Exploring the metabolic phenotypes associated with different host inflammation of acute respiratory distress syndrome (ARDS) from lung metabolomics in mice

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Rationale: The aim of this study was to analyze the metabolomics of lung with different host inflammation of acute respiratory distress syndrome (ARDS) for the identification of biomarkers for predicting severity under different inflammatory conditions.

Methods: Cecal ligation and puncture (CLP) and lipopolysaccharide (LPS)-intratracheal injection induced acute lung injury (ALI) were used. A mouse model was used to explore lung metabolic biomarkers in ALI/ARDS. The splenectomy model was used as an auxiliary method to distinguish between hyper- and hypo-inflammatory subtypes. Plasma, lung tissue and bronchoalveolar lavage fluid (BALF) samples were collected from mice after CLP/LPS. The severity of lung injury was evaluated. Expression of tumor necrosis factor-α (TNF-α) in mice serum and lung was tested by enzyme-linked immunosorbent assay (ELISA) and polymer chain reaction (PCR). Polymorphonuclear cells in BALF were counted. The lung metabolites were detected by gas chromatography/mass spectrometry (GC/MS), and the metabolic pathways predicted using the KEGG database.

Results: The LPS/CLP-Splen group had more severe lung injury than the corresponding ALI group; that in the CLP-Splen group was more serious than in the LPS-Splen group. TNF-α expression was significantly elevated in the serum and lung tissue after LPS or CLP, and higher in the LPS/CLP-Splen group than in the corresponding ALI group. The level of TNF-α in the CLP-Splen group was elevated significantly over that in the LPS-Splen group. Both these groups also showed significant neutrophil exudation within the lungs. During differential inflammation, more differential metabolites were detected in the lungs of the CLP group ALI mice than in the LPS group. A total of 41 compounds were detected in the lungs of the CLP and CLP-Splen groups. Contrastingly, eight compounds were detected in the lungs of the LPS and LPS-Splen groups. The LPS-Splen and CLP-Splen groups had significant neutrophil exudation in the lung. Random forest analysis of lung-targeted metabolomics data indicated 4-hydroxyphenylacetic acid, 1-aminocyclopentanecarboxylic acid (ACPC), cis-aconitic acid, and hydroxybenzoic acid as strong predictors of the hyper-inflammatory subgroup in the CLP group.
Furthermore, with splenectomy, 13 differential metabolic pathways between the CLP and LPS groups were revealed.

**Conclusions:** Hyper-inflammatory subgroups of ARDS have a greater inflammatory response and a more active lung metabolism. Combined with the host inflammation background, biomarkers from metabolomics could help evaluate the response severity of ARDS.

## 1 | INTRODUCTION

Acute respiratory distress syndrome (ARDS) is an acute inflammatory lung injury, associated with increased pulmonary vascular permeability, increased lung weight, and loss of aerated lung tissue.\(^1\) Although 50 years have passed since the first description of ARDS,\(^2\) the overall mortality is still more than 40%.\(^3,4\) Unfortunately, it is a clinical feature of very different mechanisms, with complex syndromes, and biological and clinical heterogeneity. Because of the heterogeneity of the host response, it is difficult to find the key to curing every patient with ARDS. The clinical and biomarker characteristics of ARDS patients demonstrated hypo-inflammatory and hyper-inflammatory effects.\(^5\) Specific subsets of critically ill patients have higher risk of disease-related outcome or differential responses to therapy.\(^6,7\) Therefore, the different inflammatory sub-phenotypes of ARDS may indicate varied risks related to the disease.\(^8\) Metabolic phenotypes, which represent different pathways important to the pathophysiology of ARDS, could potentially be used to identify the subgroup that may benefit from certain targeted therapies.\(^9\) About one-third of hyper-inflammatory ARDS patients have a higher plasma level of inflammatory biomarkers.\(^5\) Some biological indicators, such as endocan,\(^10\) SRAGE and Ang-2,\(^9\) are closely related to the hyper-inflammatory sub-phenotype of ARDS.

Tumor necrosis factor-α (TNF-α) is an important inflammatory factor that can induce T cells to produce various inflammatory factors, and then promote the occurrence of inflammatory reactions. It has been reported that TNF-α is a potential biomarker for ARDS, as well as for mortality in patients with obesity and Coronavirus (COVID-19).\(^11\) In addition, studies have found that 4-hydroxybenzoic acid has anti-catabolism and anti-inflammatory effects, and prevents the upregulation of pro-inflammatory markers, including metalloproteinases and cyclooxygenase 2.\(^12\) Whether 1-aminocyclopentanecarboxylic acid (ACPC), as a small molecule, also has such potential needs further study. Thus, classification of patients with ARDS into hyper- and hypoinflammatory sub-phenotypes using plasma biomarkers may facilitate more effective targeted therapy.\(^13\)

Metabolism has increasingly been acknowledged as a potential target for therapies aimed at modulating the immune system either to enhance or to suppress immunological responses.\(^14\) Due to the coupling of inflammation with metabolism, the novel “inflammation-immunity-metabolism axis” may be another useful way to propose new therapeutic implications and deeper understanding for ARDS. Metabolomics is a rapidly expanding field of systems biology that provides the ability to generate a “snapshot” measurement of all small molecules and metabolites in a given sample.\(^15,16\) The burgeoning field of metabolomics lies in its application to acute lung diseases, specifically pneumonia and ARDS.\(^17\) The application of untargeted metabolomics for biomarker discovery is well suited to the complexity of ARDS because metabolomics can detect several hundreds of metabolites, depending on the analytical platform, from a single sample, with minimal bias and no prior knowledge of the sample composition.\(^16,18,19\) We have found that specific compounds related to hypoxia may serve as early biomarkers for ARDS, while metabolites with significant correlations with the partial pressure of arterial oxygen (PaO\(_2\)/percentage of inspired oxygen (FiO\(_2\)) may play a role in determining its severity.\(^19\) However, it is difficult to find specific metabolic evidence in clinical samples related to differences in inflammatory hosts.

The spleen is a site where immune responses that are deleterious to the host can be regulated.\(^20\) The white pulp of the spleen is a secondary lymphoid organ with key functions in immune response initiation and regulation. Various immune cells (macrophages, dendritic cells, subsets of B and T lymphocytes) of the white pulp trap antigens and generate an antigen-specific response against invading pathogens (bacteria, viruses and fungi). Patients without a spleen (resulting from traffic accidents, trauma, etc.) have severe inflammation and a high risk of death in sepsis. Therefore, we think that patients without a spleen may form acute lung injury (ALI) models with “all or nothing” different subtypes of inflammatory hosts. We have also confirmed that this ALI lack-of-spleen model will not die quickly as a result of complete immunodeficiency.\(^21\) In order to explore differences of metabolism in host inflammation of ARDS, we established a special ALI mode (with spleen or without spleen, induced by lipopolysaccharide (LPS)-tracheal infusion, or cecal ligation and puncture (CLP)). Gas chromatography/mass spectrometry (GC/MS) metabolomics was then used to determine the endogenous metabolites in the lung tissues.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

C57BL/6 mice (male, 8–12 weeks old) from the Laboratory Animal Center of Chongqing Medical University (CQMU, Chongqing, China) were used. Previous studies had shown that estrogen provides a protective effect in ARDS.\(^22\) As, however, the role of hormones is not the research aim in this study, we chose only male mice for the studies.\(^23\) The mice were acclimatized to the new environment for 7
days at 22°C with free access to water and food and with a 12 h light/dark cycle before experiments. The study was performed according to international, national and institutional rules concerning animal experiments, clinical studies and biodiversity rights. The study protocol was approved by the Ethics Committee of our institute.

2.2 Chemicals and reagents

LPS (Escherichia coli, serotype 055:B5) was provided by Sigma-Aldrich (St Louis, MO, USA); TNF-α enzyme-linked immunosorbent assay (ELISA) kits by R&D Systems (Minneapolis, MN, USA); TRIzol reagent by TaKaRa Bio (Tokyo, Japan); and Brilliant SYBR Green qPCR Master Mix by TaKaRa Bio. SpeedVac vacuum concentrators were supplied by Thermo Scientific Savant (Waltham, MA, USA) and TNF-α PCR primers by TaKaRa Bio.

2.3 Animal models

2.3.1 Preparation of splenectomy model

The mice were positioned in dorsal recumbency and anesthetized with i.p. ketamine (150 mg/kg) and acetylpromazine (13.5 mg/kg). After shaving and aseptic preparation of the surgical site, along the left side of the abdominal line to help transverse incision into the abdominal cavity, ligation of the splenic artery and vein, and removal of all the spleen were performed. The abdominal incision was then closed. In a “sham” operation on mice, the abdominal wall was cut, the spleen was only slightly spit, and there was no resection. The mice were given subcutaneous injections of ceftriaxone (20 μg/g) for 3 days after surgery. Fourteen days later, these splenectomy mice were given subcutaneous injections of ceftriaxone (20 mg/g) for 3

2.3.2 LPS-induced model

LPS-induced ALI was performed to establish an ALI mouse model, using the same anesthesia method as described above. A 1 mg/mL solution of LPS was injected into mice through intratracheal instillation, and the control group was injected with the same volume of sterile phosphate-buffered saline (PBS). The mice were randomly divided into three groups: control group (n = 12), LPS group (LPS, n = 12), and splenectomy group (LPS-Splen, n = 12). The mice were euthanized by carbon dioxide box anesthesia 6 h, 12 h, and 24 h after being challenged with LPS or PBS, and blood and lung tissues were harvested for analysis.

2.3.3 CLP-induced model

Mice were randomly divided into three groups: control group (n = 12), CLP group (CLP, n = 12), and splenectomy group (CLP-Splen, n = 12) and the anesthesia method was followed as previously described. The mice were positioned in dorsal recumbency. After shaving and aseptic preparation of the surgical site, a ventral midline incision (1 cm) was made to allow exteriorization of the cecum. The cecum was identified and was penetrated through-and-through with a 21-gauge needle with a 3-0 silk suture at 75% from the tip. After being punctured, the cecum was gently squeezed to extrude a small amount of feces and returned to the abdominal cavity. The abdominal incision was then closed. Sham-operated control mice (Sham group) were subjected to the same surgical laparotomy after anesthesia, where the cecum was exteriorized and manipulated as described but not ligated or punctured. Immediately after surgery, the animals were resuscitated with 50 mL/kg saline injected subcutaneously. At the end of the experiment, the mice were euthanized by carbon dioxide box anesthesia 6 h, 12 h, and 24 h after the CLP procedure to collect whole blood and lung tissues for analysis.

2.4 Histopathology

Lung tissue samples were collected 6 h post-challenge with LPS or CLP. The superior lobe of the right lungs was fixed with 10% formalin in PBS for 24 h, dehydrated in a graded ethanol series, and embedded in paraffin. Paraffin sections were then stained with hematoxylin and eosin (H&E) followed by microscopic assessment and photographic documentation. Lung injury scores were estimated: the higher the score, the more severe the injury. The four following indicators of lung injury were used to arrive at this score: alveolar congestion; bleeding; gap or vascular wall neutrophil infiltration or aggregation; and alveolar septal thickening or transparent membrane formation. The marking system was: 0 marks, no or very slight damage; 1 mark, mild injury; 2 marks, moderate injury; 3 marks, severe injury; and 4 marks, very severe damage. The cumulative increase in the number of lesions related to the total score yields the pathological score of lung injury.

2.5 Polymorphonuclear cell counts in BALF

The blood vessels leading to the lungs and the left bronchus were ligated 6 h post-challenge with LPS or CLP. Then 200 μL of PBS was injected into the right lung through the trachea and, 10 s later, the PBS was removed and the bronchoalveolar lavage fluid (BALF) was collected. These lavages were carried out twice, and a total of 400 μL of BALF was collected. The serum, BALF, spleen, and lung tissues were harvested for analysis.

2.6 Expression of TNF-α in mice serum and lung homogenate

Blood and lung tissue samples were taken 6 h post-challenge with LPS or CLP. Blood was drawn from mice and allowed to coagulate for 1 h at room temperature. The serum was then obtained by centrifuging
the blood samples at 4 °C for 10 min at 1000 g. The lungs were ground in PBS (10 mg tissues/100 μL PBS). The serum and lung homogenate were then aliquoted and kept frozen at −80 °C until analysis. The TNF-α levels were measured by ELISA.

Total cellular RNA was extracted from lung tissue using TRIzol reagent accompanied by DNaseI digestion. Quantitative real-time polymerase chain reaction (qRT-PCR) for mouse TNF-α was performed using specific primers (designed and synthesized by TaKaRa Bio). The sequences for TNF-α were forward, 5’ TAC TGA ACT TCG GGG TGA TGT GTC C 3’, and reverse, 5’ CAG CCT TGT CCC TTG AAG AGA ACC 3’. The sequences for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were forward, 5’ AGG CCC GTG CTC AGT ATG TC 3’, and reverse, 5’ TGC CTG CTT CAC CAC CTT CT 3’. Quantitative real-time PCR was performed in a 25-μL volume with 2 μL cDNA, 400 nM of each of the sense and antisense primers, and 12.5 μL Brilliant SYBR Green QPCR Master Mix on an ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA, USA). The PCR was performed for 40 cycles with denaturation at 95 °C for 10 s, annealing at 53 °C for 30 s, and extension at 72 °C for 10 s. Gene expression normalized to GAPDH was used to determine relative target gene expression by the 2ΔΔC(t) method.

2.7 Metabolite extraction for mice lung tissue

Prepared mice lung tissue (20 mg) was added to fresh tubes, followed by the addition of 20 μL of the internal standard, L-alanine-2,3,3,3-d4 (10 mM). After adding 500 μL of cold methanol/water (50% v/v), the tissues were homogenized and centrifuged (17,000 g, 15 min) to collect the supernatant. The lung tissue was dried using a SpeedVac and stored at −20 °C prior to derivatization.

2.8 Methyl chloroformate derivatization

The volatilities of extracted metabolites were lowered using methyl chloroformate (MCF) derivatization, based on the protocol of Smart et al.25 In brief, 200 μL of sodium hydroxide (1 M) was added to the SpeedVac-dried samples and 167 mL of methanol and 34 μL of pyridine were also added as the methyl group donor and catalyst, respectively. The reaction was started by adding 20 μL MCF, followed by vortexing for 30 s and subsequently adding another 20 μL of MCF, followed by vortexing for 30 s. In order to isolate derivatized metabolites from the reactive mixture, 400 μL of chloroform and 400 μL of sodium bicarbonate (50 mM) were added and vortexed for 10 s. The chloroform phase was isolated, and excess water was removed by adding anhydrous sodium sulfate.

2.9 GC/MS analysis

The derivatized samples were analyzed using a model 7890 gas chromatograph (Agilent, Santa Clara, CA, USA) fitted with a ZB-1701 capillary column (30 m × 250 μm id × 0.15 μm with a 5-m guard column; Phenomenex, Torrance, CA, USA) coupled to an MSD 5975 single quadrupole mass spectrometer (Agilent) operating in electron ionization mode at 70 eV. The GC and MS procedures followed the protocol outlined by Smart et al.25 The isolated chloroform phase was injected at 290 °C in pulsed splitless mode with helium carrier gas at a flow rate of 1 mL/min. The program temperature was as follows: initial temperature of 80 °C, ramped at 25 °C/min to 200 °C, then at 3 °C/min to 215 °C, and finally at 2 °C/min to 230 °C. The auxiliary temperature, quadrupole mass analyzer temperature and source temperature were set to 250, 230 and 150 °C, respectively. The mass range was m/z 30–550, the scan speed 1.562 m/z units/s and the solvent delay 5.5 min.

2.10 Statistical analysis

Compound deconvolution and identification were performed by the automated mass spectral deconvolution and identification system (AMDIS) software (NIST, Gaithersburg, MD, USA), using our internal methyl chloroformate derivatization mass spectra library of metabolite standards. The compounds were identified based on two criteria: >85% match with the library spectrum and within a 1-min bin of the respective chromatographic retention time. The relative abundance of the metabolites was extracted via our in-house MassOmics software, using the peak height of the highest reference ion mass. The metabolite values were normalized by the abundance of the internal standard (L-alanine-2,3,3,3-d4) and total ion count, in order to correct for experimental variability. The metabolomics data have been deposited to the EMBL-EBI MetaboLights database (https://doi.org/10.1093/nar/gks1004.)26 with the identifier MTBLS627. The abundance of identified compounds was adjusted to a Gaussian distribution via log transformation prior to statistical analysis. Multivariate analysis of variance (ANOVA) followed by Tukey’s HSD test was performed in R. The predicted metabolic activities were determined using our Pathway Activity Profiling R package based on the KEGG online database. The relative metabolic activities were transformed to have a mean of zero and a standard deviation of one (z-score). Subsequently, the metabolic pathways were classified according to their cellular processes, and only the predicted metabolic pathways with p values and q values less than 0.05 were displayed. The metabolic activities were first normalized by log transformation and Pareto scaling, followed by ranking of the metabolic pathways using a random forest model to capture their contribution to the classification accuracy demonstrated in a VIP plot. The metabolic network was constructed according to a pathway-based framework provided by MetScape that connected the KEGG human metabolic pathways with our identified metabolites via the Kamada-Kawai layout, which relates the layout of metabolites to minimize metabolic reactions between metabolites within a metabolic network. All the illustrations and figures displayed were plotted using the ggplot2 R package, GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA), and SPSS 20.0 (IBM, Amonk, NY, USA).
3 RESULTS

3.1 Splenectomy establishes differential host inflammatory response against ALI in mice

Mice (with or without splenectomy) were euthanized 6 h post-challenge with LPS or CLP. The administration of LPS or CLP led to severe lung injury, compared with the controls (Figure 1A). The lung injury scores showed that splenectomy can aggravate lung injury directly in either the LPS or the CLP group, $p < 0.0001$ (Figure 1A). All parts of the lung injury degree index, such as thickened alveolar wall, hemorrhage in the alveolus, alveolar collapse, and inflammatory cell infiltration in the LPS-Splen group or CLP-Splen group, were more severe than in the corresponding ALI group. Moreover, the lung injury in the CLP-Splen group is more serious than in the LPS-Splen group, $p < 0.05$ (Figure 1A; Table S1, supporting information). The concentrations of TNF-$\alpha$ were significantly elevated in the serum and lung tissue after LPS or CLP compared with the controls, $p < 0.001$ (Figures 1B and 1C). In addition, the expression of TNF-$\alpha$ in the LPS-Splen or CLP-Splen groups was higher than in the corresponding ALI (LPS or CLP) groups. The levels of TNF-$\alpha$ in the CLP-Splen group were significantly elevated compared with the LPS-Splen group, $p < 0.05$ (Figures 1B and 1C). Subsequently, infiltration of neutrophils was confirmed with neutrophil numbers in BALF (Figure 1D). After splenectomy, the LPS (Splen) and CLP (Splen) groups have significant neutrophil exudation in the lungs (Figure 1D).

3.2 Analysis of ALI animal models based on the different modeling methods

The PLS-DA and leave-one-out cross-validation results are shown in Figure 2. The supervised PLS-DA showed that the four different groups were well-clustered, with specific metabolic profiles for each (Figure 2B). After ANOVA was performed for the CLP and CLP-Splen groups, a total of 41 compounds were detected in the lungs, including metabolites from organic acids (15/41, 36.59%), amino acids (8/41, 19.51%), amino acid derivatives (5/41, 12.20%), and others (Figure 3A; Figure S1, supporting information). In the random forest (RF) analysis the “mean decrease accuracy” indicates how much a certain metabolite contributes to separation of the groups, and the overall “predictive accuracy” is indicative of the accuracy of a set of metabolites in discriminating spleen status.28 RF analysis of lung-targeted metabolomics data defined a set of 15 metabolites that constitute the best predictors of differences in host inflammation status: in particular, increased 4-hydroxyphenylacetic acid.

![Figure 1](wileyonlinelibrary.com)
1-aminocyclopentanecarboxylic acid (ACPC), and cis-aconitic acid. Tridecane and hydroxybenzoic acid were strong predictors of the hyper-inflammatory subgroup in CLP-induced ALI (Figure 3B). After ANOVA was performed for the LPS and LPS-Splen groups, only 8 compounds were detected in the lungs. The organic acids (4/8, 50%) make up the largest category among the subgroups of different inflammation in ALI, which was induced by LPS (Figure 3C). Consequently, under differential inflammation (hyper- vs hypo-), the CLP-induced ALI lungs will detect more differential metabolites than the LPS-induced ALI lungs (Figures 3A and 3C). Detailed information on this is displayed in Table S2 (supporting information).

When clustering the hyper-inflammatory and hypo-inflammatory classes separately, differences in the lung metabolites between CLP and LPS were found. Under splenectomy-based conditions, there were 19 differential metabolites between the CLP and LPS groups; the organic acids (10/19, 52.63%) and TCA cycle intermediates (4/19, 21.05%) were the two largest categories (Figure 3D). Under no-splenectomy-based conditions, there were only 7 differential
metabolites between the CLP and LPS groups and the organic acids (4/7, 57.14%) formed the largest category (Figure 3E).

3.3 Analysis of predicting metabolic pathways of ALI animal models based on the different modeling methods

While identifying the linked metabolic pathways, the ANOVA test was used to extract significant pathways from them. For the CLP and CLP-Splen groups, the KEGG alignment revealed 80 pathways that were linked to the above-detected metabolites. Amino acid metabolism (10/80, 12.50%), chemical structure transformation maps (8/80, 10.00%), biosynthesis of other secondary metabolites (8/80, 10.00%), metabolism of other amino acids (7/80, 8.75%), and carbohydrate metabolism (6/80, 7.50%) were the first five largest categories (Figure 4A). For the LPS and LPS-Splen groups, however, only six pathways linked to the above-detected metabolites were revealed from KEGG. The benzoic acid family, bisphenol degradation, and folate biosynthesis were revealed in the 12 h-post-LPS-intervention group. In addition, tropane, piperidine and pyridine alkaloid biosynthesis, biosynthesis of phenylpropanoids, and phenylalanine metabolism were revealed in the 24 h-post-LPS-intervention group (Figure 4B). The hyper-inflammatory and hypo-inflammatory groups were then clustered to predict the metabolic pathways separately. Under splenectomy-based conditions, there were 13 differential metabolic pathways between the CLP groups and the LPS groups (Figure 4C). However, we could not find differential metabolic pathways between the CLP groups and the LPS groups under no-splenectomy-based conditions.

4 DISCUSSION

ARDS is a clinically and biologically heterogeneous disorder associated with effects such as trauma, shock, infection, and sepsis. Failure of clinical therapeutic trials prompted the investigation and subsequent discovery of two distinct phenotypes of ARDS (hyper-inflammatory and hypo-inflammatory) that have different biomarker profiles and clinical courses and respond differently to management strategies. The hyper-inflammatory subgroup (about one-third of all) shows higher mortality, higher severity of illness, and worse clinical outcomes. Even in COVID-19, the hyper-inflammatory response is closely related to the ARDS of critical COVID-19 pathogenesis. A major issue is that ARDS is such a heterogeneous, multi-factorial, end-stage condition that the strategies for “lumping and splitting” are critical. Metabolic phenotypes, representing different pathways important in the pathophysiology of ARDS, can be used to identify the subgroups. They can also help distinguish the sub-phenotypes of ARDS (hypo-inflammatory and hyper-inflammatory) and identify the risk of developing ARDS, diagnosis, risk stratification and monitoring. The use of metabolomics as a possible diagnostic tool for ARDS has been investigated in several studies, including exhaled breath and oedema fluid analyses. We previously found that phenylalanine, aspartic acid, and carbamic acid levels were significantly different in the plasma samples of ARDS patients. Four metabolites (ornithine, caprylic acid, azetidine, and iminodiacetic acid) could serve as metabolic phenotypes to potentially predict the severity of ARDS. Due to the limitations of the research conditions, it is difficult to distinguish the subtypes of inflammation solely by metabolomics.

The spleen performs vital hematological and immunological functions. Removal of the spleen had already been established as a
routine technique to treat splenic trauma and other diseases affecting the spleen. However, splenectomized (asplenic) or hyposplenic individuals have an increased risk of infections, and this can lead to severe sepsis known as overwhelming post-splenectomy infection (OPSI), which has a very high mortality rate. A previous study showed that a higher Charlson comorbidity index score was significantly associated with severe sepsis/septic shock post-splenectomy. Moreover, splenectomy can alter the serum cytokine profile, exacerbating the systemic inflammatory responses and injury to multiple organs. The spleen is necessary for the recruitment of classical monocytes and neutrophil extravasation into the injured lungs, and it can play an important role in intestinal ischemia–reperfusion (IIR)-induced ALI. Furthermore, the spleen coordinates interleukin (IL)-6-dependent IL-10 production, which reduces lung injury during experimental acute kidney injury (AKI). Splenic factors also exacerbate severe acute pancreatitis (SAP)-associated lung injury.

In animal experiments, the splenectomy model can be used as an auxiliary method to distinguish high and low inflammatory subtypes. Therefore, an ALI animal model of host inflammation differentiation can be established after carrying out a splenectomy. The splenectomy model, which could demonstrate the significant involvement of splenic factors in lung injury, plays an important role in the experiment. However, it has a higher mortality rate under experimental conditions, and this mortality rate is significantly increased when combined with the CLP model. In our study, this mortality rate was very high after conducting the splenectomy followed by CLP and the passage of time (from 6 to 24 h). In order to ensure the homogeneity of experimental mice in metabolic analysis, the same batch of mice was used to establish models synchronously, such that they would have an effective cluster effect. It was difficult to achieve a greater number of animals at each time point and in each group because of the extremely high mortality rate, and because of the existing limitations of these special animal models. Ideally, more mice (over 10) in each group would be beneficial for repetitive data collection.

We found that the CLP-Splen and LPS-Splen groups had more severe lung damage than the corresponding non-splenectomy ALI group (Figure 1A). Moreover, the LPS-Splen and the CLP-Splen groups showed significant neutrophil exudation in the lungs after splenectomy; however, the changes in the CLP-Splen group were more significant (Figure 1D). Possible causes may be that splenectomy did not affect neutrophil extravasation in the LPS models of lung injury, as was confirmed by Rieg et al. or because of TNF-α-induced adhesion of monocytes to endothelial cells and leukocyte transmigration in ALI. We found that the expression in the LPS-Splen or the CLP-Splen group was higher than in the corresponding ALI group, and that the levels of TNF-α in the CLP-Splen group were significantly elevated compared with the LPS-Splen group, $p < 0.05$ (Figures 1B and 1C). Following splenectomy, there is an alteration in the course of systemic inflammation in the splenectomized individual. A previous study showed that immunoreactive binding protein (LBP) accumulates in lung lavage fluids with lung injury and enhances LPS-stimulated TNF-α gene expression in alveolar macrophages, depending on the CD14 receptor. The sensitizing effect of LPS stimulation aggravates lung damage. LBP may play an important role in augmenting TNF-α expression by alveolar macrophages in the lung. The duration of CLP action is prolonged, the stimulatory effect persists, and TNF-α expression is higher than with LPS-intratracheal injection (Figures 1B and 1C). On the other hand, the cholinergic anti-inflammatory pathway is completely inhibited following splenectomy. Although splenectomy itself was not associated with increased serum IL-6 or lung injury, the absence of a counter anti-inflammatory response by splenic IL-10 production results in a high proinflammatory response and lung injury. Based on the difference of inflammation (after splenectomy) in ALI, the metabolomic differences of lung tissues could be identified. PCA and OPLS-DA were used for discriminant analysis, and univariate statistical analysis was used to screen important differential metabolites in untreated (hypo-inflammatory) or splenectomy-treated (hyper-inflammatory) ALI mice, which had been subjected to different modeling methods (LPS or CLP). A total of 41 compounds and 80 pathways were found in lungs, differing between hyper- and hypo-inflammatory in the CLP groups (Figures 3A and 4A). However, only 8 compounds and 6 pathways were found to differ in the lungs between hyper-inflammatory and hypo-inflammatory in LPS (Figures 3C and 4B). This suggests that non-pulmonary ARDS (such as CLP) has more active lung metabolomics changes that are involved in inciting the differences in host inflammatory response. Moreover, continuous stimulation of CLP promoted inflammation and injury to the lungs. Organic acids form the largest group of differential metabolites in CLP and LPS under differential inflammatory conditions, and can be attributed to defects in the intermediary metabolic pathways of carbohydrates, amino acids, and fatty acid oxidation. Related physiological analysis had found that sepsis experienced a highly catabolic status. Many proteins decompose into amino acids to supply energy, which seemed to be relevant to poor prognosis. Thus, the concentration of amino acids and their derivatives (13/41, 31.71%) demonstrated a notable upward tendency in the CLP group (Figure 3A). Among the metabolic pathways, this category, including amino acid metabolism (10/80, 12.50%) and metabolism of other amino acids (7/80, 8.75%), also dominates (Figure 4A). Interestingly, RF analysis of lung-targeted metabolomics data showed that the metabolic biomarker group with 5 products was a strong predictor of the hyper-inflammatory subgroup in CLP-induced ALI (Figure 3B). 4-Hydroxyphenylacetic acid (4-HPA), as one of the major metabolites in polyphenols, is a necessary adaptive response of microbiota to the stress-induced changes in inflammation. 4-HPA could be a biomarker for quantifying leukocyte-mediated damage, and it has been confirmed to participate in the intermediate step of tyrosine degradation. ACPC is a nonmetabolized amino acid. Amino acid transport by system “A” is sodium-dependent and results in a high intracellular-to-extracellular gradient. APAC was also shown to have a high affinity for the “A” transport system of endothelial cell membranes. Sepsis specifically decreases cell membrane potential and inhibits the amino acid transport system A. This is probably because of the reduction in the
pulmonary absorption of amino acids in ARDS. Hypoxia causes an imbalance of the NADPH/NADP⁺ and NADH/NAD⁺ ratios, accompanied by the accumulation of intermediates of the TCA cycle, such as cis-aconitic acid, as was found by RF analysis.

Tridecane may be a substance derived from environmental factors. Exposure to fuels and heavy metabolites (3-tridecanone, 2-tridecanol, and 2-tetradecanol) was observed only in the lung tissues, possibly indicating that metabolism occurred in the lungs.⁵⁰ However, the aliphatic compound n-tridecane showed no cytotoxic effects on chemotactractant protein-1 (MCP-1) and IL-8 production.⁵¹ We therefore believe that tridecane cannot be a member of the metabolic biomarker group. Low-molecular-weight phenolic acids (PhAs) are the products of the degradation of aromatic amino acids and polyphenols by the intestinal microflora,⁵² and all PhAs have an impact on mitochondria and neutrophils. Low-molecular-weight PhAs of microbial origin participate in the regulation of the ROS production in both the circulation and tissues, thereby affecting the level of oxidative stress.⁵³ Therefore, there may be a group of metabolic biomarkers related to inflammation, hypoxia, infection, etc., between the hyper-inflammatory and hypo-inflammatory subgroups of ARDS. The etiology has also become a variable. When we clustered the hyper-inflammatory and hypo-inflammatory separately, there were 19 differential metabolites and 13 differential metabolic pathways between the CLP group and the LPS group in the hyper-inflammatory subgroup (Figures 3D and 4C). In the hypo-inflammatory subgroup, there were only 7 differential metabolites between the CLP and the LPS groups (Figure 3E), although no different metabolic pathways could be found in this study. Overall, the hyper-inflammatory subgroups of ARDS were observed to exert more abundant metabolism changes in the lung.

5 | CONCLUSIONS

Metabolomics has the potential to improve our understanding of ARDS biology. From the analysis of lung metabolomics, the difference in host inflammatory responses is a key link in determining the severity of ARDS. Hyper-inflammatory subgroups of ARDS have a heavier inflammatory response and a more active lung metabolism. Combined with the host inflammation background, biomarkers from metabolomics could help evaluate its severity.

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