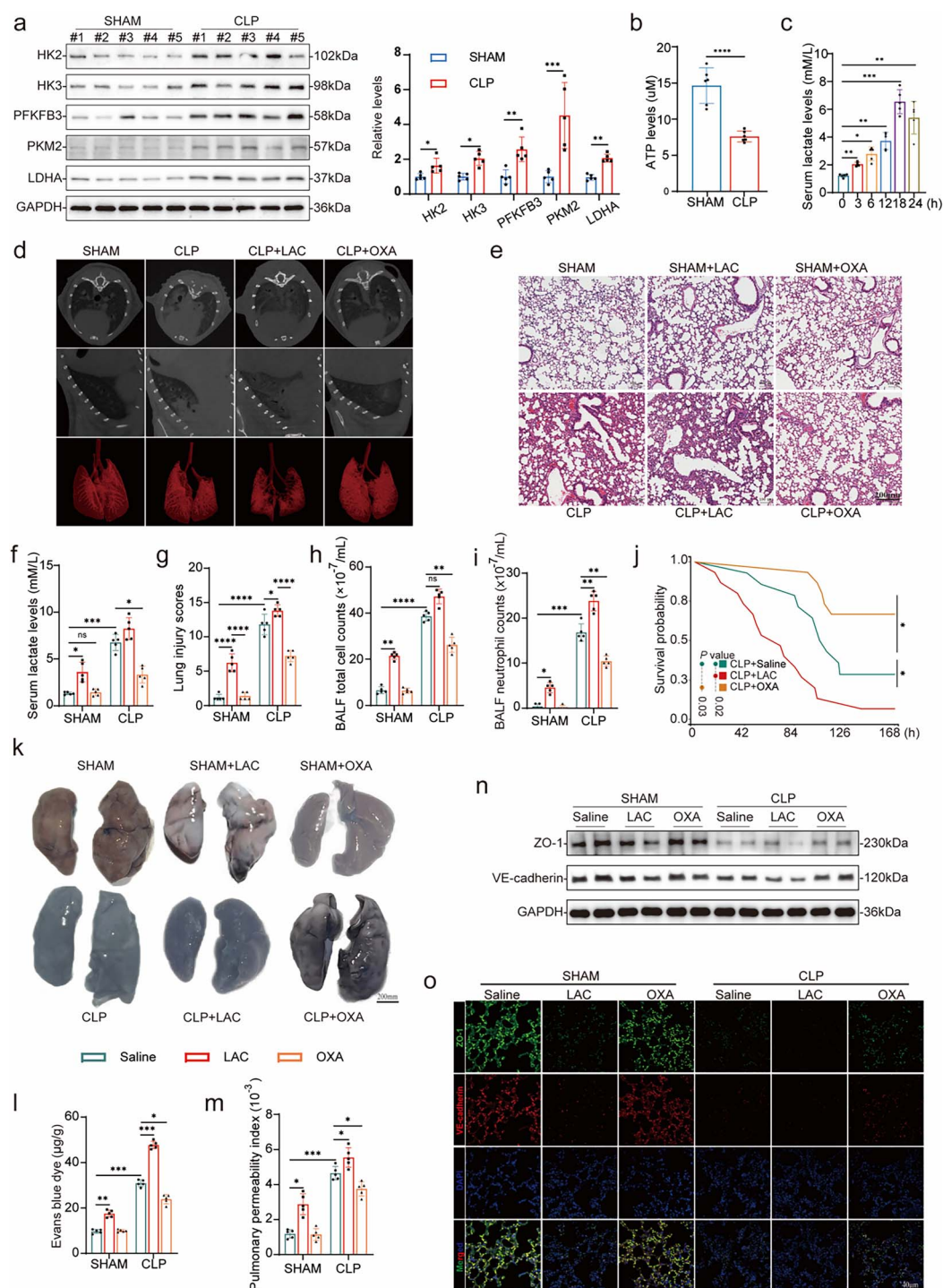


Figure 1. Lactate exacerbates acute lung injury and increases pulmonary vascular permeability in CLP mice. **(a)** The levels of glycolytic markers HK2, HK3, PFKFB3, PKM2, and LDHA in lung tissue lysates of SHAM as well as CLP mice were examined using western blot ($n = 5$ per group). **(b)** ATP levels in lung tissue of SHAM, CLP mice were assessed by commercially available ATP kits ($n = 6$ per group). **(c)** Serum levels of lactate in mice at different time points after CLP were assessed by commercially available lactate kits ($n = 5$ per group). **(d)** Representative images of SHAM, CLP, CLP + LAC, and CLP + OXA mouse lung—mouse microcomputed tomography. **(e)** Representative images of SHAM, SHAM + LAC, SHAM + OXA, CLP, CLP + LAC, and CLP + OXA mouse lung—H&E staining. Scale bar: 200 μ m. **(f)** Serum levels of lactate in SHAM + Saline, SHAM + LAC, SHAM + OXA, CLP + Saline, CLP + LAC, and CLP + OXA mice were assessed by commercially available lactate kits ($n = 5$ per group). **(g)** Lung injury in SHAM, SHAM + LAC, SHAM + OXA, CLP, CLP + LAC, and CLP + OXA mice was assessed based on H&E staining results ($n = 5$ per group). **(h, i)** Total cell counts and neutrophil counts in alveolar lavage fluid of SHAM + Saline, SHAM + LAC, SHAM + OXA, CLP + Saline, CLP + LAC, and CLP + OXA mice ($n = 5$ per group). **(j)** CLP + Saline, CLP + LAC, and CLP + OXA mice 7-day survival analysis (Kaplan–Meier method, $n = 15$ per group). **(k, l)** Vascular permeability in mouse lung tissue was examined by EBD staining and analysed quantitatively ($n = 5$ per group). **(m)** Lung permeability index (the lung permeability index was calculated as the BALF protein content divided by the plasma protein content) was calculated in SHAM + Saline, SHAM + LAC, SHAM + OXA, CLP + Saline, CLP + LAC, and CLP + OXA mice ($n = 5$ per group). **(n, o)** Levels of the vascular connectivity marker VE-cadherin, ZO-1, were measured using western blot as well as immunofluorescence analysis on lung tissues from SHAM + Saline, SHAM + LAC, SHAM + OXA, CLP + Saline, CLP + LAC, and CLP + OXA mice, scale bar: 40 μ m. All data were represented as the means $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant. CLP caecal ligation and puncture, H&E hematoxylin and eosin, EBD Evans Blue Dye, SD standard deviation, OXA oxamate

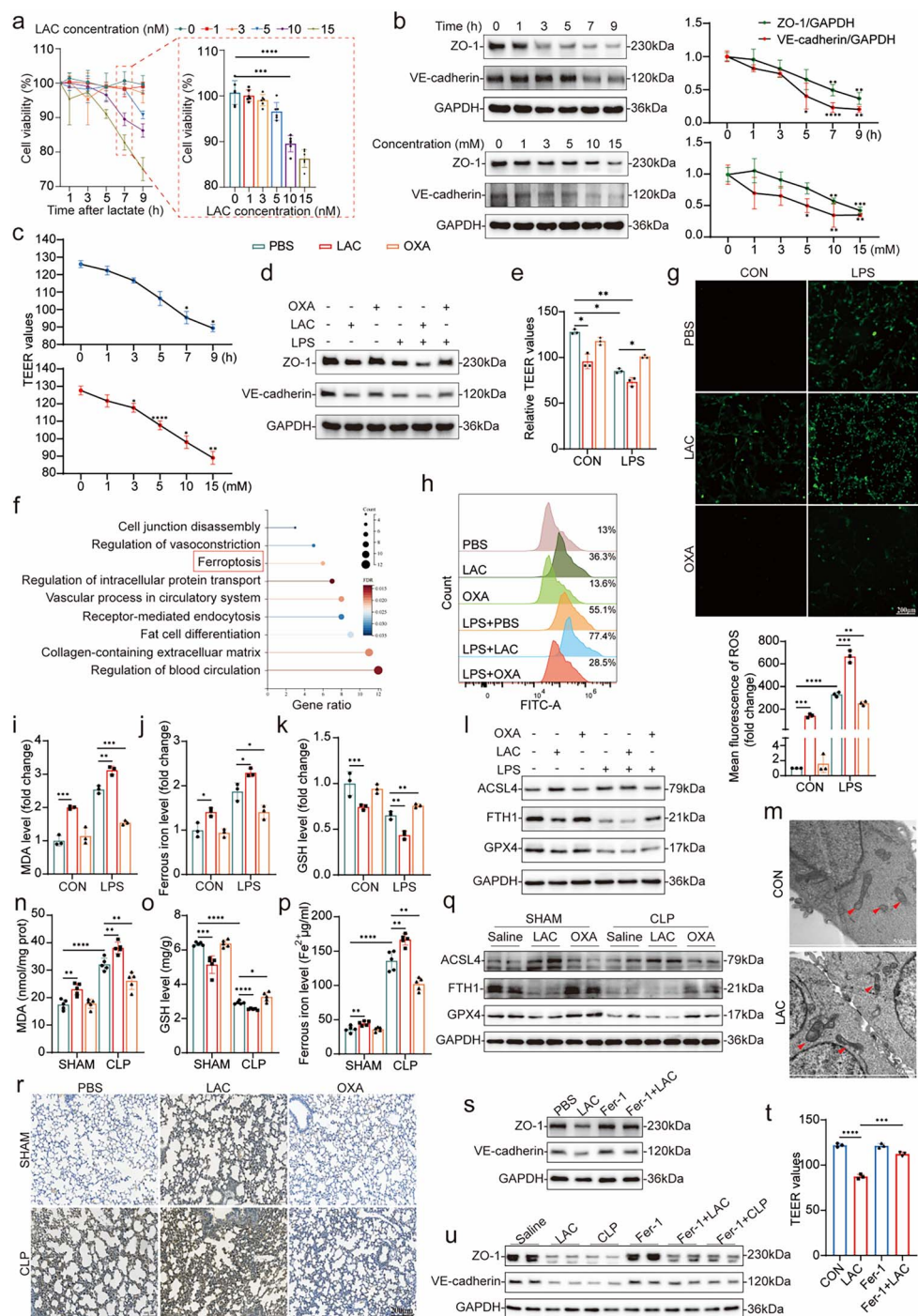


Figure 2. Lactate exacerbates pulmonary vascular hyperpermeability by inducing ferroptosis in MPMVECs. **(a)** Cell viability of MPMVECs was analysed by commercially available cell viability kits at different concentrations as well as time gradients of lactate treatment ($n=3$ per group). **(b)** MPMVECs VE-cadherin, ZO-1 levels were analysed by western blot at different concentrations as well as temporal gradients of lactate treatment ($n=3$ per group). **(c)** The level of trans-endothelial electrical resistance of MPMVECs was measured at different concentrations as well as time gradient lactate treatment ($n=3$ per group). **(d)** The effects of OXA and lactate on VE-cadherin, ZO-1 in control cells or LPS-treated MPMVECs were analysed by western blot ($n=3$ per group). **(e)** The effects of OXA and lactate on trans-endothelial electrical resistance in control cells or LPS-treated MPMVECs ($n=3$ per group). **(f)** KEGG enrichment analysis. **(g)** ROS were detected by immunofluorescence in differently treated MPMVECs. Green: ROS, scale bar: 200 μm . **(h)** C11-BODIPY 581/591 content of differently treated MPMVECs detected by flow cytometry. **(i-k)** MDA, Fe²⁺, and GSH levels were detected in MPMVECs from different treatments ($n=3$ per group). **(l)** The levels of ACSL4, FTH1, and GPX4 were detected by western blot in MPMVECs from different treatments ($n=3$ per group). **(m)** Transmission electron microscopy ultrastructural representative images of mitochondria from normal MPMVECs and MPMVECs after lactate treatment, scale bar: 500 nm. **(n-p)** MDA, Fe²⁺, and GSH levels were detected in lung tissues of mice with different treatments ($n=5$ per group). **(q)** ACSL4, FTH1, and GPX4 levels were detected by western blot in lung tissues of mice with different treatments ($n=6$ per group). **(r)** Representative images of DAB-enhanced Perls' Prussian Blue staining of mouse lung tissue from different treatments, scale bar: 200 μm . **(s, t)** MPMVECs were treated with lactate (10 nM) or Fer-1 (2 μM), and the levels of VEPage cadherin and ZO-1 were analysed by western blot as well as determining the levels of trans-endothelial electrical resistance levels ($n=3$ per group). **(u)** The effects of Fer-1 and lactate on VE-cadherin, ZO-1 in lung tissues of normal or post-CLP mice were analysed by western blot ($n=5$ per group). All data were represented as the means $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant. MPMVEC Mouse pulmonary microvascular endothelial cell, LPS lipopolysaccharide, OXA oxamate, MDA malondialdehyde, ACSL4 long-chain acyl-CoA synthetase 4, KEGG Kyoto Encyclopedia of Genes and Genomes, CLP caecal ligation and puncture, SD standard deviation

damage. To test this hypothesis, we examined the effects of lactate on intracellular oxidative stress and iron accumulation in an LPS-induced sepsis cell model. Compared with those in the Con group, the levels of reactive oxygen species (ROS) were higher in the LPS and lactate groups, as were the levels of lipid hydroperoxide (LPO) measured using BODIPY and the lipid peroxidation marker MDA (Figure 2g–i). The levels of ferrous ions in the cells also significantly increased (Figure 2j), whereas the levels of the antioxidant marker GSH decreased (Figure 2k). Additional lactate administration in the LPS group further exacerbated intracellular oxidative stress and iron accumulation, whereas OXA treatment alleviated LPS-induced intracellular oxidative stress and iron accumulation (Figure 2g–k). We also assessed the expression of the ferroptosis-related proteins GPX4, ACSL4, and FTH1. We found that under LPS stimulation, the protein level of ACSL4 increased in MPMVECs, whereas the protein levels of GPX4 and FTH1 decreased (Figure 2l, Figure S2e, see online supplementary material). Interestingly, lactate treatment upregulated ACSL4 expression while downregulating FTH1 expression but did not affect GPX4 protein levels. Even under LPS stimulation, lactate or OXA administration did not affect GPX4 protein levels (Figure 2l, Figure S2e, see online supplementary material). Transmission electron microscopy revealed that, compared with the control treatment, lactate treatment caused mitochondria swelling, a reduction in mitochondrial cristae, and deeper mitochondrial staining (Figure 2m). We subsequently assessed the effect of lactate on ferroptosis *in vivo*. As expected, lactate supplementation increased oxidative stress and iron accumulation in lung tissues, as evidenced by elevated MDA and Fe^{2+} levels in lung tissue lysates, upregulated ACSL4 protein expression, decreased GSH levels, and downregulated ferritin protein expression in both the SHAM and CLP groups (Figure 2n–q, Figure S2f, see online supplementary material). DAB-enhanced Prussian blue staining of lung tissue indicated increased iron deposition (Figure 2r). We also observed that under lactate treatment, GPX4 protein expression in lung tissue lysates was downregulated, an effect that may be related to lactate reducing GPX4 expression in lung epithelial cells [40]. To investigate the impact of lactate-induced ferroptosis on the MPMVEC barrier, we treated cells with the ferroptosis inhibitor ferrostatin-1 (Fer-1). Under lactate stimulation, pretreatment with Fer-1 significantly reversed the downregulation of vascular junction protein expression induced by lactate in MPMVECs (Figure 2s, Figure S2g, see online supplementary material), reduced the TEER values for MPMVEC monolayers in the lactate group, and increased cell viability (Figure 2t, Figure S2h, see online supplementary material). Similarly, in *in vivo* experiments, the intraperitoneal injection of Fer-1 (5 mg/kg body weight) reduced the lung permeability indices under CLP or lactate challenge and upregulated the expression of vascular junction proteins (Figure S2i–j, Figure 2u, see online supplementary material). These results support the hypothesis that lactate induces ferroptosis in pulmonary microvascular endothelial cells, leading to vascular barrier dysfunction.

Lactate induces ferroptosis in murine microvascular endothelial cells by promoting lipid peroxidation

Excessive lipid peroxidation and iron homeostasis dysregulation are core triggers of ferroptosis and play dominant

roles in ferroptosis initiation and progression [41–43]. Iron-dependent lipid ROS accumulation is involved in all known ferroptosis pathways [44]. Polyunsaturated fatty acids (PUFAs) are sensitive to lipid peroxidation, and PUFA-containing membrane phospholipids are potential targets for ROS [45, 46]. We used liquid chromatography–mass spectrometry (LC–MS/MS) to analyse the compositional changes in lipids in lactate-stimulated murine microvascular endothelial cells. Principal component analysis of untargeted lipidomic data, BasePeak spectra, and partial least squares discrimination confirmed the reliability of the sequencing data (Figure 3a, b, Figure S3a, see online supplementary material). Lactate treatment altered the intracellular lipid profile (Figure 3c). Compared with the control, after lactate treatment, the membrane phospholipid components, represented by PE, PC, PA, and PG, significantly increased (Figure 3d), and the lipid chain length and degree of unsaturation also increased (Figure 3b–i, see online supplementary material). Phospholipids containing arachidonic acid (AA) (C20:4), adrenic acid (AdA) (22:4), and eicosapentaenoic acid (EPA) are preferential substrates for oxidation [47]. For subsequent differential abundance analysis of lipid subclasses, we focused on the differences in these lipids. We found that the abundance of phospholipids containing AA, AdA, and EPA was significantly greater in the lactate-treated cells than in the control cells (Figure 3e–l). ACSL4 and lysophosphatidylcholine acyltransferase 3 (LPCAT3) are involved in the biosynthesis and remodelling of phospholipids, represented by PEs, leading to the accumulation of peroxidized lipid substrates in cells, thereby triggering ferroptosis [48]. Our transcriptome sequencing revealed that ACSL4 transcription levels were significantly elevated, whereas LPCAT3 transcription levels did not significantly change (Figure 3m). Therefore, we speculate that lactate promotes lipid peroxidation by upregulating ACSL4 expression, leading to ferroptosis. In this study, ACSL4 expression increased with increasing duration and concentration of lactate (Figure 3n, Figure S3j, see online supplementary material). To confirm whether ACSL4 is the key enzyme mediating lactate-induced lipid peroxidation, we used siRNA to knock down ACSL4 expression in MPMVECs (Figure S3k, see online supplementary material). The results revealed that ACSL4 knockdown alleviated the lactate-induced reduction in cell viability, increased the GSH level, and significantly reduced the MDA, ROS, and LPO levels (Figure 3o–s). Interestingly, ACSL4 knockdown had little effect on Fe^{2+} levels (Figure 3t). Additionally, we observed that ACSL4 knockdown reduced the effects of lactate on TEER (Figure 3u) and prevented damage to the vascular junction proteins VE-cadherin and ZO-1 (Figure 3v, Figure S3l, see online supplementary material). These results suggest that lactate promotes lipid peroxidation in MPMVECs by upregulating ACSL4 expression, inducing ferroptosis, and thereby increasing vascular endothelial permeability.

Lactate promotes ferroptosis in murine microvascular endothelial cells through ferritin autophagy

Lactate stimulation significantly increased intracellular iron levels in MPMVECs, indicating the dysregulation of intracellular iron homeostasis. However, ACSL4 knockdown did not rescue the dysregulation of intracellular iron homeostasis.

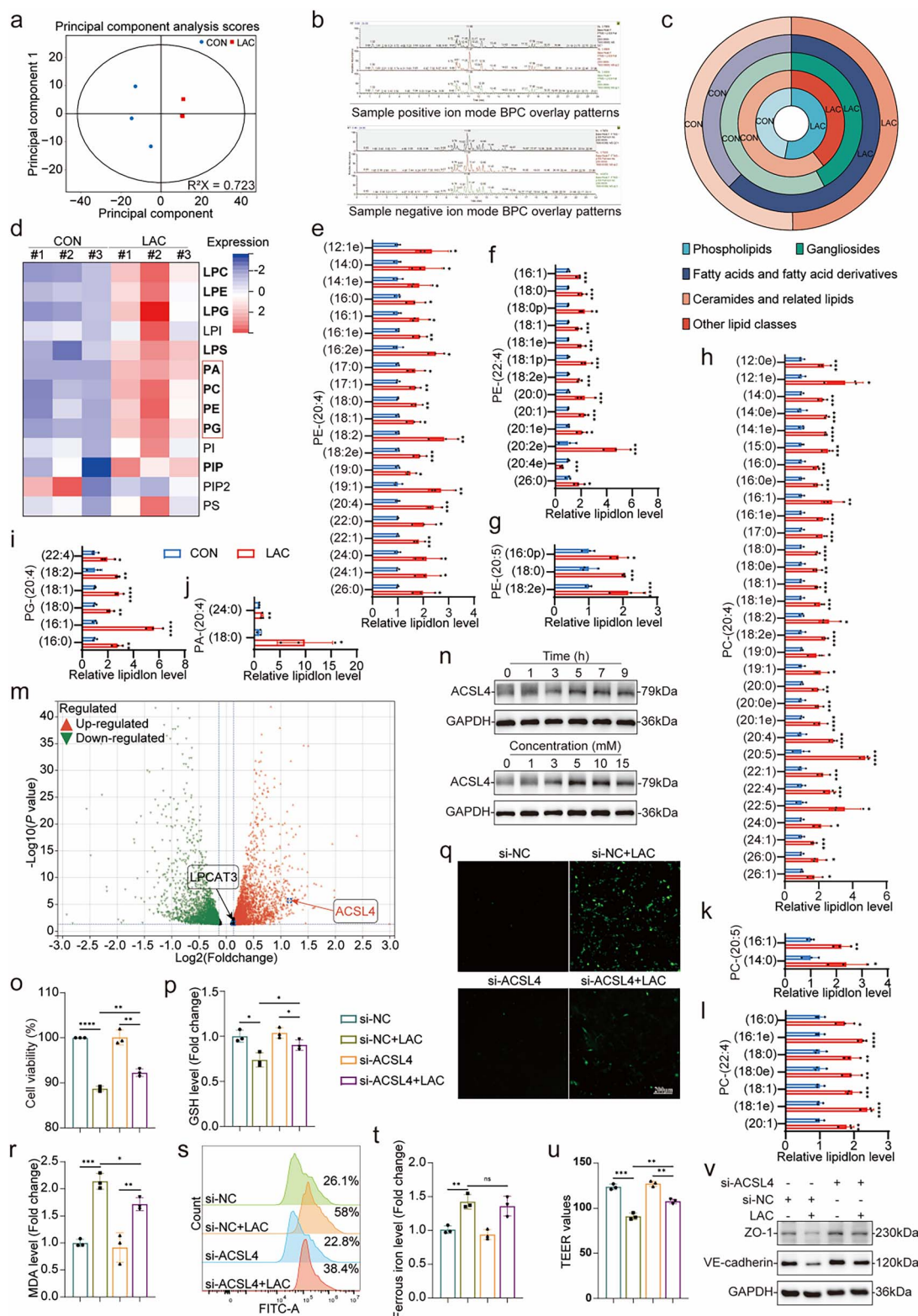


Figure 3. Lactate induces ferroptosis in murine microvascular endothelial cells by promoting lipid peroxidation. **(a, b)** BasePeak spectra and partial least squares discrimination for the untargeted lipid metabolome. **(c)** Changes in lipid composition were analysed by LC-MS/MS in normal MPMVECs as well as in lactate-treated MPMVECs ($n=3$ per group). **(d)** Heatmap showing the expression of phospholipids in the lipid metabolome analysis of different groups of MPMVECs ($n=3$ per group). **(e–l)** Relative expression of phospholipid subclasses in different groups of MPMVECs in lipid metabolome analysis ($n=3$ per group). **(m)** Differential gene expression analysis of control MPMVECs as well as lactate-treated MPMVECs was performed using RNA-seq. **(n)** ACSL4 levels in MPMVECs under different concentrations of lactate treatment were examined by western blot ($n=3$ per group). **(o–u)** Effect of lactate treatment on cell viability, GSH, ROS, MDA, C11-BODIPY 581/591, Fe²⁺, and trans-endothelial electrical resistance in MPMVECs knocked down and not knocked down by ACSL4 ($n=3$ per group). **(v)** The effect of lactate treatment on VE-cadherin, ZO-1 levels in MPMVECs with and without knockdown of ACSL4 was analysed by western blot ($n=3$ per group). All data were represented as the means $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant. MPMVEC mouse pulmonary microvascular endothelial cell, ACSL4 long-chain acyl-CoA synthetase 4, LC-MS/MS liquid chromatography–mass spectrometry, ROS reactive oxygen species, MDA malondialdehyde, SD standard deviation

Intracellular iron homeostasis is regulated primarily by TF- and TFR-mediated iron uptake, FPN1-mediated iron export, IRP2-mediated iron metabolism, and ferritin-mediated iron storage [49]. Interestingly, the qPCR results indicated that lactate treatment did not affect the mRNA levels of TF, TFR, FPN1, IRP2, or FTH1 (Figure 4a). Subsequent WB analysis revealed that only FTH1 protein expression decreased after lactate treatment, while the protein levels of TF, TFR, FPN1, and IRP2 were unaffected by lactate (Figure 4b, Figure S4a, see online supplementary material). FTH1 is essential for intracellular iron storage, and its proteolytic degradation leads to the rapid release of free iron [50]. These results suggest that ferritin undergoes posttranslational degradation. The caspase, ubiquitin–proteasome, and autophagy–lysosome pathways are the three major systems controlling protein degradation in eukaryotic cells [51–53]. To determine which pathways are involved in lactate-induced ferritin degradation, cells were treated with dimethyl sulfoxide (DMSO) as a control and then with Z-VAD (a pan-caspase inhibitor), MG132 (a proteasome inhibitor), or 3-MA (an autophagy inhibitor) after lactate stimulation. As shown in Figure 4c and Figure S4b, FTH1 expression was preserved in cells treated with 3-MA but not in cells treated with LAC, Z-VAD, or MG132. These data suggest that lactate degrades FTH1 via the autophagy–lysosome pathway. Recent studies have confirmed that ferritinophagy plays a critical role in the process of ferroptosis [53, 54]. NCOA4, as a cargo receptor for ferritin autophagy degradation, mediates ferritinophagy, leading to ferritin degradation, increased intracellular iron levels, and subsequent ferroptosis [54, 55]. LC3, as a structural protein of autophagosomes, binds to NCOA4 to form autophagosomes and participates in ferritinophagy [56]. We observed via RNA-seq that NCOA4 and LC3 (including LC3a and LC3b) transcript levels increased in the lactate-treated group (Figure 4d). Subsequent WB analysis revealed that NCOA4 and LC3 levels increased with increasing duration or concentration of lactate, whereas the trend for the autophagy substrate P62 was opposite. Additionally, the conversion of LC3-I to LC3-II significantly increased (Figure 4e–f, Figure S4c–d, see online supplementary material). Pretreatment with the autophagy inhibitor 3-MA significantly reduced lactate-induced increases in the levels of LC3, SQSTM1, and LC3-II/LC3-I (Figure S4e, see online supplementary material). Cellular immunofluorescence revealed significant increases in the colocalization of NCOA4, FTH1, and lysosomes after lactate stimulation (Figure 4g). Electron microscopy revealed a significant increase in the number of autophagosomes after lactate treatment (Figure 4h). In the LPS-stimulated cell model, additional lactate significantly increased NCOA4 and LC3 protein levels. OXA treatment to inhibit lactate production reduced NCOA4 and LC3 protein levels and decreased the interaction between NCOA4 and ferritin (Figure 4i, j, Figure S4f, see online supplementary material). Consistent results were obtained in the CLP mouse model with increased lactate or lactate inhibition (Figure 4k, Figure S4g, see online supplementary material). To elucidate the relevance of NCOA4 and LC3 in lactate-induced ferritinophagy, we constructed NCOA4-knockdown murine microvascular endothelial cells. NCOA4 silencing resulted in increased FTH1 levels due to impaired ferritinophagy induced by lactate and rescued the lactate-induced decrease in cell viability, with a significant decrease in cellular iron ion levels and a decrease in LPO and ROS levels (Figure 4l–r, Figure S4h, see online

supplementary material). Inhibition of NCOA4 attenuates lactate damage to the permeability of monolayer MPMVECs (Figure 4s). In the CLP mouse model, the expression of ACSL4 and NCOA4 in lung tissues increased significantly along with the disease course, and positively correlated with serum lactate levels (Figure S4i–k, see online supplementary material). Taken together, these results suggest that lactic acid increases ferritin autophagy via NCOA4, leading to the dysregulation of intracellular iron homeostasis, which in turn induces iron death in cells, leading to increased vascular permeability.

Lactate mediates the positive regulation of ACSL4, NCOA4, and LC3 by increasing histone H3K18 lactylation

Recent studies have shown that lactate can induce lactylation by providing substrate for adding lactyl groups to lysine (K) residues in histones, thereby regulating gene transcription [57]. We found that with increasing lactate treatment time and concentration, the level of L-lactyl-lysine (Pan K1a) in cells tended to increase (Figure 5a). In the LPS-stimulated cell model, LPS increased Pan K1a levels, and additional lactate further increased Pan K1a levels, whereas OXA significantly inhibited the LPS-induced increase in Pan K1a levels (Figure 5b). To validate the impact of histone lactylation on ACSL4, NCOA4, and LC3, the lactylation inhibitor C646 was used to reverse the lactate-induced upregulation of Pan K1a, ACSL4, NCOA4, and LC3 expression, as well as the binding of NCOA4 to FTH1 (Figure 5c, d, Figure S5a, see online supplementary material). The enzymes mediating lactylation are mainly acetyltransferases [58], and C646 used in this study is one such representative. We examined the effect of lactate on acetylation. Pan Kac levels did not change with variations in lactate concentration or treatment time. In *in vivo* experiments, in CD31-marked vascular areas in mouse lung tissues, Pan K1a expression was significantly increased in CLP mice, whereas OXA treatment significantly reduced Pan K1a expression (Figure 5e, f). Additionally, we observed that under lactate or LPS stimulation, Pan K1a expression was highest at ~15 kDa, corresponding to the molecular weights of histones H3 and H4.

Recent research on histone lactylation has focused mainly on H3 and H4, which undergo lactylation, leading to a series of effects. Therefore, we focused on the lactylation of histones H3 and H4. We assessed the fluorescence colocalization of K1a with histones H3 or H4 in CD31-labelled blood vessels in the lungs of mice. Compared with K1a and histone H4, K1a and histone H3 were colocalized at higher levels in the SHAM and CLP groups (Figure 5g). In CLP lungs, in CD31-marked blood vessels, the intensity of K1a and histone H3 from a to b showed strong colocalization (Figure S5b, see online supplementary material). Multiple lactylation modification sites have been identified on histone H3 [58]. We used CPLM (<http://cplm.biocuckoo.cn/index.php>) and UniProt (<https://www.uniprot.org/>) to predict lactylation sites on histone H3 in both mice and humans. We found that in both species, the confidence for the lactylation of histone H3 at K18, K23, and K27 was high (Figure 5h, Figure S5c, see online supplementary material). WB analysis of CLP mouse lung tissues revealed that among H3K18la, H3K23la, and H3K27la levels, only H3K18la was significantly increased compared with that in the SHAM group (Figure 5i). In MPMVECs, H3K18la protein expression increased with

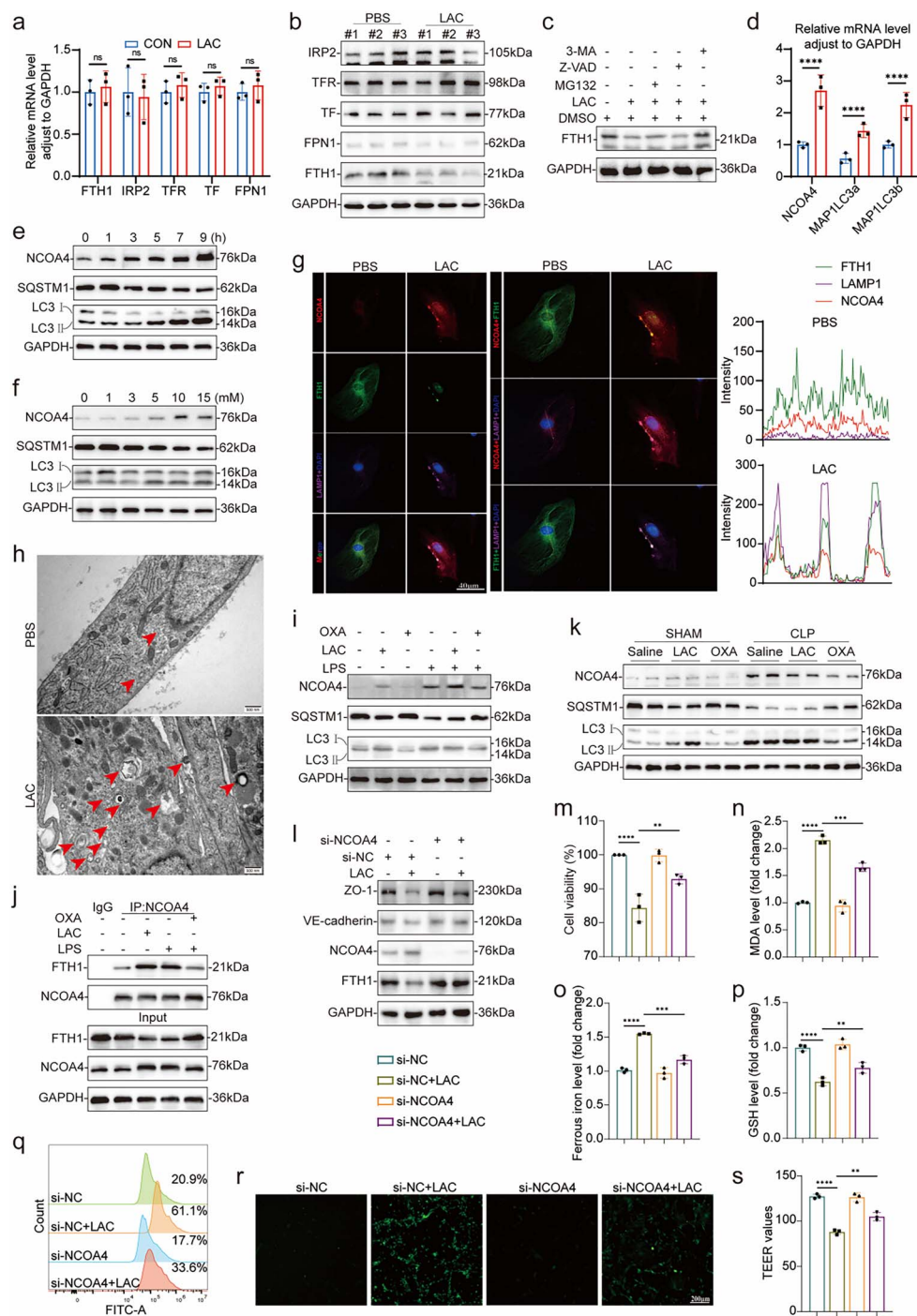


Figure 4. Lactate promotes ferroptosis in murine microvascular endothelial cells through ferritin autophagy. **(a)** The effect of lactate treatment on FTH1, IRP2, TFR, TF, and FPN1 mRNA expression in MPMVECs was detected by qPCR ($n = 3$ per group). **(b)** The effect of lactate treatment on the expression of FTH1, IRP2, TFR, TF, and FPN1 in MPMVECs after treatment was analysed by western blot ($n = 3$ per group). **(c)** The effects of Z-VAD, MG132, and 3-MA on lactate-induced FTH1 degradation. Cells were treated with DMSO or Z-VAD (20 μ M), MG132 (10 μ M), or 3-MA (5 mM) for 6 h, and then treated with lactate for 7 h. Cells were harvested for protein blotting to analyse FTH1 expression ($n = 3$ per group). **(d)** The effect of lactate treatment on NCOA4, MAP1LC3a, and MAP1LC3b mRNA levels in MPMVECs was detected by qPCR ($n = 3$ per group). **(e, f)** NCOA4, LC3, and SQSTM1 levels in MPMVECs treated with different concentrations of lactate as well as temporal gradients of lactate were analysed by western blot ($n = 3$ per group). **(g)** NCOA4 and FTH1 levels of MPMVECs under lactate treatment were analysed by immunofluorescence. Co-localization levels of NCOA4 with FTH1, NCOA4 with LAMP1, and FTH1 with LAMP1 were also examined (NCOA4: red, FTH1: green, LAMP1: purple), scale bar: 40 μ m. **(h)** Representative images of the ultrastructure of autophagic lysosomes in transmission electron microscopy of MPMVECs in the normal group and MPMVECs after lactate treatment; red arrows indicate autophagic lysosomes, scale bar: 500 nm. **(i)** NCOA4, LC3, and SQSTM1 levels were analysed by western blot in MPMVECs from different treatments ($n = 3$ per group). **(j)** Cell lysates of differently treated MPMVECs were prepared by RIPA lysis and coimmunoprecipitated with NCOA4 antibody with IgG as a negative control. **(k)** NCOA4, LC3, and SQSTM1 levels were analysed by western blot in lung tissues of mice with different treatments ($n = 6$ per group). **(l)** The effect of lactate treatment on VE-cadherin, ZO-1, and FTH1 levels in MPMVECs with and without knockdown of NCOA4 was analysed by western blot ($n = 3$ per group). **(m–s)** The effect of lactate treatment on cell viability, GSH, ROS, MDA, C11-BODIPY 581/591, Fe^{2+} , and trans-endothelial electrical resistance in MPMVECs knocked down and not knocked down for NCOA4 ($n = 3$ per group). All data were represented as the means $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant. MPMVEC mouse pulmonary microvascular endothelial cell, qPCR quantitative PCR, ROS reactive oxygen species, MDA malondialdehyde, SD standard deviation, DMSO dimethyl sulfoxide

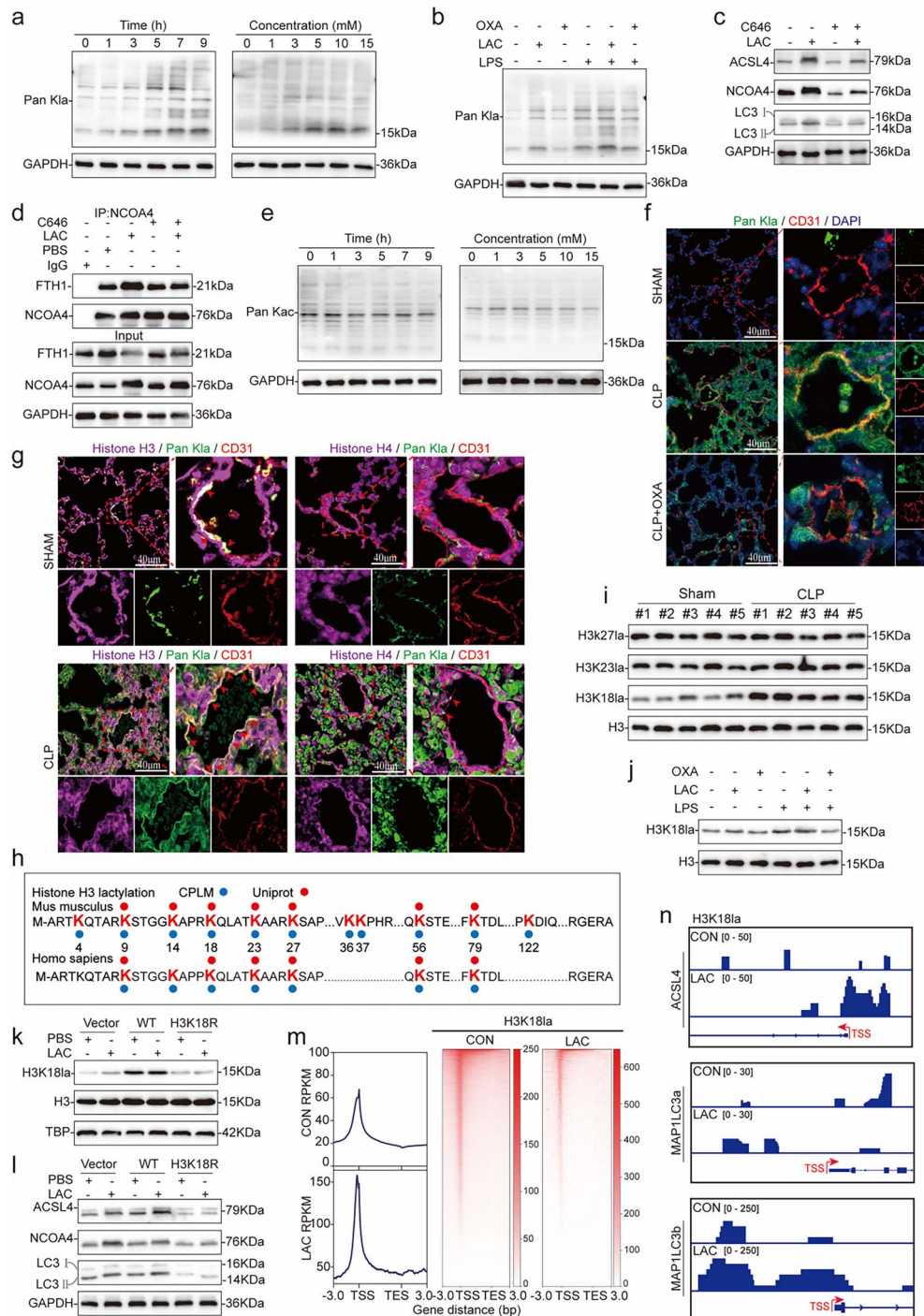


Figure 5. Lactate mediates the positive regulation of ACSL4, NCOA4, and LC3 by increasing histone H3K18 lactylation. **(a)** The effect of different concentrations of lactate or different times of treatment on Pan K1a levels in MPMVECs ($n = 3$ per group). **(b)** The effects of different treatment times of lactate on the Pan K1a levels in MPMVECs ($n = 3$ per group). **(c)** The effect of C646 on ACSL4, NCOA4, and LC3 levels in MPMVECs after 7 h of lactate stimulation was examined by western blot ($n = 3$ per group). **(d)** Co-immunoprecipitation using NCOA4 antibody with IgG as a negative control was used to detect NCOA4 binding to FTH1 in MPMVECs after 7 h of lactate stimulation by C646 treatment. **(e)** The effect of different concentrations of lactate or different times of treatment on Pan Kac levels in MPMVECs ($n = 3$ per group). **(f)** Representative images of immunofluorescence co-localization of Pan K1a and the vascular cell marker CD31 in the lungs of SHAM, CLP, CLP + OXA mice, scale bar: 40 μm. **(g)** Representative images of immunofluorescence co-localization of Pan K1a, Histone H3, Histone H4, and the vascular cell marker CD31 in the lungs of SHAM and CLP mice, with arrows indicating significant areas of co-localization, scale bar: 40 μm. **(h)** Lactylation sites on histone H3 were predicted on CPLM (<http://cplm.biocuckoo.cn/index.php>) and UniProt (<https://www.uniprot.org/>). **(i)** H3K27la, H3K23la, and H3K18la levels in lung tissue lysates of SHAM, CLP mice were analysed by western blot ($n = 5$ per group). **(j)** The effects of OXA and lactate on H3K18la expression in MPMVECs with or without LPS stimulation ($n = 3$ per group). **(k, l)** Vector, wild-type, or H3K18R overexpressing MPMVECs were constructed by overexpression plasmid, and the effect of lactate treatment on the levels of H3K18la, H3, ACSL4, NCOA4, and LC3 in each group of MPMVECs was analysed by western blot ($n = 3$ per group). **(m)** Binding density of H3K18la within ±3 kb of the TSS region of MPMVECs from CUT&Tag data visualized by deepTools. **(n)** Representative traces of CUT&Tag show the effect of lactate on the enrichment of H3K18la in the TSS region of ACSL4, MAP1LC3a, and MAP1LC3b genes. All data were represented as the means ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant. MPMVEC mouse pulmonary microvascular endothelial cell, CLP caecal ligation and puncture, ACSL4 long-chain acyl-CoA synthetase 4, NCOA4 nuclear receptor coactivator 4, OXA oxamate, CUT&Tag cleavage under target and tagmentation

increasing duration and concentration of lactate, whereas H3K18ac levels did not change (Figure S5d, see online supplementary material). Compared with the control, LPS increased H3K18la protein expression, and additional lactate further elevated H3K18la levels, whereas OXA significantly inhibited the LPS-induced increase in H3K18la levels (Figure 5j, Figure S5e, see online supplementary material). We subsequently constructed lentiviral plasmids expressing wild-type H3 (H3-WT) and a mutant H3 in which K18 was replaced with arginine (H3-K18R). This mutation was validated by Sanger sequencing (Figure S5f, see online supplementary material) and Western blot analysis (Figure 5k). Compared with the H3-WT group, in the H3-K18R mutant group, lactate treatment significantly reduced the expression of ACSL4, NCOA4, and LC3 (Figure 5l). Studies have shown that histones can be directly enriched at specific gene promoter regions, increasing their transcription levels [57]. To determine whether H3K18la directly binds to the promoter regions of ACSL4, NCOA4, and LC3, we performed cleavage under target and tagmentation (CUT&Tag) to extract and sequence gene fragments bound by H3K18la in MPMVECs treated with PBS or lactate. Compared with the control, lactate significantly increased the number of H3K18la-binding regions, with a notable increase in the enrichment of gene promoters (Figure 5m). We also observed the enrichment of H3K18la in the promoter regions of ACSL4 and LC3 (including MAP1LC3a and MAP1LC3b), with lactate treatment significantly increasing the extent of H3K18la enrichment (Figure 5n). To validate the CUT&Tag results, we performed chromatin immunoprecipitation-PCR (ChIP-qPCR) and RT-qPCR analyses, and the results confirmed the enrichment of H3K18la in the promoter regions of ACSL4 and LC3 and the increased transcription levels of ACSL4 and LC3 after lactate treatment (Figure S5g–h, see online supplementary material). However, we did not observe H3K18la enrichment in the NCOA4 promoter region. The ChIP-PCR results further confirmed that H3K18la does not directly bind to the NCOA4 promoter region (Figure S5h, see online supplementary material). These findings indicate that H3K18la does not directly regulate the transcription of NCOA4.

H3K18la promotes NCOA4 expression by regulating the transcription of the transcription factor GATA2

Studies have shown that histone lactylation can indirectly regulate downstream gene expression by modulating the transcription of upstream transcription factors. To explore the mechanism by which histone H3K18la regulates NCOA4 expression, we used Promo (https://algen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), TFDB (<https://link.zhihu.com/?target=https%3A/guolab.wchscu.cn/AnimalTFDB4/>), and ARCHS4 (<https://maayanlab.cloud/archs4>) to predict transcription factors for NCOA4 and intersected the results with the RNA-seq and H3K18la-bound fragment sequencing data, which led to the identification of the transcription factor GATA2 (Figure 6a). Lactate treatment significantly increased the enrichment of H3K18la in the GATA2 promoter region (Figure 6b). We performed ChIP-qPCR and RT-qPCR analyses, and the results confirmed the enrichment of H3K18la in the GATA2 promoter region and the increased GATA2 transcription levels following lactate treatment (Figure 6c, d).

Dual-luciferase reporter assays further validated the transcriptional activation of NCOA4 (Figure 6e). In MPMVECs, GATA2 protein expression increased with increasing duration and concentration of lactate treatment (Figure 6f, Figure S6a, see online supplementary material). Moreover, GATA2 mRNA levels in cells significantly increased after lactate stimulation (Figure 6g). Compared with the control, LPS increased GATA2 protein expression, and the addition of lactate further elevated GATA2 levels, whereas OXA significantly inhibited the LPS-induced increase in GATA2 levels (Figure 6h, Figure S6b, see online supplementary material). In the CLP mouse model, GATA2 protein expression increased in lung tissues as the duration of CLP increased (Figure 6i). Increased or inhibited lactate levels produced consistent results in both cells and tissues (Figure 6j, k, Figure S6c, see online supplementary material). When C646 was used to inhibit histone lactylation, the lactate-induced upregulation of GATA2 expression was significantly suppressed (Figure 6l, Figure S6d, see online supplementary material). Similar effects were observed in cells expressing the H3-K18R mutant (Figure 6m). We subsequently constructed GATA2 knockdown and overexpression cell models to explore the effects of GATA2 knockdown and overexpression on NCOA4 expression in MPMVECs. GATA2 knockdown significantly inhibited the lactate-induced upregulation of NCOA4 expression (Figure 6n). GATA2 overexpression reversed the C646-induced reduction in NCOA4 expression (Figure 6o). In conclusion, these results suggest that H3K18la promotes NCOA4 expression by enhancing GATA2 transcription in MPMVECs.

P300 gene knockdown attenuates acute lung injury in sepsis and pulmonary vascular hyperpermeability

To better understand the role of histone lactylation in the ferroptosis of pulmonary microvascular endothelial cells *in vivo*, we constructed a P300 knockdown model using AAV6, and the model was validated by Western blotting (Figure 7a, Figure S7a–b, see online supplementary material). Subsequently, CLP was randomly performed on WT and P300-knockdown mice, and lung tissues were collected 24 h post-surgery. Compared with the WT, P300 knockdown significantly reduced acute lung injury in the CLP model mice (Figure 7b–d). Additionally, P300 inhibition significantly improved pulmonary hyperpermeability in CLP mice (Figure 7e–g), reduced endothelial damage (Figure 7h), and rescued the loss of vascular adhesion proteins (Figure 7i–k). Compared with the WT, P300 knockdown significantly suppressed Pan K1a and H3K18la levels in the lung tissues of CLP mice and reduced ACSL4, NCOA4, LC3, and GATA2 protein levels, although there was no change in the LC3II/LC3I ratio (Figure 7l, Figure S7c, see online supplementary material). We also observed that after P300 knockdown, iron accumulation and oxidative stress levels in the lung tissues of CLP mice significantly decreased, as indicated by lower Fe²⁺ and MDA levels (Figure 7m, n), decreased GSH levels (Figure 7o), and enhanced lung tissue DAB-enhanced Perls' Prussian Blue staining, indicating aggravated iron deposition (Figure 7p). Survival analysis revealed that inhibiting P300 *in vivo* increased the 7-day survival rate of CLP mice (Figure 7q).

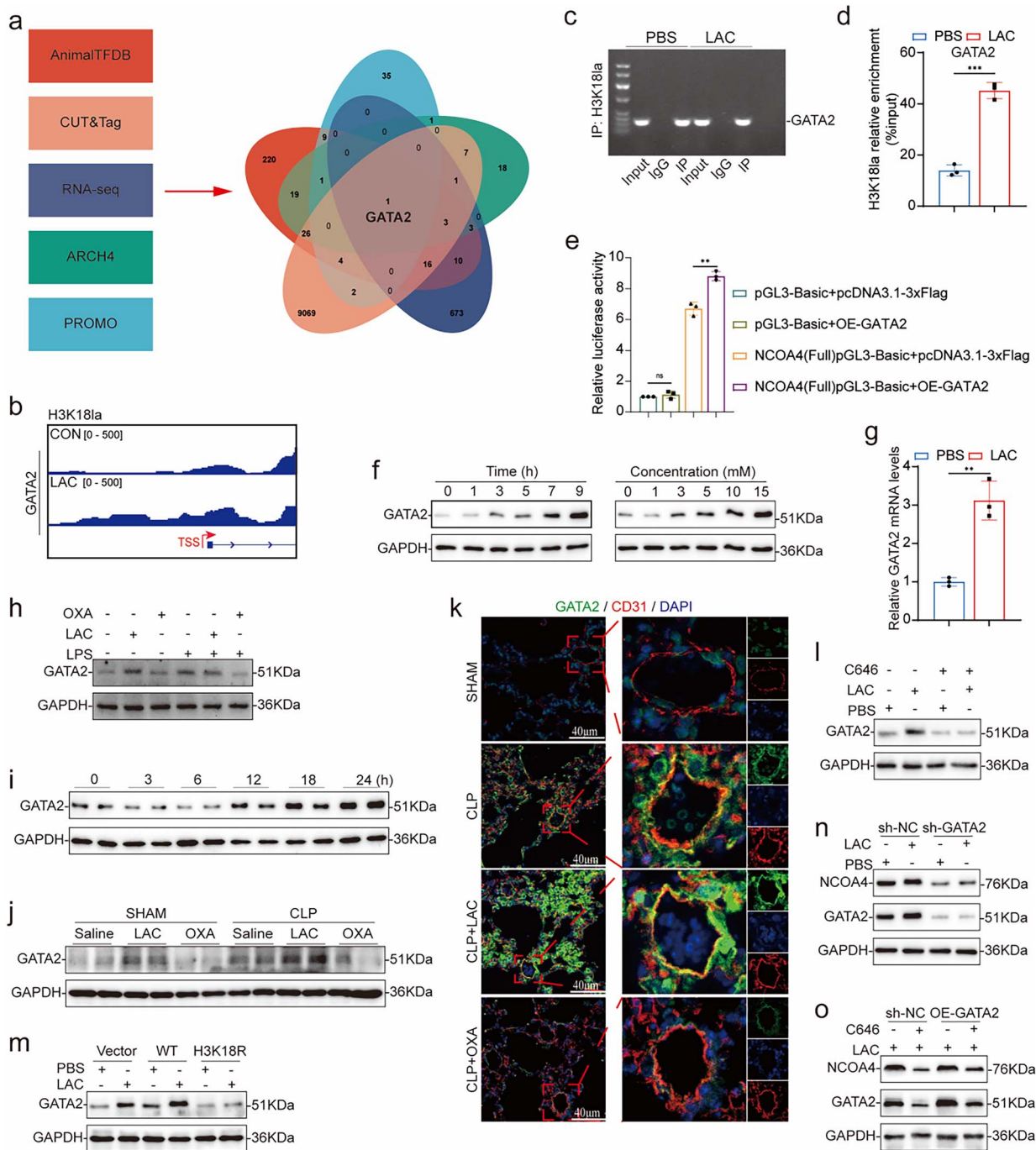


Figure 6. H3K18la promotes NCOA4 expression by regulating the transcription of the transcription factor GATA2. **(a)** NCOA4 upstream transcription factors were predicted at three online prediction sites, AnimalTFDB, ARCH4, and PROMO, and intersected with differential genes from RNA-seq and CUT&Tag data. GATA2 was screened and visualized using Venn diagrams. **(b)** Representative traces of CUT&Tag show the effect of lactate on the enrichment of H3K18la in the TSS region of GATA2. **(c, d)** ChIP-PCR as well as ChIP-qPCR validation of H3K18la in the GATA2 promoter region. **(e)** A luciferase reporter gene vector containing the NCOA4 promoter sequence was constructed and transfected into MPMVECs, and a GATA2 overexpression vector was constructed to measure luciferase activity. **(f)** The effect of different concentrations of lactate or different time treatments on GATA2 levels in MPMVECs ($n=3$ per group). **(g)** The effect of lactate treatment (10 mM for 7 h) on GATA2 mRNA levels in MPMVECs ($n=3$ per group). **(h)** GATA2 levels in MPMVECs from different treatments were analysed by western blot ($n=3$ per group). **(i)** GATA2 levels in mouse lung tissue lysate at different time points after CLP were analysed by western blot ($n=6$ per group). **(j)** GATA2 levels in lung tissue lysates from mice with different treatments were analysed by western blot ($n=6$ per group). **(k)** Representative images of immunofluorescence co-localization of GATA2 and the vascular cell marker CD31 in the lungs of SHAM, CLP, CLP + LAC, and CLP + OXA mice, with the arrowheads indicating significant areas of co-localization, scale bar: 40 μm . **(l)** The effect of C646 on GATA2 levels in MPMVECs after 7 h of lactate stimulation was examined by western blot ($n=3$ per group). **(m)** Vector, wild-type, or H3K18R overexpressing MPMVECs were constructed by overexpression plasmid, and the effect of lactate treatment on the levels of GATA2 in each group of MPMVECs was analysed by western blot ($n=3$ per group). **(n)** A model of GATA2 knockdown MPMVECs was constructed by plasmid and the effect of lactate treatment on GATA2 levels in MPMVECs in each group was analysed by western blot ($n=3$ per group). **(o)** GATA2 overexpressing MPMVECs were constructed by overexpression plasmid, and the effect of C646 treatment on GATA2 levels in each group of MPMVECs was analysed by western blot after 7 h of lactate stimulation ($n=3$ per group). All data were represented as the means $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant. H3K18la histone H3 lysine 18 lactylation, NCOA4 nuclear receptor coactivator 4, CUT&Tag cleavage under target and tagmentation, ChIP-qPCR chromatin immunoprecipitation-PCR, CLP caecal ligation and puncture, SD standard deviation, OXA oxamate, MPMVEC mouse pulmonary microvascular endothelial cell

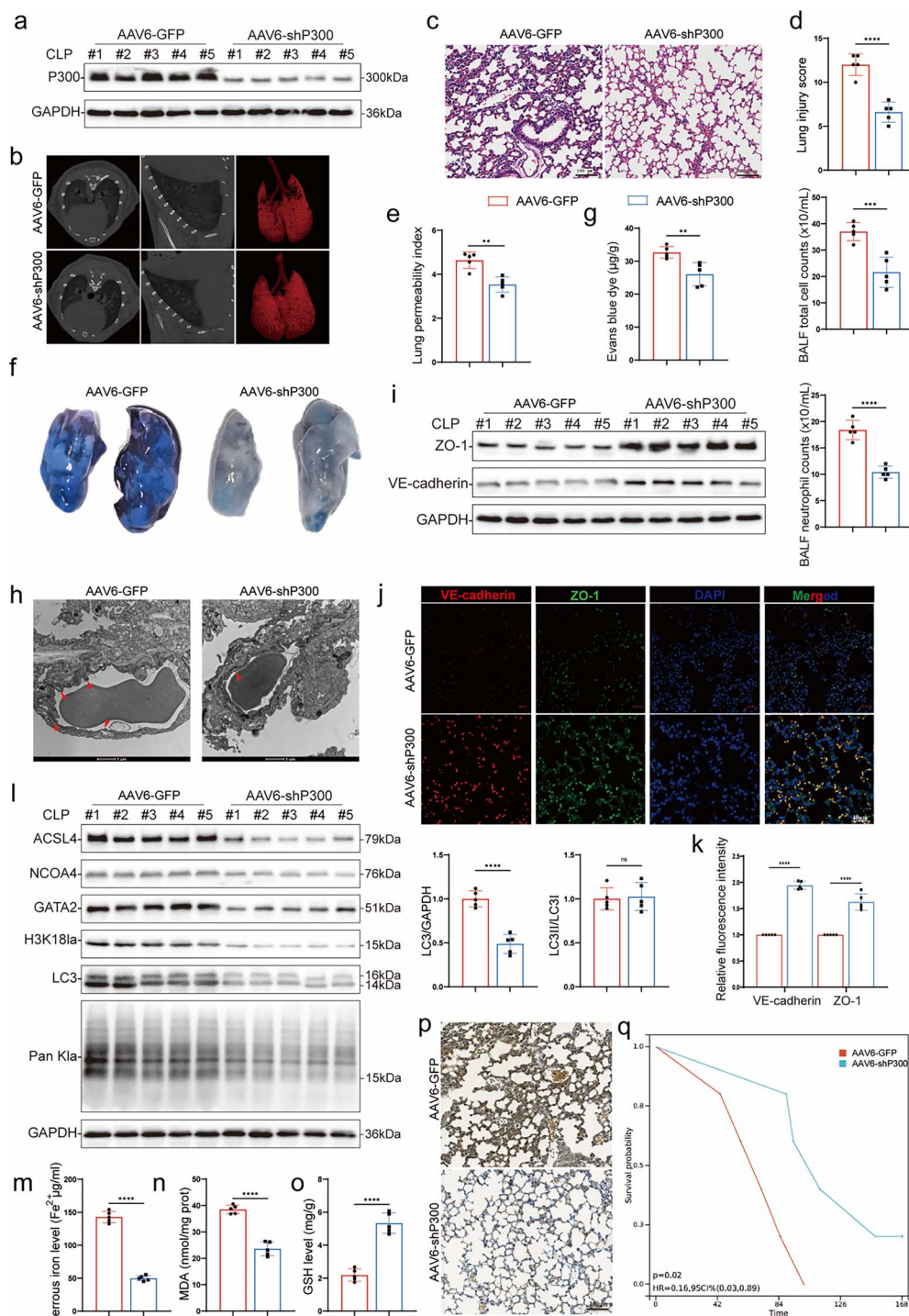


Figure 7. P300 gene knockdown attenuates acute lung injury in sepsis and pulmonary vascular hyperpermeability. **(a)** Construction of a P300 knockout CLP mouse model by tail vein injection of AAV6-shP300 or AAV6-GFP validated by western blot. **(b)** Representative images of lung mouse microcomputed tomography 24 h after tail vein injection of AAV6-shP300 or AAV6-GFP 4 weeks after mouse CLP. **(c, d)** representative images of H&E staining of paraffin sections of lung tissues from mice 24 h after CLP after tail vein injection of AAV6-shP300 or AAV6-GFP for 4 weeks, while lung injury was calculated for each group based on the results of H&E ($n = 5$ per group), scale bar: 100 μm . **(e)** Vascular permeability in mouse lung tissue was detected by calculating the lung permeability index after 24 h of CLP in mice after 4 weeks of tail vein injection of AAV6-shP300 or AAV6-GFP ($n = 5$ per group). **(f, g)** Vascular permeability in mouse lung tissue was examined by Evans blue dye (EBD) staining and analysed quantitatively ($n = 5$ per group). **(h)** Representative images of the ultrastructure of the vascular endothelium by transmission electron microscopy of lung tissue in mice 24 h after CLP after tail vein injection of AAV6-shP300 or AAV6-GFP for 4 weeks; red arrows indicate damaged endothelium, scale bar: 1 μm . **(i–k)** VE-cadherin, ZO-1 levels were measured using western blot as well as immunofluorescence analysis on lung tissues of mice 24 h after CLP after 4 weeks of tail vein injection of AAV6-shP300 or AAV6-GFP ($n = 5$ per group), scale bar: 40 μm . **(l)** Levels of ACSL4, NCOA4, GATA2, H3K18la, LC3, and Pan KLa were analysed by western blot in lung tissues of mice 24 h after CLP after tail vein injection of AAV6-shP300 or AAV6-GFP for 4 weeks ($n = 5$ per group). **(m–o)** MDA, Fe^{2+} , and GSH levels were detected in lung tissues of mice with different treatments ($n = 5$ per group). **(p)** Representative images of paraffin sections of differently treated mouse lung tissue DAB-enhanced Perl's Prussian Blue staining. **(q)** Mice were injected with AAV6-shP300 or AAV6-GFP in the tail vein for 4 weeks, and mice were analysed for 7-day survival after CLP (Kaplan–Meier method, $n = 5$ per group). All data were represented as the means $\pm$ SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$; ns, not significant. CLP caecal ligation and puncture, H&E hematoxylin and eosin, EBD Evans Blue Dye, SD standard deviation, MDA malondialdehyde, NCOA4 nuclear receptor coactivator 4, ACSL4 long-chain acyl-CoA synthetase 4

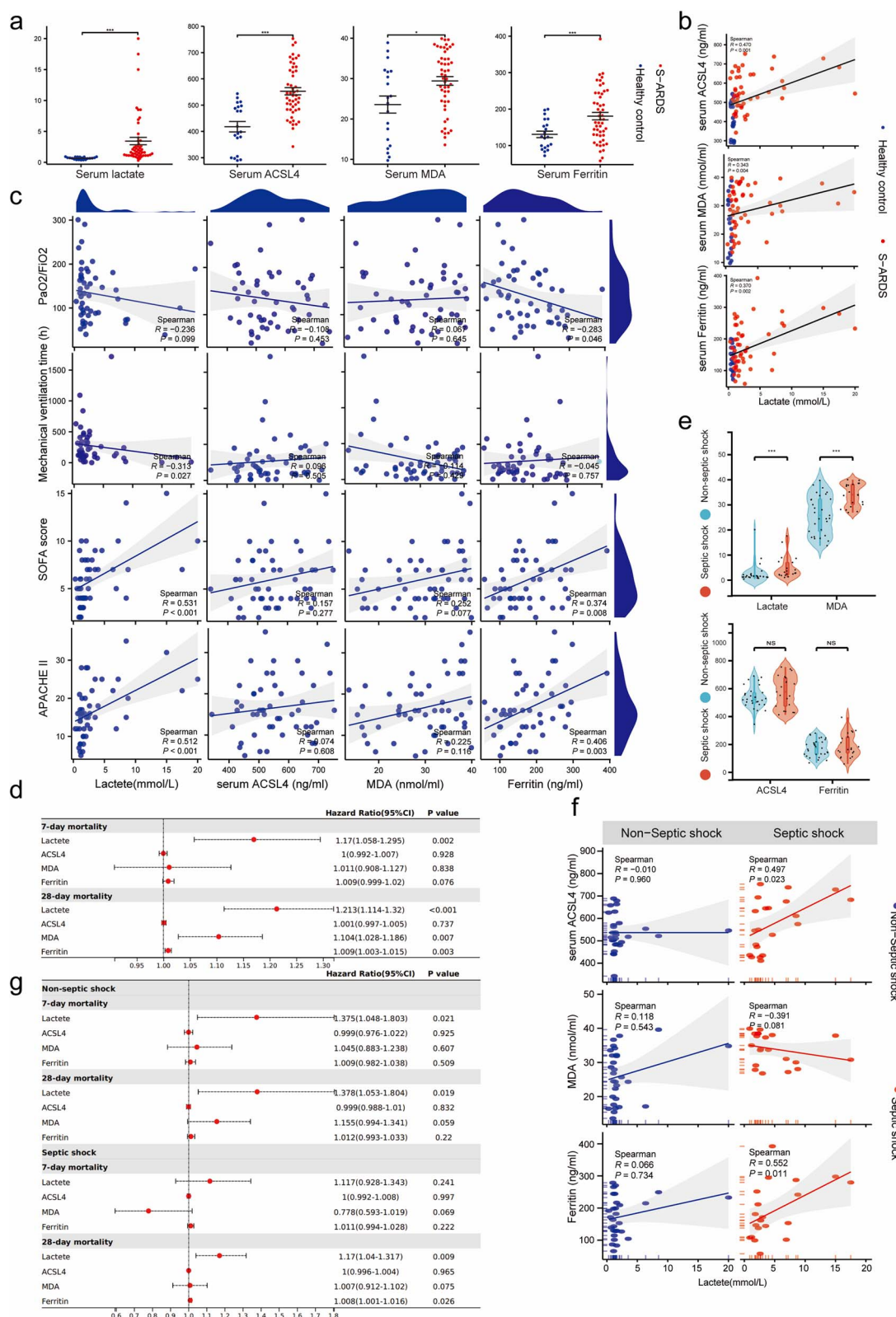


Figure 8. Serum lactate is correlated with ferroptosis levels in patients with S-ARDS. **(a)** Comparison of blood concentrations of lactate, ACSL4, MDA, or ferritin between healthy subjects and patients with S-ARDS. **(b)** Correlation analysis of serum lactate with ACSL4, MDA, or ferritin concentrations in healthy subjects and S-ARDS patients. **(c)** Correlation analysis of blood lactate, ACSL4, MDA, or ferritin concentrations with PaO₂/FiO₂, mechanical ventilation time, SOFA, and APACHE II in patients with S-ARDS. **(d)** Forest plots based on multivariate Cox regression analyses visualized the HR (95% CI) for the risk of 7-day as well as 28-day ICU death in patients with S-ARDS at different initial blood lactate levels. **(e)** Comparison of blood concentrations of lactate, ACSL4, MDA, or ferritin between patients with and without shock S-ARDS. **(f)** Correlation analysis of blood lactate levels with ACSL4, MDA, or ferritin concentrations in patients with or without shock S-ARDS. **(g)** Forest plots based on multivariate Cox regression analyses visualized the HR (95% CI) of the risk of 7-day as well as 28-day ICU death in patients with or without shock S-ARDS at different initial blood lactate levels. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant. ACSL4 long-chain acyl-CoA synthetase 4, MDA malondialdehyde, S-ARDS sepsis-associated acute respiratory distress syndrome, SOFA Sequential Organ Failure Assessment, APACHE II Acute Physiology and Chronic Health Evaluation II, ICU intensive care unit

Serum lactate is correlated with ferroptosis levels in patients with sepsis-associated acute respiratory distress syndrome (S-ARDS)

To clinically validate the role of lactate-mediated ferroptosis in S-ARDS, we recruited S-ARDS patients and healthy controls. After the inclusion and exclusion criteria were established, a total of 50 S-ARDS patients and 20 healthy controls were enrolled. Blood samples and medical histories were collected on the day that S-ARDS patients were admitted to the ICU, and patient outcomes were tracked. The clinical information of the S-ARDS patients included in the analysis is presented in a table (Table S3). We measured the serum levels of lactate, MDA, ACSL4, and ferritin in all participants. Compared with healthy controls, S-ARDS patients presented significantly higher serum levels of lactate, ACSL4, MDA, and ferritin (Figure 8a). We then analysed the correlation between lactate and each marker, revealing a positive correlation between serum lactate and ACSL4, MDA, and ferritin (Figure 8b). In S-ARDS patients, we assessed the correlations between these markers and the oxygenation index, duration of invasive mechanical ventilation, SOFA score, and APACHE II score. We observed a negative correlation between ferritin and the oxygenation index and between lactate and the duration of invasive mechanical ventilation. Lactate and ferritin were positively correlated with the SOFA and APACHE II scores (Figure 8c). We further evaluated the associations between these markers and the prognosis of S-ARDS patients. After adjusting for age and sex, Cox regression analysis revealed that lactate was an independent risk factor for 7-day or 28-day mortality in S-ARDS patients, whereas MDA and ferritin were independent risk factors for 28-day mortality (Figure 8d). Subgroup analysis revealed that patients with septic shock had increased levels of lactate and MDA (Figure 8e). Furthermore, the correlations between the serum lactate level and the levels of ACSL4 and ferritin were detected only in patients with septic shock (Figure 8f). Multivariate Cox regression analysis revealed that, in non-septic shock patients, only the serum lactate level was an independent risk factor for 7-day or 28-day mortality. On the other hand, both the serum lactate level and ferritin level were independent risk factors for 28-day mortality in septic shock patients (Figure 8g). A recent study revealed elevated plasma NCOA4 levels in sepsis patients, which were positively correlated with the SOFA score and lactate level [59]. These findings suggest increased ferroptosis levels in sepsis patients. There is a strong association between lactate and ferroptosis in sepsis patients, and both are independent risk factors for the prognosis of S-ARDS patients.

Discussion

Lactate, a byproduct of energy metabolism, is significantly elevated in the serum of S-ARDS patients and is associated with poor prognosis. Previous studies have shown that lactylation, an emerging epigenetic modification, is closely related to metabolic function, the immune response, and inflammatory regulation in the pathophysiology of sepsis [60]. However, further research is needed to elucidate the exact mechanism of lactylation in sepsis. In this study, we first revealed that elevated lactate levels during sepsis promoted ferroptosis in PMVECs, leading to increased vascular permeability and aggravated ALI. On the one hand, increased lactate-induced H3K18la enhances the transcription of ACSL4 in PMVECs,

leading to excessive lipid peroxidation within cells. On the other hand, elevated H3K18la directly promotes the transcription of the autophagy marker LC3 and indirectly upregulates NCOA4 expression through the transcription factor GATA2, thereby increasing ferritinophagy. Inhibiting lactate and histone lactylation can reduce ferroptosis in PMVECs and increase the survival rate of mice. These findings reveal a novel mechanism by which lactate promotes vascular injury through histone lactylation.

The increase in and accumulation of lactate during sepsis result from hyperactive glycolysis and are positively correlated with the incidence and mortality of septic shock [61]. Those observations are consistent with our findings; we also found that serum lactate levels are related to the duration of mechanical ventilation and disease severity. Previous studies have confirmed that lactate can disrupt endothelial junctions via the GPR81 receptor, leading to increased vascular permeability and organ dysfunction [22]. The mechanism by which high levels of lactate impair endothelial function during sepsis requires further investigation, considering the complexity of vascular endothelial regulation [62, 63]. Therefore, we isolated and cultured MPMVECs and used *in vivo* and *in vitro* experiments to elucidate the mechanism by which lactate affects pulmonary vascular permeability. After ruling out the impact of lactate pH, we observed that lactate exposure resulted in decreased MPMVEC viability and increased cell death. Inhibiting apoptosis did not improve the lactate-induced decrease in MPMVEC viability. We subsequently performed KEGG enrichment analysis and found the enrichment of ferroptosis-related genes in MPMVECs following lactate exposure. We further observed that ferroptosis was significantly inhibited in CLP- and LPS-stimulated MPMVECs after the addition of lactate inhibitors. We also found a significant correlation between serum lactate levels and ferroptosis markers such as ACSL4, MDA, and ferritin in S-ARDS patients. In addition, inhibiting ferroptosis significantly improved the lactate-induced decline in cell viability and reduced vascular permeability. These results indicate that ferroptosis plays a crucial role in lactate-induced vascular injury.

Studies have confirmed that ferroptosis plays a role in the progression of sepsis [64, 65]. Endothelial cell ferroptosis can lead to increased vascular permeability [66]. Ferroptosis is a unique form of cell death characterized by iron-dependent phospholipid peroxidation. Therefore, in this study, we focused on the mechanisms by which lactate influences lipid peroxidation and iron overload during ferroptosis in MPMVECs. Excessive lipid peroxidation serves as the primary signal triggering ferroptosis [67]. Massive production of LPO is essential for the execution of iron pituitary disease. ACSL4 catalyses the formation of fatty acyl-CoA through the incorporation of coenzyme A (CoA) into PUFAs, such as AA, EPA, docosahexaenoic acid, epoxyeicosatrienoic acid, and hydroxyeicosatetraenoic acid [68–70]. These fatty acyl-CoAs either provide cellular energy via β -oxidation or are esterified and incorporated into membrane phospholipids (PLs) through the action of LPCAT3 [71]. Notably, insufficient GPX4/GSH-mediated reductive capacity leads to the accumulation of fatty acyl-CoAs in cell membranes, resulting in hyperperoxidation, membrane rupture, and subsequent ferroptosis [48, 72]. Additionally, the oxidation of excess AA-CoA into hydroxyeicosatetraenoic acids (HETEs) was catalysed, such as 5-HETE, which further contributes to ferroptosis. Intriguingly, this study

demonstrates that AA may also downregulate ACSL4 by promoting its ubiquitination and proteasomal degradation, suggesting a potential bidirectional regulatory loop between ACSL4 and AA [73]. We observed changes in the lipid composition in MPMVECs after lactate administration. PLs, represented by PEs, presented significant increases in content, chain length, and unsaturation after lactate stimulation. Moreover, the levels of AA, AdA, and EPA in PLs also increased significantly. In the RNA-seq data, only ACSL4, which is among the key regulatory enzymes ACSL4 and LPCAT3, showed significant changes, and the WB results revealed an increase in ACSL4 protein levels after lactate treatment. Therefore, we hypothesize that lactate promotes ferroptosis in MPMVECs by upregulating ACSL4 expression. The finding that ACSL4 was subsequently knocked down *in vitro* supported our hypothesis. After ACSL4 inhibition and knockdown *in vitro*, although there was a downwards trend in the intracellular ferrous iron level, it was not significant and could not fully explain the iron overload caused by lactate exposure. Intracellular iron homeostasis is mainly maintained by iron uptake, activation, and storage. The disruption of these processes can lead to an imbalance in iron homeostasis [49]. In this study, we found that lactate promotes the degradation of ferritin, releasing stored iron and causing iron overload. We subsequently observed that ferritin is primarily degraded through the autophagy-lysosome pathway. The expression of NCOA4, a specific receptor that regulates ferritinophagy, was upregulated under lactate stimulation, accompanied by decreased levels of the autophagy substrate SQSTM1, indicating increased autophagic flux [55].

Recent studies have reported that lactate can participate in the epigenetic regulation of gene transcription through histone modification and plays a critical role in immune dysregulation and organ damage associated with sepsis [28, 29, 57]. Lactylation is directly regulated by lactate [21]. A clinical cohort study observed differences in H3K18la levels in peripheral blood samples from healthy volunteers and critically ill patients. H3K18la was significantly correlated with severity and prognosis in critically ill patients and could differentiate between infectious and non-infectious shock [27]. In this study, the overall level of lactylation in the lung tissue of septic mice increased. Subsequent CUT&Tag analysis demonstrated that H3K18la directly binds to the promoter regions of ACSL4 and LC3 and is enriched in the promoter region of the transcription factor GATA2 upstream of NCOA4. Both interfering with lactate production and inhibiting P300 reduced H3K18la levels, lipid peroxidation, and iron overload. Interestingly, studies have shown that lipid peroxidation can increase H3K18la levels in the arteries of atherosclerosis patients [74]. In this study, lactate increased ACSL4 expression via histone H3K18la, thereby increasing lipid peroxidation, suggesting that there may be a positive feedback loop between these two processes. Wu *et al.* reported that during sepsis, histone H3K18la increases ACSL4 mRNA stability by regulating N6-methyladenosine modification levels, thereby inducing ferroptosis in lung epithelial cells [40]. However, we found that histone H3K18la can directly upregulate ACSL4 transcription, leading to increased ACSL4 protein expression. Recent studies indicate a close connection between lactate and autophagy, with lactylation serving as a bridge between lactate and autophagy [75]. Lactylation can promote autophagic flux

and stabilize lysosomal activity to maintain cell homeostasis and inhibit ageing [76, 77]. We observed that H3K18la can directly upregulate LC3 and NCOA4 expression, promoting ferritinophagy and ferroptosis, providing new evidence of a connection among lactate, lactylation, and autophagy. GATA2, a zinc finger transcription factor, is a key regulator of the development and maintenance of haematopoietic stem cells [78]. GATA2 has been reported to promote inflammation and oxidative stress [79, 80]. This study revealed that lactate administration significantly increased GATA2 expression and that GATA2, a novel ferroptosis-promoting factor, increased NCOA4 transcription, thereby promoting ferritinophagy.

Like histone lactylation, nonhistone lactylation plays notable roles and has intriguing regulatory mechanisms in physiological and pathological processes [81]. He *et al.* reported that the lactylation of NCOA4 at the K450 site promotes ferritinophagy in hippocampal neurons [82]. Unfortunately, this study focused primarily on the mechanism of the impact of histone lactylation on ferroptosis and did not explore the effect of NCOA4 lactylation on MPMVEC ferroptosis during sepsis. Whether ACSL4, a key protein in ferroptosis, can also undergo lactylation and thus regulate lipid peroxidation remains an issue that should be addressed in future studies. Notably, P300 is involved not only in lactylation but also in acetylation. However, after 10 h of lactate stimulation, we did not observe any changes in acetylation, suggesting that histone acetylation may occur earlier than lactylation. Furthermore, studies have shown that GATA2 can recruit P300 and promote H3 acetylation [83]. GATA2 may also promote H3 lactylation through the recruitment of P300, forming a positive feedback loop; however, further research is needed to confirm this mechanism.

Several limitations warrant consideration. First, our clinical cohort comprised only 50 patients with sepsis-associated ARDS. Although this sample allowed us to identify correlations between circulating lactate and ferroptosis markers, it is insufficiently powered for robust multivariable modelling or for meaningful stratification by aetiology (infectious vs non-infectious triggers) or by severity subgroups. Second, our mechanistic experiments focused on pulmonary microvascular endothelial cells. Whether alveolar epithelial cells, circulating leukocytes, or fibroblasts undergo similar lactate-driven, H3K18la-mediated ferroptosis—and thereby contribute to the overall lung injury phenotype—remains untested. Third, our *in vitro* conditions employed fixed lactate concentrations and durations that do not fully reproduce the fluctuating metabolic milieu encountered *in vivo*. These constraints temper the generalizability of our findings and should guide the design of larger, multicentre translational studies.

Inhibiting histone lactylation is an appealing therapeutic concept. In our murine sepsis model, genetic silencing of p300 via AAV6 and pharmacological blockade with C646 both reduced lung injury and improved survival. Yet, several hurdles complicate clinical translation. The shared catalytic site of p300/CBP for acetyl-CoA and lactyl-CoA means that current inhibitors lack the selectivity required to disrupt lactylation without simultaneously perturbing global acetylation [58]; isoform-specific compounds remain unavailable. Histone lactylation also peaks early during the hyperinflammatory phase, so any intervention must be precisely timed to avoid interfering with protective innate immunity. Systemic suppression could further impair beneficial lactylation-dependent programmes in immune or reparative cells,

underscoring the need for cell-targeted delivery strategies. Equally missing are bedside-ready assays that quantify H3K18la in peripheral blood or bronchoalveolar lavage fluid, without which patient stratification for precision trials is impossible. Finally, the long-term epigenetic consequences of sustained p300 inhibition have not been assessed. Collectively, these considerations indicate that therapeutic exploitation of histone lactylation will hinge on the development of highly selective inhibitors, validated biomarkers, and rigorously phased safety studies.

Conclusions

In summary, our study provides new insights and potential therapeutic mechanisms regarding the critical role of histone lactylation derived from high lactate levels in PMVEC ferroptosis and the development of ALI during sepsis. Specifically, we explored the mechanisms by which histone H3K18la regulates lipid peroxidation and iron overload. Additionally, we revealed that the transcription factor GATA2, a previously unrecognized regulator of ferroptosis, can upregulate NCOA4 transcription to promote ferritinophagy. These findings increase our understanding of the role of lactylation in regulating ferroptosis and affirm the biological function of lactate in causing vascular dysfunction and lung injury during septicemia.

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Author contributions

Pu Fang (Data curation [equal], Methodology [equal], Project administration [equal], Validation [equal], Visualization [lead], Writing—original draft [lead]), Shuai Li (Data curation [equal], Methodology [equal], Project administration [equal], Validation [equal], Visualization [supporting], Writing—original draft [supporting]), Zong-Qing Lu (Data curation [equal], Investigation [equal], Methodology [equal], Project administration [equal], Validation [equal], Visualization [supporting], Writing—original draft [supporting]), Dun-Lin Xia (Methodology [supporting], Validation [supporting]), Meng-Meng Xu (Funding acquisition [supporting], Methodology [supporting], Validation [supporting]), Jing-Jing Pan (Investigation [equal], Validation [supporting]), Lin Fu (Conceptualization [equal], Funding acquisition [equal], Supervision [equal], Writing—review & editing [equal]), Geng-Yun Sun (Conceptualization [equal], Funding acquisition [equal], Supervision [equal], Writing—review & editing [equal]), and Qing-Hai You (Conceptualization [equal], Funding acquisition [equal], Supervision [equal], Writing—review & editing [equal])

Ethics approval and consent to participate

The clinical study was approved by the Research Ethics Committee of the Anhui Chest Hospital (No. KJ2023-40). All animal experiments were approved by the Ethics Committee of the Institute of Health and Medicine, Hefei National Integrated Science Centre (approval number: IHM-AP-2020-004). Informed consent was obtained from all subjects involved in the study.

Conflict of interest

None declared.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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