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Identification and Validation of Liver Transplantation-Induced Acute Lung Injury Biomarkers Using a Bioinformatics and Experimental Approach

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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ABCDEFG 1-5

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CDE 1,3-5

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Conflict of interest:

None declared

Background:

Acute lung injury (ALI) after liver transplantation (LT) is a critical complication negatively affecting transplant outcomes and patient survival. However, effective biomarkers for early diagnosis remain unidentified. This study aimed to identify hub biomarkers and signaling pathways involved in post-LT ALI through integrated bioinformatics and machine-learning analyses and to validate their diagnostic potential.

Material/Methods:

Differential gene expression analysis identified 27 differentially expressed genes (DEGs). Functional enrichment analyses revealed significant involvement in cytokine-mediated signaling, particularly within the NF- κ B and TNF pathways. Single-sample gene set enrichment analysis (ssGSEA) evaluated immune infiltration. Machine-learning algorithms identified crucial biomarkers for ALI prediction. Transcription factor–hub gene and competitive endogenous RNA (ceRNA) networks were constructed. Single-cell RNA sequencing (scRNA-seq) and enzyme-linked immunosorbent assay (ELISA) analyses validated biomarker expression patterns in relation to ALI.

Results:

Hub biomarkers identified included CXCL3, CD48, and IRAK3. ssGSEA revealed prominent macrophage and neutrophil infiltration associated with ALI. Machine-learning models confirmed CXCL3, CD48, and IRAK3 as reliable predictive biomarkers, which were incorporated into a robust diagnostic nomogram. scRNA-seq analysis showed cell-type–specific expression patterns for CXCL3, CD48, and IRAK3, with heterogeneous associations across datasets. ELISA validated significantly altered protein levels of CXCL3, CD48, and IRAK3 in post-transplant ALI cases compared with controls.

Conclusions:

CXCL3, CD48, and IRAK3 are novel and promising diagnostic biomarkers for predicting post-LT ALI. These findings provide foundational insights that could support improved diagnosis, prevention strategies, and targeted therapeutic interventions, ultimately enhancing patient outcomes after liver transplantation.

Keywords:

Biomarkers • Cysteamine • Organ Transplantation • Sick Building Syndrome

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Introduction

Acute lung injury (ALI) is primarily diagnosed based on classical radiologic criteria, with acute respiratory distress syndrome (ARDS) representing its most severe manifestation [1]. ARDS is characterized by impaired gas exchange, which can progress to multiple organ dysfunction syndrome (MODS) [1]. For patients with end-stage liver disease (ESLD), liver transplantation (LT) remains the only curative treatment and has become a routine clinical procedure [2]. Advances in surgical techniques and perioperative care have significantly improved LT success and survival rates [3]. However, post-LT complications remain a major concern. The incidence of post-LT ALI has been reported to range from 60% to 80% [4], making it a leading cause of morbidity and mortality after LT. ALI and ARDS significantly affect LT outcomes, with post-LT ARDS incidence varying between 1% and 30%, depending on the diagnostic criteria used [5,6]. Once ARDS develops, mortality rates can exceed 50% [7]. The pathogenesis of ALI/ARDS after LT is complex and multifactorial, involving both pulmonary and non-pulmonary factors. Triggers include pneumonia, sepsis, massive blood transfusions, and trauma, all of which contribute to an exaggerated inflammatory response in the lungs [8,9]. This response is characterized by acute hypoxia, bilateral pulmonary infiltrates, pulmonary edema, extensive neutrophil infiltration, and alveolar epithelial damage, leading to high morbidity, mortality, and healthcare costs [1,8,9].

ALI/ARDS is a dynamic condition driven by multiple cell types, molecular mechanisms, and signaling pathways. Neutrophils are hub contributors, particularly through the release of neutrophil extracellular traps (NETs), which exacerbate inflammation and lung injury [10,11]. Additionally, macrophage polarization has been recognized as a critical factor in ALI/ARDS pathogenesis [12,13]. Several signaling pathways, including TLR4/NF- κ B and Keap1/Nrf2/HO-1, have been implicated in disease progression [14,15]. Despite advances in understanding these mechanisms, early detection and targeted interventions remain challenging, with current diagnostic strategies relying primarily on blood gas analysis and imaging [1,16]. The absence of specific and reliable biomarkers for post-LT ALI often results in delayed diagnosis, limited opportunities for early intervention, and persistently high mortality rates. Supportive care, including mechanical ventilation and positive end-expiratory pressure (PEEP), remains the cornerstone of treatment, as effective targeted therapies are still lacking [17]. Over the past decade, advancements in high-throughput sequencing and microarray technologies have revolutionized genetic and epigenetic research, enhancing disease diagnosis and treatment strategies [18]. Predictive accuracy can be significantly improved by integrating multiple hub genes into diagnostic models [18]. However, constructing reliable multi-gene models remains challenging due to the complexity and variability

of gene expression data. Machine-learning algorithms have facilitated the standardization and analysis of these data, enabling the identification of disease-specific molecular markers and improving diagnostic precision [19,20]. Nevertheless, few studies have focused specifically on identifying biomarkers that are relevant to post-liver transplantation ALI, and even fewer have integrated multi-dataset analysis with experimental validation. This gap underscores the need for studies that combine bioinformatics, machine-learning, and clinical evaluation to discover clinically translatable biomarkers.

This study aimed to identify and validate potential molecular biomarkers and therapeutic targets for post-LT ALI/ARDS. To achieve this, 5 ALI-related datasets were retrieved from the Gene Expression Omnibus (GEO) database. Bioinformatics analyses, including Limma differential expression analysis, Gene Ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and single-sample gene set enrichment analysis (ssGSEA), were performed to identify differentially expressed genes (DEGs). Three machine-learning algorithms were applied to screen and prioritize hub diagnostic genes for post-LT ALI/ARDS. These hub genes were subsequently used to construct a diagnostic nomogram and develop transcription factor (TF)-hub gene and competing endogenous RNA (ceRNA) networks. Furthermore, to enhance the robustness and translational relevance of the findings, we validated the expression and cellular distribution of these genes using scRNA-seq data, an independent external dataset, and clinical samples from LT recipients. This comprehensive approach aims to enhance the early diagnosis, prevention, and treatment of post-LT ALI/ARDS. Because publicly available transcriptomic datasets specific to liver transplantation-associated ALI are limited, the discovery phase of this study used multiple ALI/ARDS-related GEO datasets to identify conserved inflammatory signatures. Liver transplantation specificity was subsequently addressed through validation in liver transplant recipients using bronchoalveolar lavage fluid (BALF) and serum samples, thereby linking the computational findings to the post-transplant clinical context.

Material and Methods

ALI-Related Gene Datasets and Data Processing

To investigate potential molecular markers associated with acute lung injury (ALI), 5 raw datasets were obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). The datasets included GSE172222 [23], GSE2322 [24], GSE3037 [25], GSE68610 [26], and GSE10361 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi>). The included GEO datasets were generated on multiple microarray platforms. Therefore, probe identifiers were first mapped to gene symbols within each dataset, after which datasets were merged at the

gene level and batch effects were corrected. According to the original study descriptions, 3 datasets (GSE2322, GSE3037, GSE68610) were derived from human peripheral blood or blood-associated cells in clinical ALI/ARDS cohorts, while 2 datasets (GSE10361 and GSE172222) were generated from lung-related cell lines exposed to injurious stimuli. This combined strategy was used to capture systemic inflammatory signatures (from blood-based datasets) and core mechanistic responses occurring at the cellular level (from cell-line models), providing a more comprehensive overview of ALI pathobiology. Importantly, among the 5 datasets, GSE172222 models ischemia–reperfusion injury in a simulated transplant-related lung injury setting. The remaining datasets were included to identify conserved transcriptional alterations broadly shared across ALI/ARDS, which may also be relevant to the inflammatory milieu after liver transplantation. This has now been explicitly highlighted as the dataset most closely related to post-LT ALI. The remaining datasets were included to identify conserved transcriptional alterations broadly shared across ALI/ARDS, which may also be relevant in the post-transplant context.

To assess data reproducibility, principal component analysis (PCA) was performed. Additionally, GSE222810 and GSE255726 datasets were used to validate hub gene expression at the single-cell RNA sequencing (scRNA-seq) level. The probe expression matrix was converted into a gene expression matrix using the platform annotation file, and array data were normalized using the robust multichip average (RMA) method. For microarray data, background correction and normalization were performed using the robust multichip average (RMA) method where raw CEL files were available; otherwise, normalized series matrix files were used as provided. When multiple probes mapped to the same gene symbol, the probe with the highest average expression across samples was retained. The 5 datasets were then merged, and batch effects were mitigated using the “ComBat” function from the sva package. PCA was re-applied to evaluate and visualize the merged data.

Differential Expression Analysis of ALI-Related Genes

DEGs were identified using the R package “Limma.” The screening criteria were set to an adjusted P value of <0.05 and $|\log_2(\text{fold change [FC]})| > 0.5$. Volcano plots and heatmaps were generated using the Enhanced Volcano and heatmap packages in R, respectively.

GO and KEGG Enrichment Analyses of ALI-Related DEGs

Gene Ontology (GO) analysis was conducted to classify DEGs into biological process (BP), cellular component (CC), and molecular function (MF) categories, providing a systematic framework for understanding gene functions in high-throughput genomic and transcriptomic data [27]. Kyoto Encyclopedia of

Genes and Genomes (KEGG) pathway analysis was performed to identify biological pathways associated with ALI, offering insights into genome functions in relation to diseases, pharmacological agents, and molecular interactions [28]. Both analyses were conducted using the clusterProfiler package in R, with an adjusted P value <0.05 and a minimum of 5 enriched genes required to ensure the biological significance of functional annotations. The results were visualized using the GOplot package, and enrichment results were graphically represented using ggplot2, generating bubble and bar plots.

Immune Infiltration Analysis

The extent of immune cell infiltration in ALI samples was analyzed using the single-sample gene set enrichment analysis (ssGSEA) algorithm implemented in the GSVA R package, focusing on 28 distinct immune cell types. Enrichment scores were calculated for each immune cell type based on gene expression profiles, using predefined immune signature genes. Differences in immune infiltration between ALI and non-ALI cohorts were assessed using the Wilcoxon rank-sum test. Multiple testing was controlled using the Benjamini–Hochberg method, with FDR <0.05 considered statistically significant. ssGSEA enrichment scores were computed for each immune cell signature, and group differences were evaluated as described above. The results were considered significant if they exhibited a false discovery rate (FDR) <0.05 and an absolute NES >1 . The results were visualized using the Vioplot and pheatmap R packages.

Identification of Potential Biomarkers Through Machine-Learning Algorithms

To identify hub diagnostic biomarkers for ALI, 3 machine-learning approaches were applied: least absolute shrinkage and selection operator (LASSO) regression, support vector machine-recursive feature elimination (SVM-RFE), and random forest (RF) analysis. LASSO logistic regression was implemented using the glmnet package, selecting optimal predictors while addressing multicollinearity. The parameters were set to $\alpha=1$ and $n\lambda=1000$, with $\lambda_{\min}$ identified as the optimal penalty value. SVM-RFE, a support vector machine-based method for feature selection, was applied using the e1071 package to iteratively remove less informative genes. The RF algorithm, which constructs decision trees through recursive partitioning, was performed using the randomForest package, with 500 trees and the Gini index used to evaluate feature importance. Genes identified as significant by all 3 algorithms were considered potential ALI biomarkers.

Modeling and Testing of an ALI Diagnostic Nomogram

To assess the diagnostic performance of identified biomarkers, receiver operating characteristic (ROC) curves were generated

using the pROC R package, and the area under the curve (AUC) was calculated to evaluate predictive accuracy. A diagnostic nomogram was constructed using the rms package, incorporating hub genes to estimate post-LT ALI probability. The overall risk score was determined as the sum of individual gene risk scores. The nomogram's diagnostic performance was further validated through calibration plots, decision curve analysis (DCA), and ROC curve evaluation.

Single-Cell RNA Sequencing (scRNA-seq) Analysis

Raw data for GSE222810 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi>) were obtained from the GEO database and processed using the Seurat package in R Studio. Cells with fewer than 100 detected genes, more than 5000 genes, or mitochondrial gene expression exceeding 20% were excluded. Batch effects were corrected using the harmony package. The FindVariableFeatures function was used to select the top 2000 most variable genes. Cell annotation was performed using the SingleR package. Highly variable genes were identified using variance stabilization transformation (VST) and scaled with the ScaleData function. Principal component analysis (PCA) was conducted using RunPCA, specifying 20 dimensions. Cell clustering was performed using FindNeighbors and FindClusters, and visualization was generated using RunUMAP. Biomarker expression was evaluated across cell types using the VlnPlot and FeaturePlot functions.

Construction of Transcription Factor–Hub Gene and Competitive Endogenous RNA Networks

Transcription factors (TFs) interacting with hub genes were identified using TRRUST, a manually curated database of transcriptional regulatory interactions [29]. Target microRNAs (miRNAs) were predicted using miRTarBase [30], StarBase [31], and TargetScan [32], retaining only those identified by all 3 databases to enhance accuracy. The Enrichr database (<http://amp.pharm.mssm.edu/Enrichr/>) was used to predict TFs associated with hub genes, with P value <0.05 as the selection criterion. The TF–hub gene network was visualized using Cytoscape. Additionally, target miRNAs were identified using miRanda, miRWalk, miRDB, and TargetScan, incorporating only miRNAs present in all 4 databases. Since long non-coding RNAs (lncRNAs) can regulate miRNAs by competing for miRNA response elements, potential lncRNA–miRNA interactions were predicted using StarBase 3.0, applying stringent filtering criteria (hg19 genome, CLIP-Data ≥ 5). The resulting competitive endogenous RNA (ceRNA) network was visualized in Cytoscape [33].

Verification of Hub Genes Using External GEO Datasets

To validate hub gene expression, the GSE255726 dataset (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi>) containing 6 ALI and 6 non-ALI samples was analyzed. Statistical analyses were

performed using ggplot2 and ggpubr in R. The t test was used to compare gene expression levels between ALI and non-ALI groups. ROC curves and AUC values were calculated to assess the diagnostic potential of hub genes using IBM SPSS Statistics 25.

Patients and Clinical Samples

Bronchoalveolar lavage fluid (BALF) and blood serum samples were prospectively collected from patients who underwent liver transplantation (LT) at our institution. Samples were obtained on the second postoperative day. Patients were categorized into ALI (acute lung injury) and non-ALI groups based on the ratio of arterial oxygen partial pressure to inspired oxygen fraction (PaO₂/FiO₂); patients with a PaO₂/FiO₂ ≤ 300 were defined as having ALI [34]. Collected samples were immediately centrifuged at 3000 rpm for 10 minutes, and the supernatants were stored at -80°C until further analysis. The study adhered strictly to the principles of the Declaration of Helsinki and received ethics approval from the Research Ethics Committee of Guangxi Medical University Cancer Hospital, Nanning, China under approval number KY-IIT-2024-63. Written informed consent was obtained from all patients or their legal guardians prior to inclusion in the study.

Enzyme-Linked Immunosorbent Assay (ELISA)

To validate the expression of hub genes identified by bioinformatics and machine-learning analyses, BALF and serum samples from 5 ALI and 5 non-ALI patients were analyzed. Commercial ELISA kits (UpingBio Technology Co., Ltd., Hangzhou, China) were used to quantitatively measure protein levels of CXCL3, CD48, and IRAK3. All procedures strictly followed the manufacturer's instructions. Absorbance values were measured using a microplate reader, and protein concentrations were calculated based on standard curves provided by the kit manufacturer.

Statistical Analysis

Statistical analyses and visualizations were conducted using R software (version 4.2) and GraphPad Prism (version 8.0.1). Differential expression between clinical cohorts was analyzed using a two-sided Wilcoxon test. The false discovery rate (FDR) was controlled using the Benjamini-Hochberg method. Correlation analysis was conducted using the Spearman test, and a P value <0.05 was considered statistically significant.

Results

Identification and Functional Enrichment Analysis of ALI-Related Differentially Expressed Genes (DEGs)

The overall research workflow is illustrated in **Figure 1**. Differential expression analysis identified 27 DEGs associated

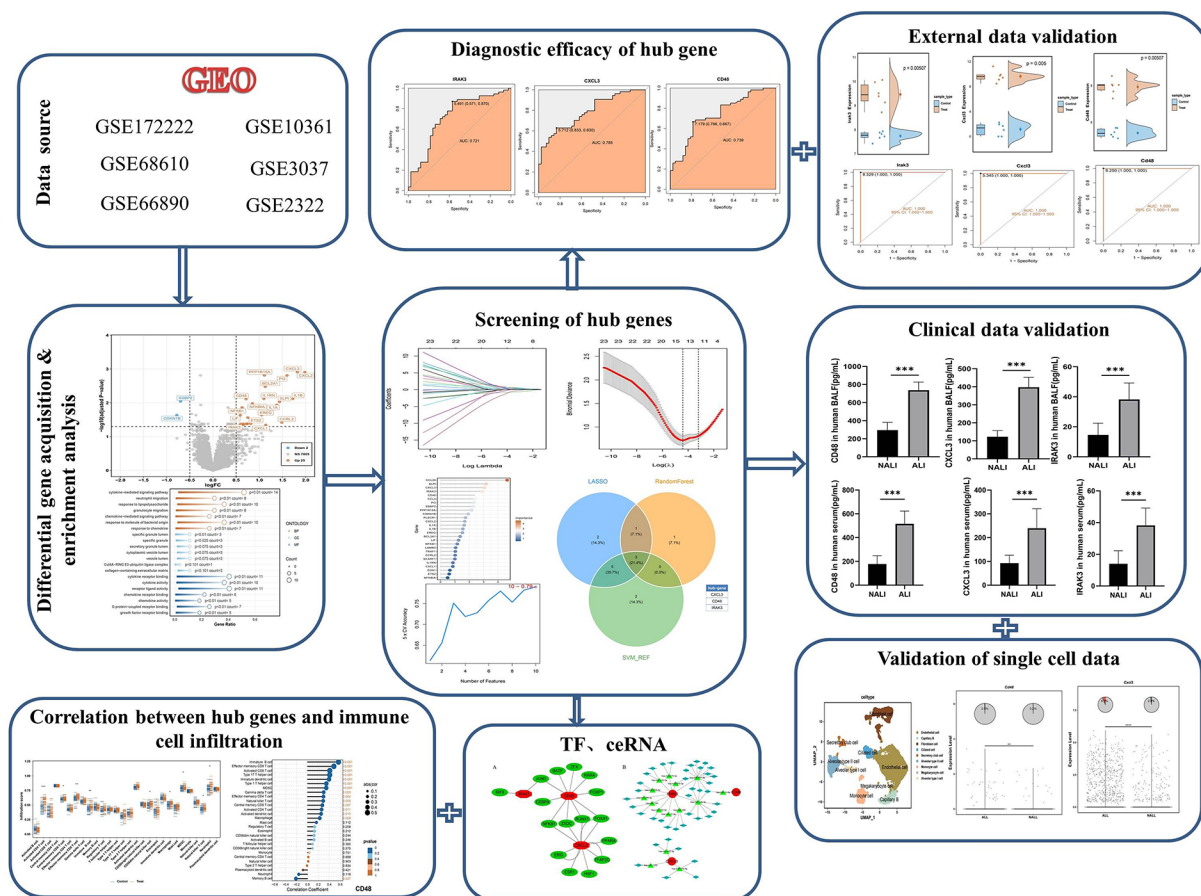


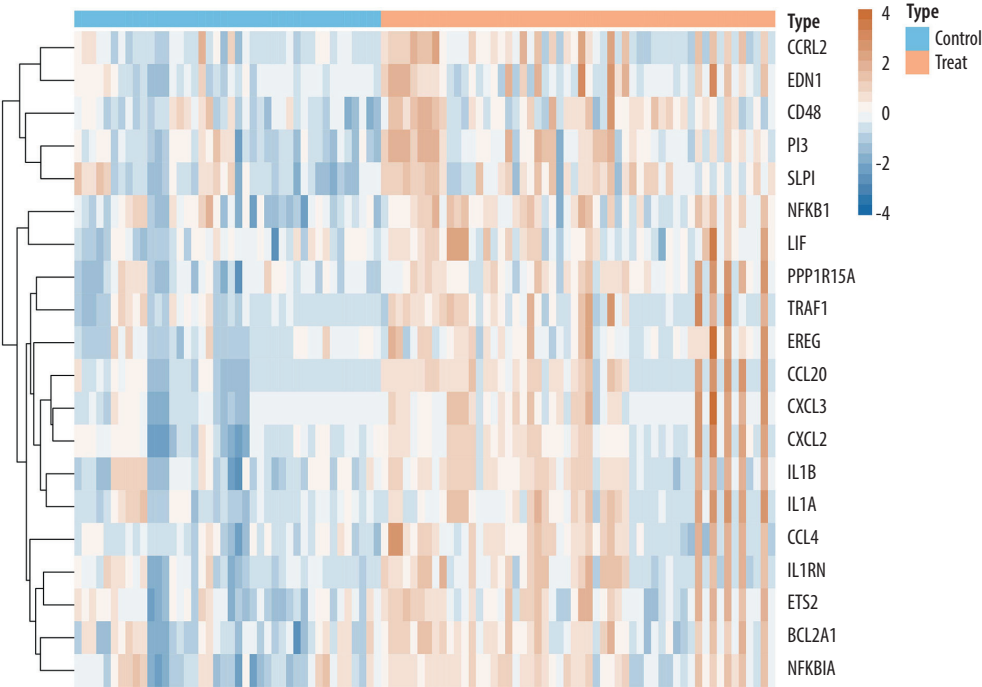
Figure 1. Flowchart of this study.

with ALI, comprising 25 upregulated and 2 downregulated genes. The expression profiles of these DEGs were visualized using a volcano plot and a heatmap, demonstrating distinct expression patterns between ALI and control samples (Figure 2A, 2B). To elucidate the biological functions and pathways implicated in ALI, GO and KEGG enrichment analyses were conducted. GO analysis revealed significant enrichment in multiple terms across 3 categories (Figure 2C). In the biological process (BP) category, DEGs were predominantly associated with cytokine-mediated signaling pathways and responses to lipopolysaccharide. The cellular component (CC) category highlighted enrichment in specific granule lumen, specific granule, and secretory granule lumen. In terms of molecular function (MF), DEGs were involved in cytokine receptor binding, receptor ligand activity, and cytokine activity. Furthermore, KEGG pathway analysis indicated that the DEGs were primarily associated with the NF- κ B signaling pathway, TNF signaling pathway, Legionellosis, and IL-17 signaling pathway (Figure 2D). These findings demonstrate that ALI is characterized by a strong pro-inflammatory transcriptional profile, consistent with dysregulated innate immune activation.

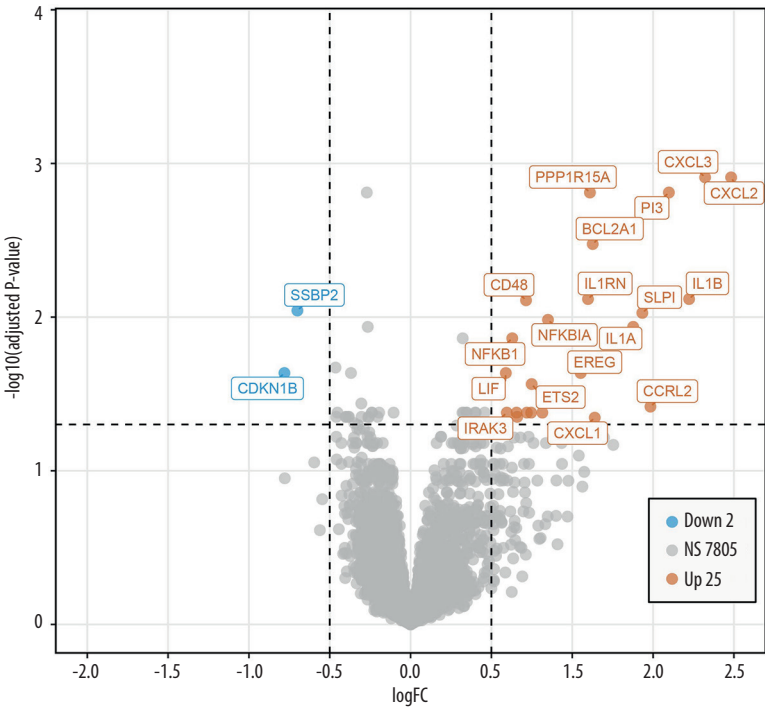
Identification of Hub Genes Through Machine-Learning Algorithms

Three distinct machine-learning algorithms – LASSO regression, SVM-RFE, and random forest (RF) analysis – were employed to identify hub genes among the 27 ALI-related DEGs. LASSO regression, with an optimal sparsity parameter $\lambda=0.0146$, identified 11 hub genes (Figure 3A). The SVM-RFE algorithm further refined this selection, identifying 10 genes that exhibited the highest accuracy and the lowest error rate (Figure 3B, 3C). Additionally, RF analysis determined 5 genes with an importance score greater than 1 (Figure 3D, 3E). A Venn diagram integrating results from all 3 algorithms revealed 3 hub genes – CXCL3, CD48, and IRAK3 – as the most critical biomarkers for ALI (Figure 3F). The convergence of 3 complementary machine-learning approaches underscores the robustness and novelty of identifying CXCL3, CD48, and IRAK3 as potential diagnostic biomarkers.

A



B



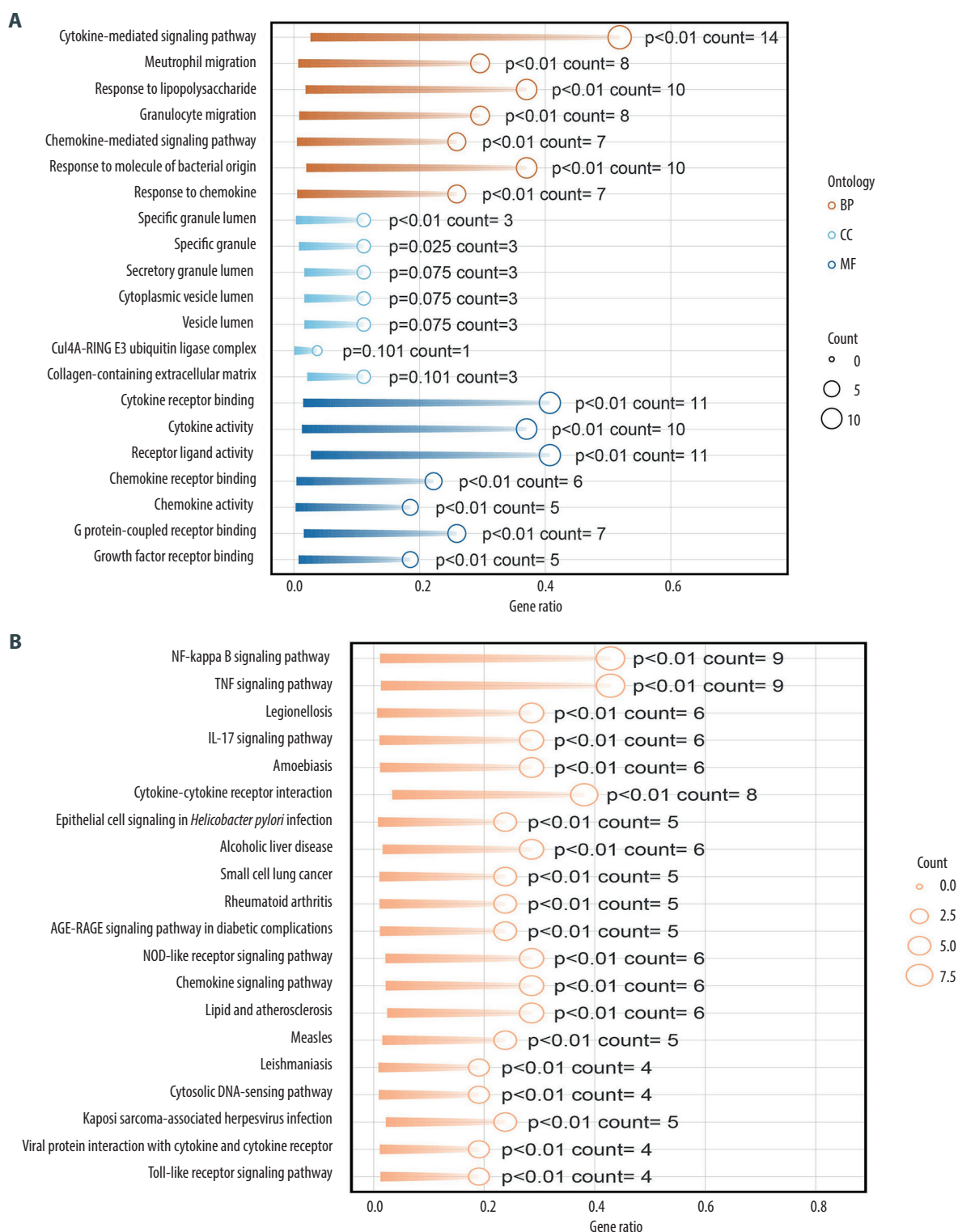


Figure 2. Differentially expressed ALI-related genes (DEGs) in ALI and healthy samples. (A) Heat map of 20 DEGs in ALI and healthy samples. (B) Volcano plot of the 27 DEGs. The red dots represent the significantly upregulated genes and the blue suggest the significantly downregulated genes. (C) Gene Ontology (GO) enrichment analysis of 27 DEGs. (D) Kyoto Encyclopedia of Genes Genomes (KEGG) analysis of 27 DEGs. BP – biological process; CC – cellular component; MF – molecular function.

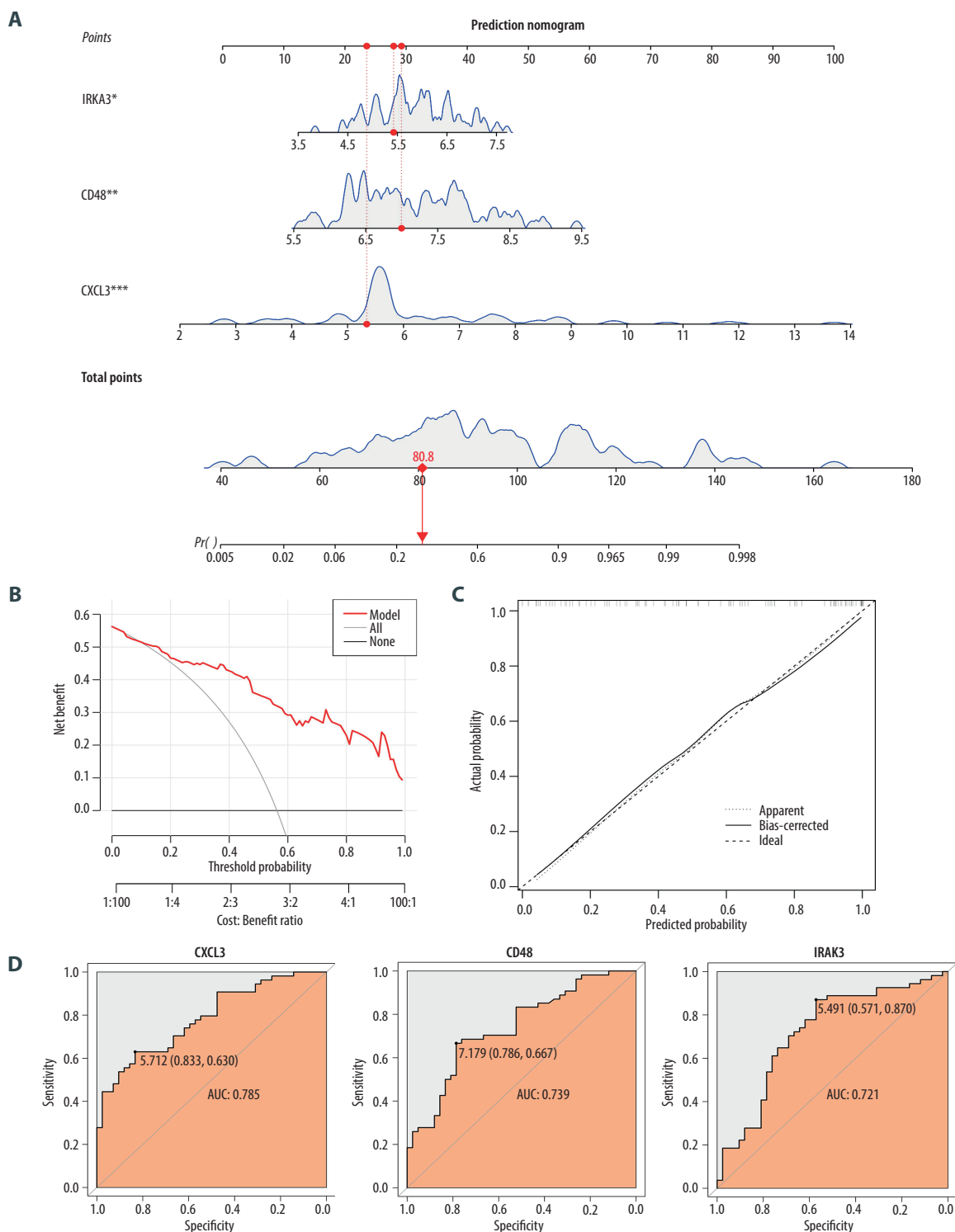


Figure 3. Screening and verification of hub genes. (A) The performance of least absolute shrinkage and selection operator (LASSO) to screen hub genes. (B, C) Support vector machine-recursive feature elimination (SVM-RFE) algorithm to screen hub genes. (D) Random forest (RF) algorithm to screen hub genes. (E) The DEGs recognized in RF. Importance showed the rank of genes in accordance with their relative importance. (F) Venn diagram demonstrates the intersection of hub genes acquired by the 3 algorithms.

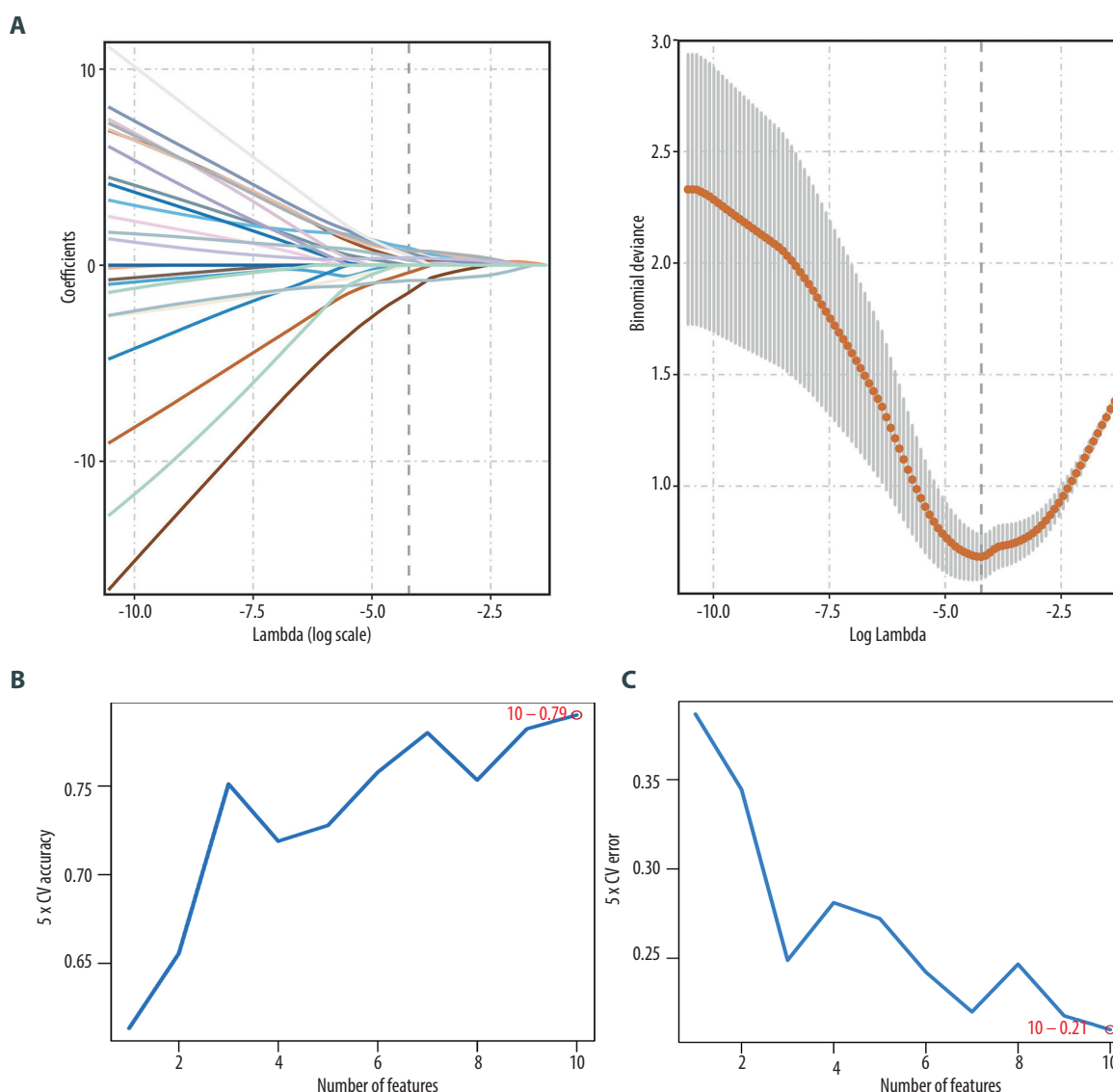
Construction and Validation of an ALI Diagnostic Nomogram

A diagnostic nomogram was developed based on the 3 identified hub genes (CXCL3, CD48, and IRAK3) to predict ALI occurrence (Figure 4A). Decision curve analysis (DCA) demonstrated that the nomogram provided clinical utility and net benefit, supporting its practical applicability in ALI diagnosis (Figure 4B). Bias-corrected calibration curves indicated strong agreement between predicted and observed outcomes, confirming the model's reliability (Figure 4C). The predictive performance of the nomogram was assessed using receiver operating characteristic (ROC) curves, which yielded area under the curve (AUC) values of 0.785 for CXCL3, 0.739 for CD48, and 0.721 for IRAK3, suggesting moderate diagnostic accuracy for

these biomarkers in ALI (Figure 4D-4F). These results indicate that integrating the 3 biomarkers enhances diagnostic precision compared with single-gene assessment, highlighting their potential clinical utility.

Correlation Between Immune Infiltration and ALI

To investigate the immune landscape associated with ALI, ssGSEA was used to assess infiltration levels of 28 immune cell types. The immune infiltration profile revealed varying levels of immune responses among different cell types. Notably, 9 immune cell types demonstrated a positive correlation with ALI, including macrophages, neutrophils, effector memory CD8⁺ T cells, immature B cells, central memory CD8⁺ T cells, myeloid-derived suppressor cells (MDSCs), CD56dim natural killer (NK)



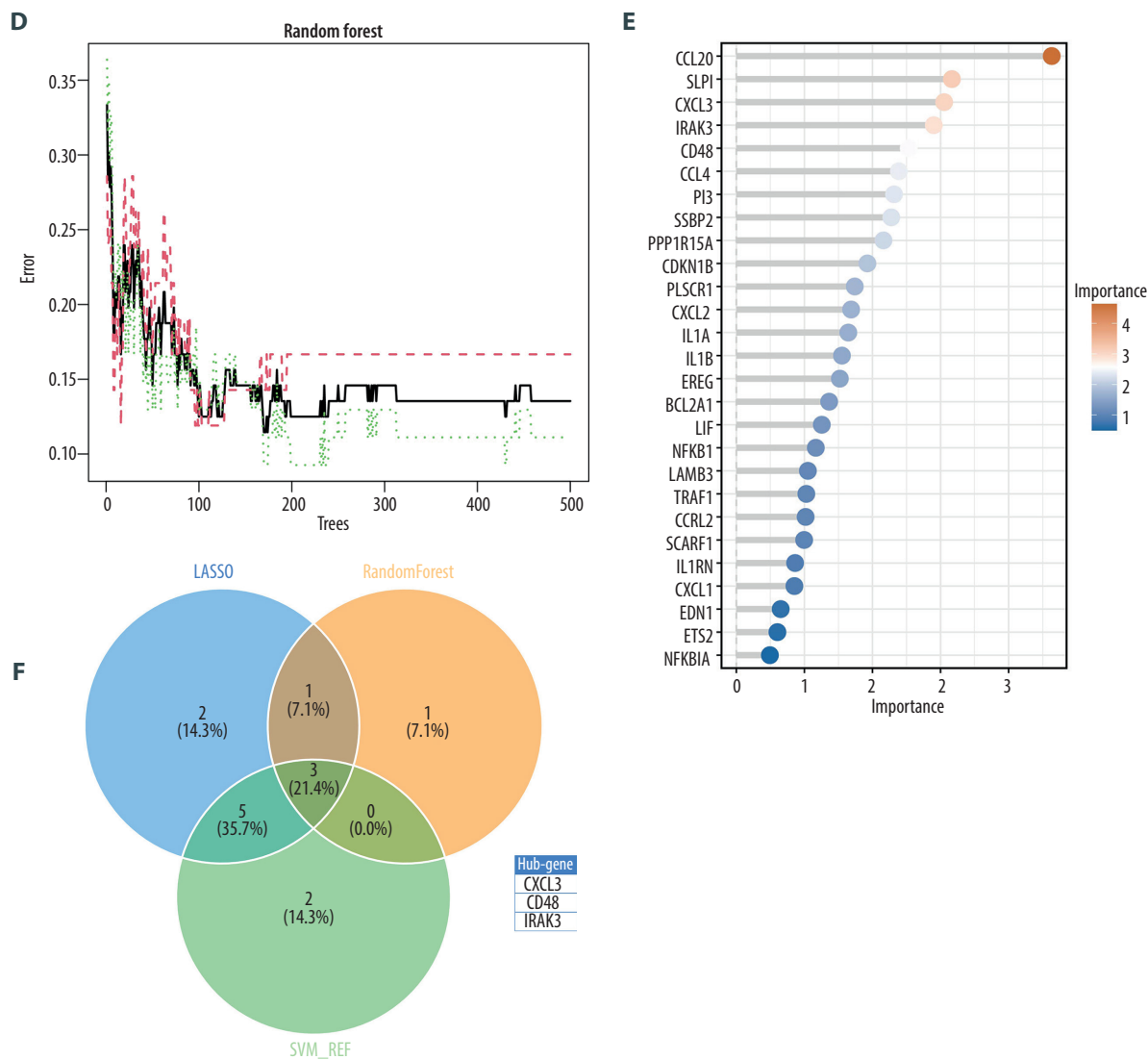


Figure 4. Validation of the efficacy of the hub genes diagnostic. (A) Nomogram of the hub genes. (B) The Decision Curve Analysis (DCA) of the nomogram. (C) Calibration curve of the nomogram. (D-F) The Receiver Operating Characteristic (ROC) curve of the hub genes: CXCL3, CD48 and IRAK3.

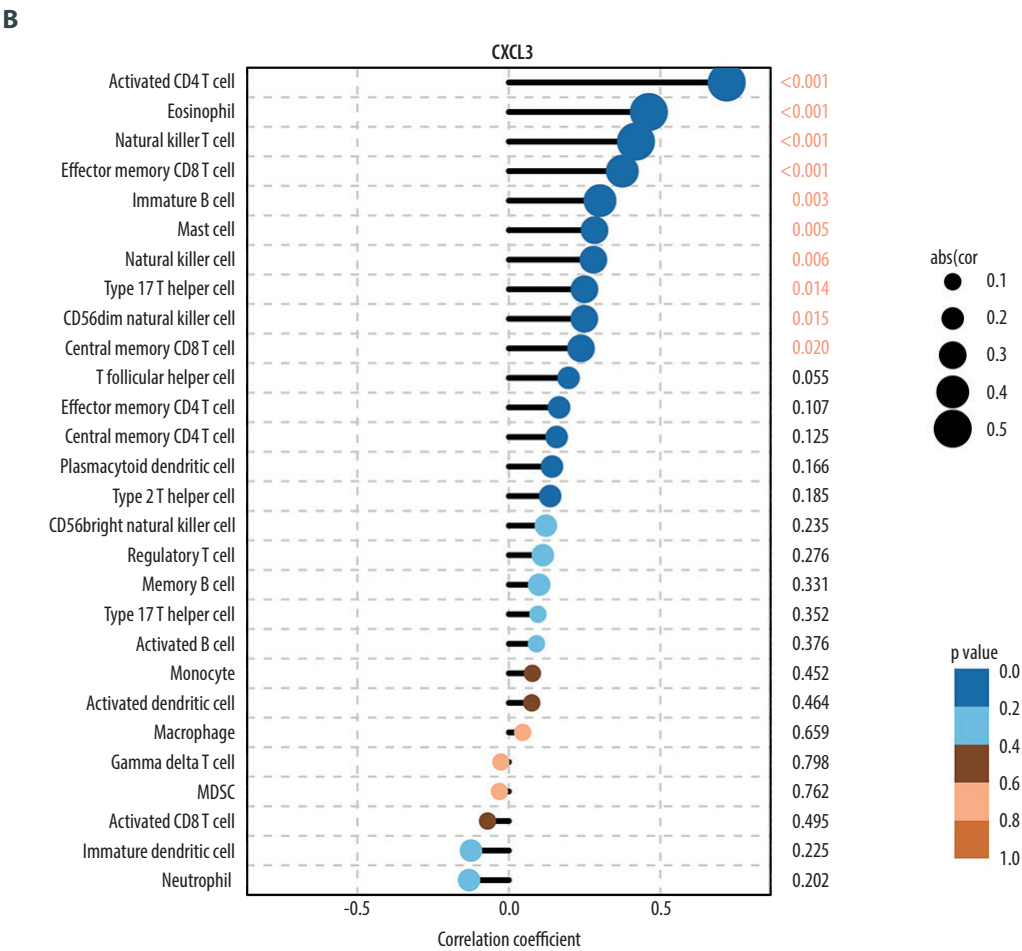
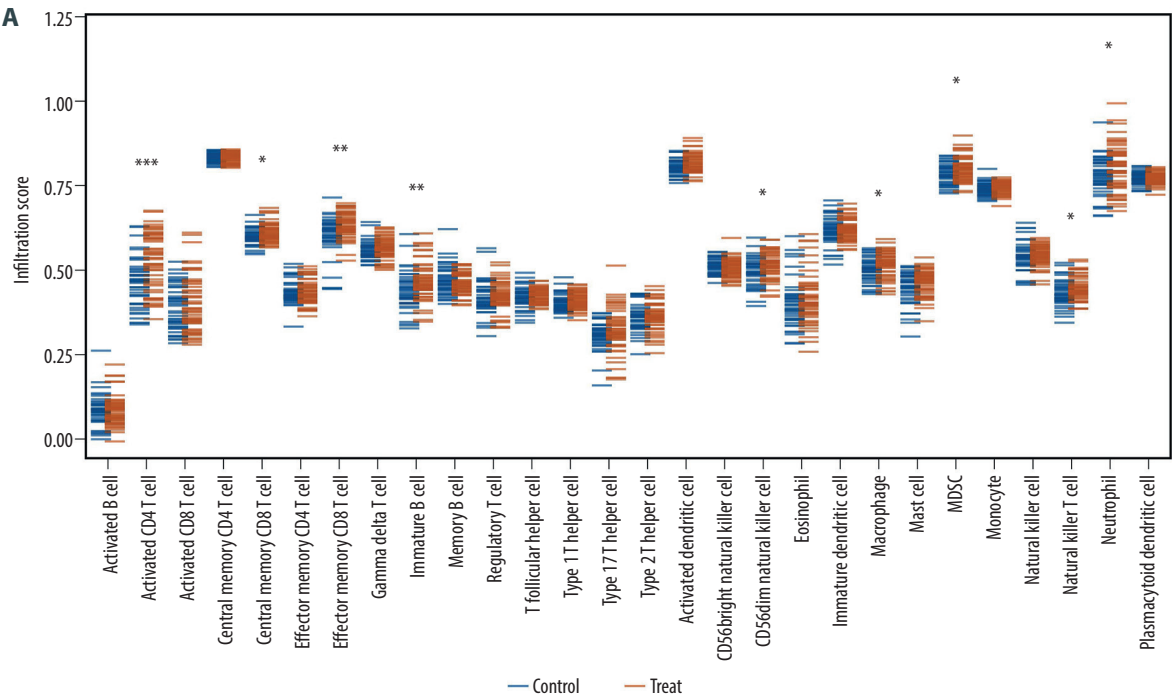
cells, activated CD4⁺ T cells, and natural killer T cells (Figure 5A). This pattern reflects a coordinated activation of both innate and adaptive immune responses during ALI.

Further analysis explored the correlation between immune infiltration and the 3 hub genes. CXCL3 showed a significant positive correlation with 10 immune cell types, while CD48 correlated positively with 14 immune cell types and negatively with one (Figure 5B, 5C). In contrast, IRAK3 exhibited a positive correlation with 7 immune cell types and a negative correlation with one (Figure 5D). The strong immune–gene correlation further supports the involvement of CXCL3, CD48, and IRAK3 in ALI-related immune dysregulation.

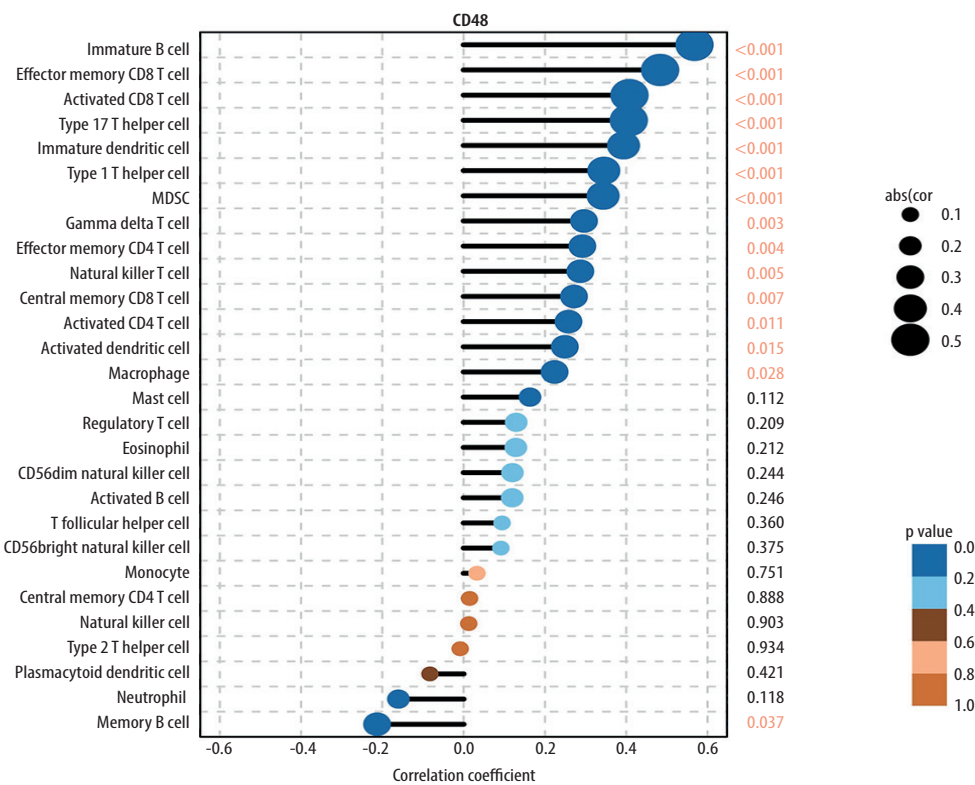
Single-Cell RNA Sequencing (scRNA-seq) Analysis of Hub Genes

To further validate the expression patterns of the 3 hub genes at the single-cell level, scRNA-seq data from both ALI-positive and ALI-negative samples were analyzed (Figure 6A, 6B). Cell annotation identified 9 major cell types, including endothelial cells, monocytes, megakaryocytes, fibroblasts, ciliated cells, alveolar type I cells, alveolar type II cells, secretory club cells, and capillary cells (Figure 6C, 6D).

Expression pattern analysis revealed that CXCL3 exhibited a strong positive correlation with ALI, while CD48 displayed a



C



D

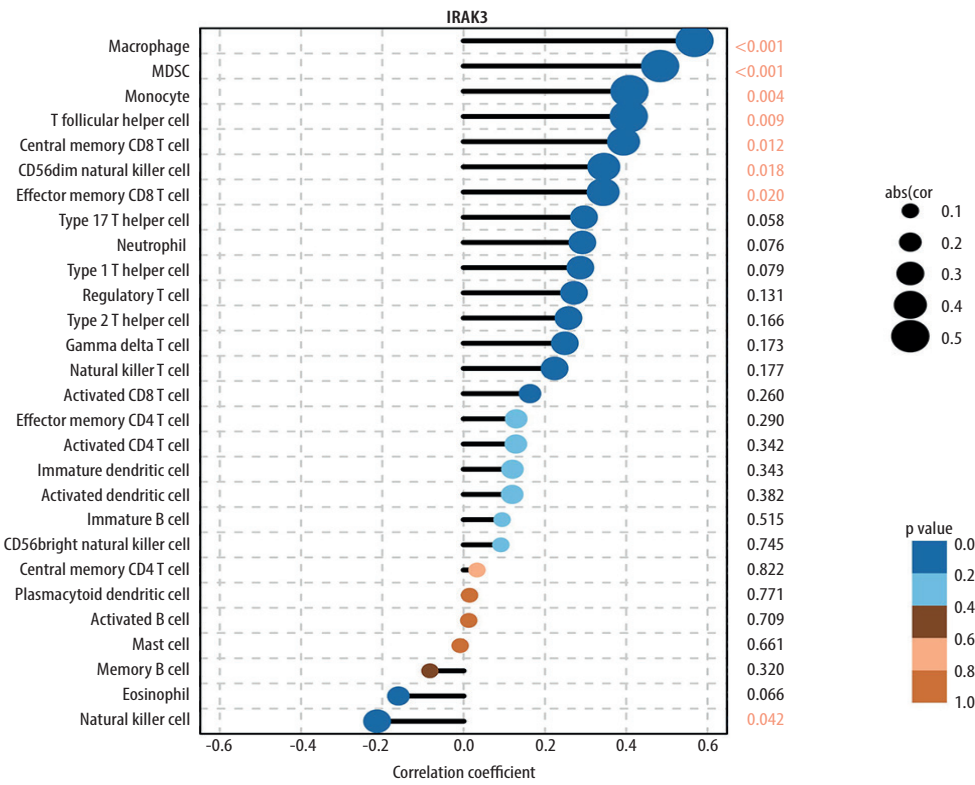
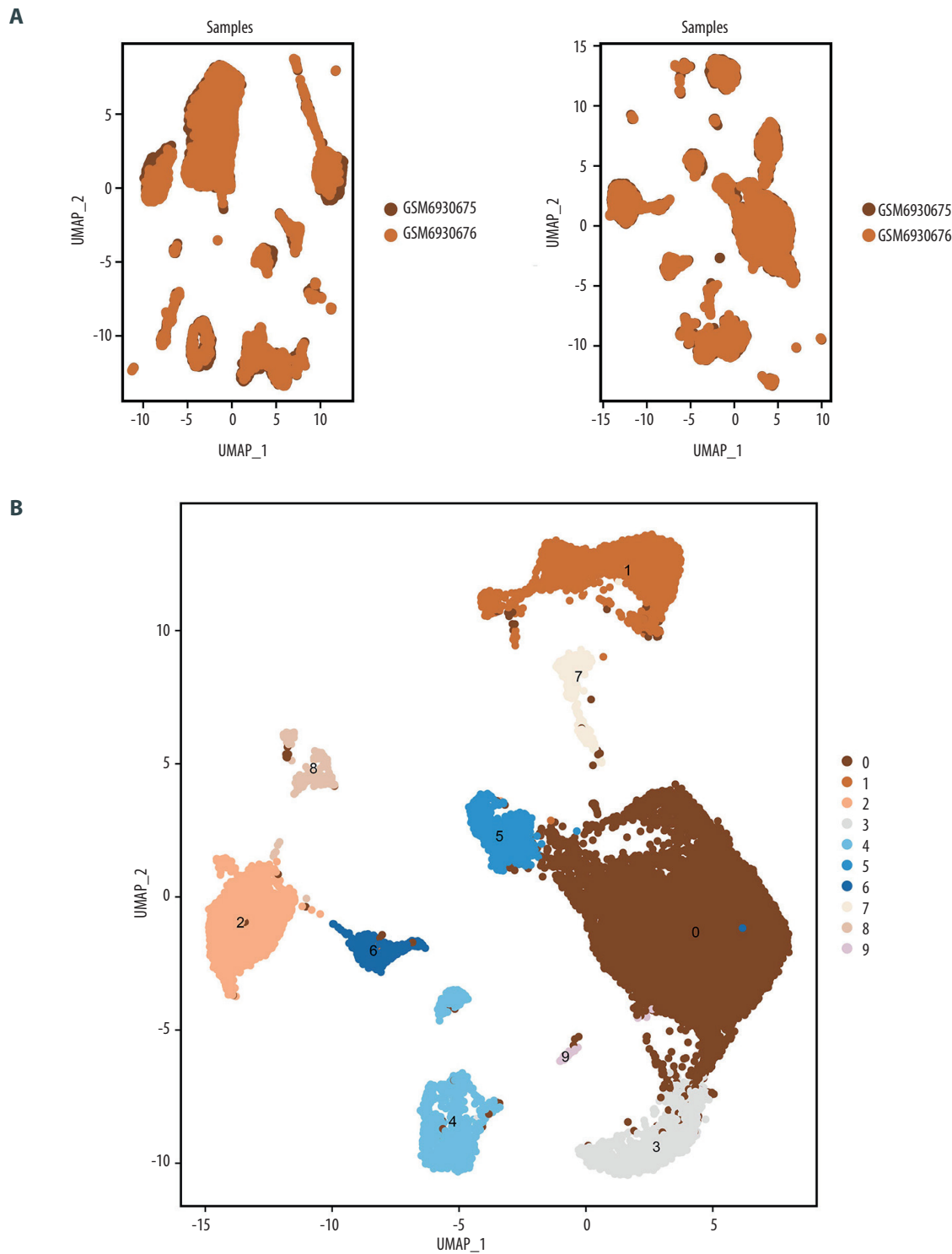
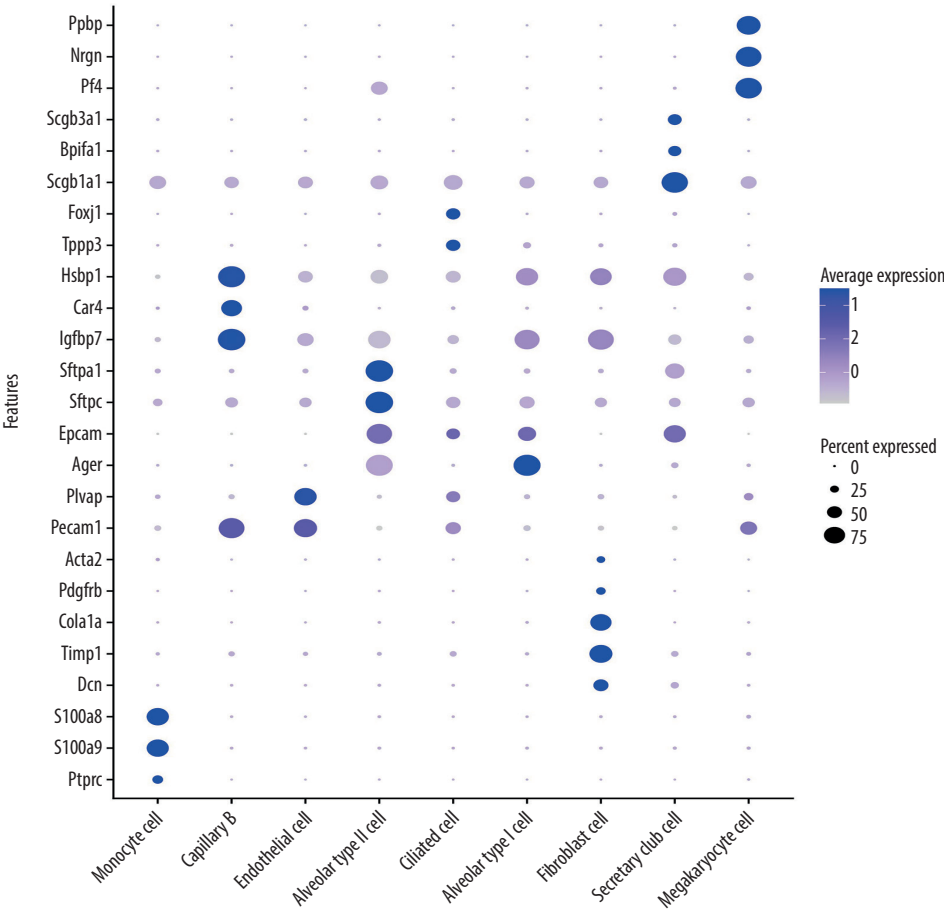


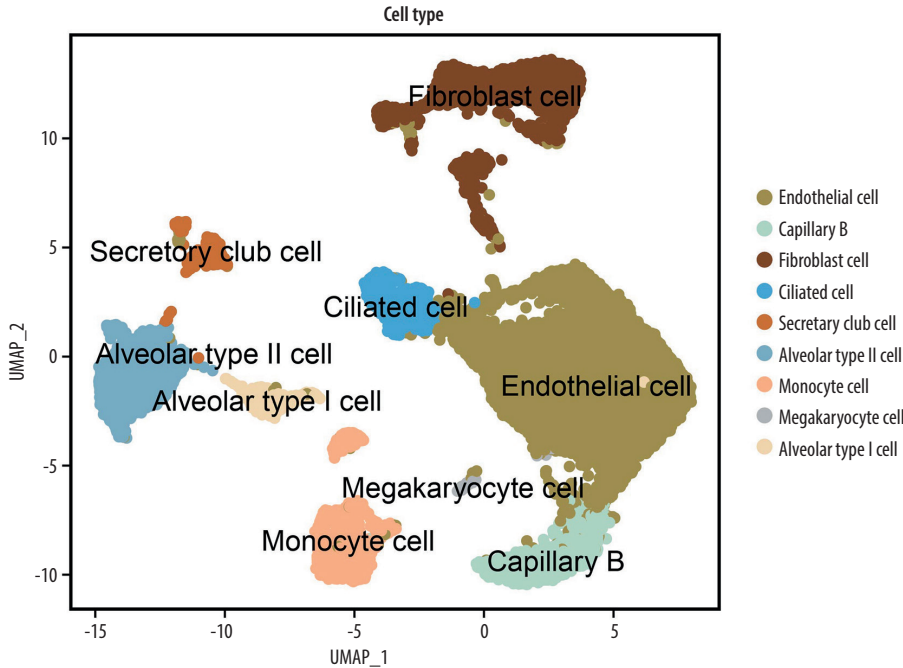
Figure 5. Identification of hub immune cell types correlated with the ALI. (A) The boxplot of 28 immune–gene-sets content. (B-D) Lollipop plots showed the correlations of CXCL3, CD48, and IRAK3 and immune cell types in ALI.



C



D



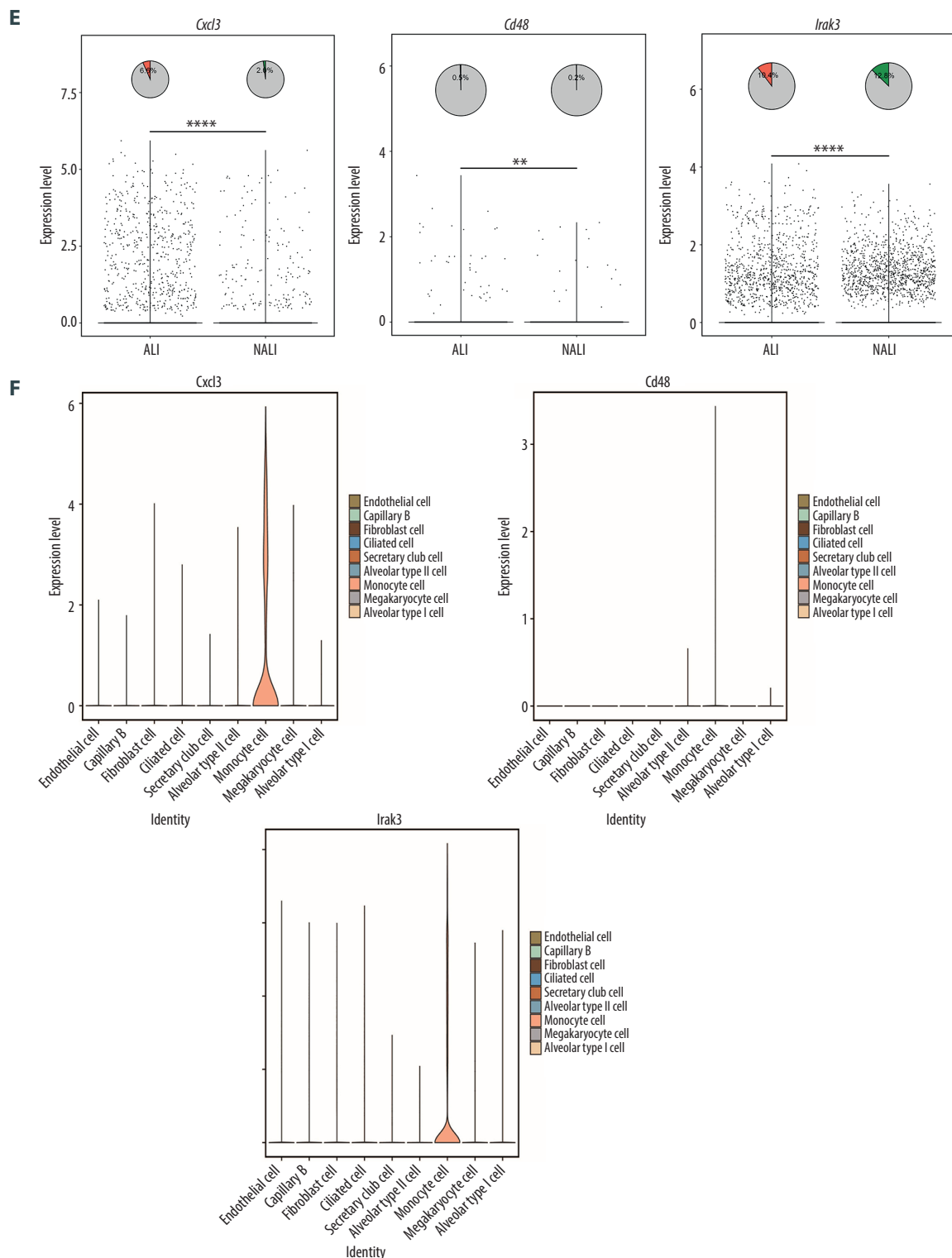
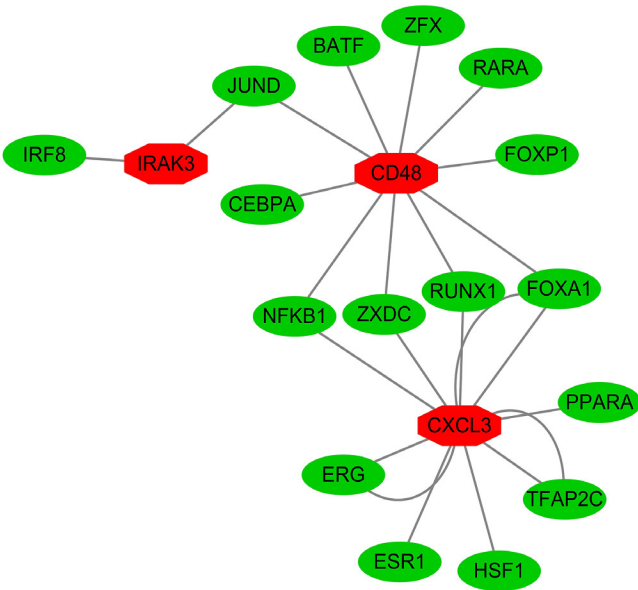


Figure 6. Single-cell RNA sequencing analysis. (A, B) UMAP of Seurat clusters. (C) Dot plot of cell population annotation according to different signatures. (D) UMAP cell types visualization of the single-cell RNA seq dataset GSE222810. Different colors indicate distinct clusters. (E) The expression level of CXCL3, CD48, and IRAK3 in ALI and NALI samples in GSE222810. (F) The expression level of CXCL3, CD48, and IRAK3 in different immune cells.

A



B

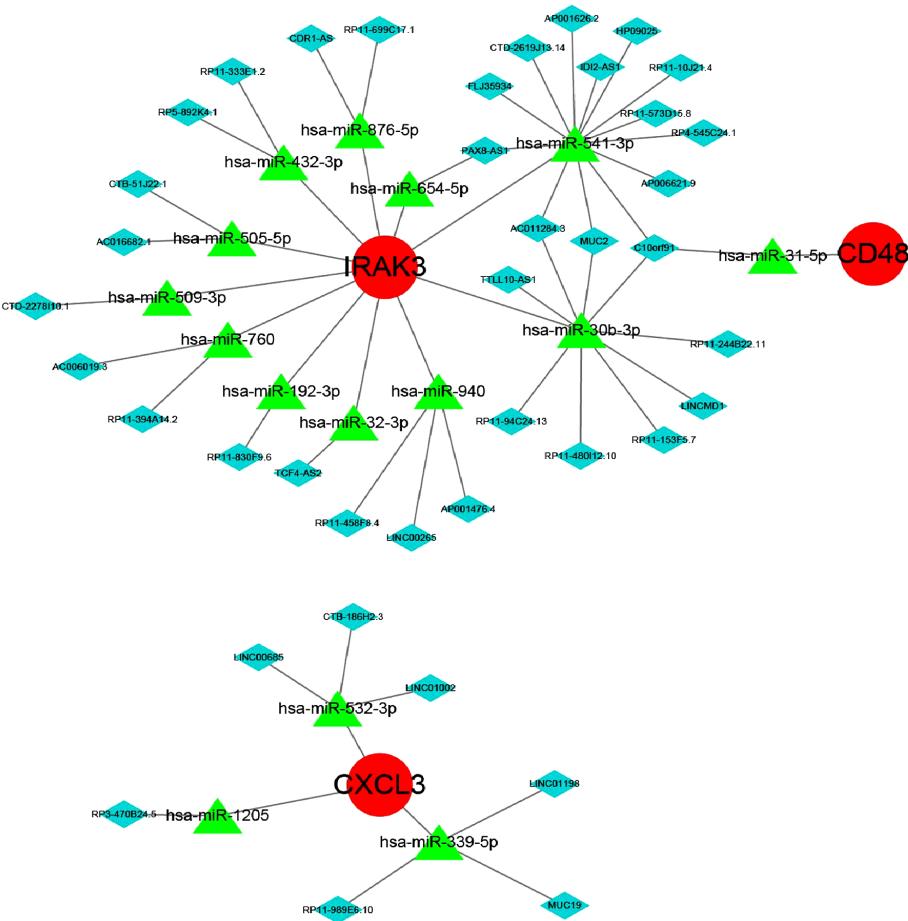


Figure 7. TF-hub gene interaction network and ceRNA network. (A) TF-hub gene network; (B) ceRNA network.

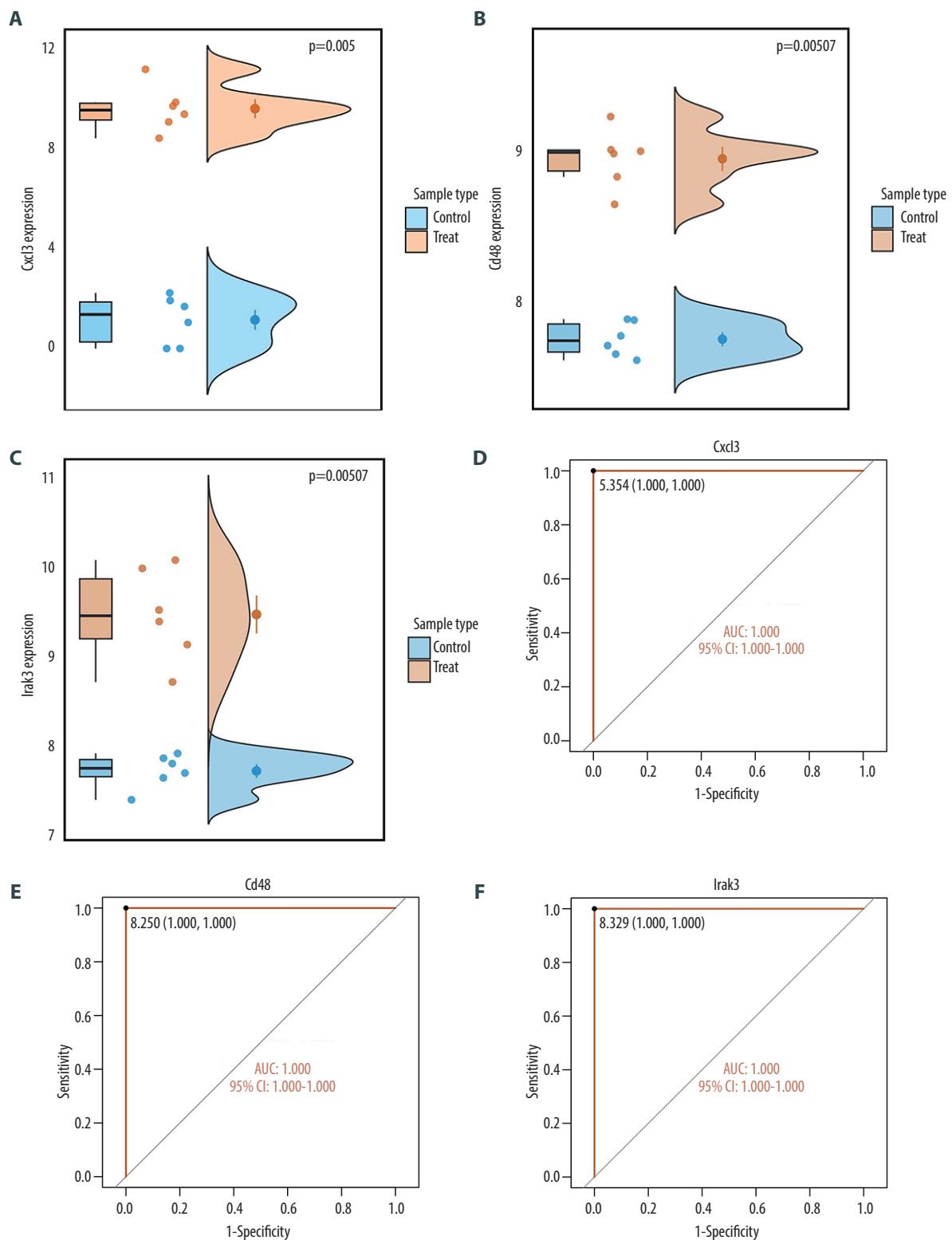


Figure 8. Validation of hub genes expression in external single-cell data. (A-C) Differential expression analysis of hub genes in ALI and NALI groups of external validation set GSE255726. (D-F) AUC of predicted outcomes of CXCL3, CD48, and IRAK3 in GSE255726.

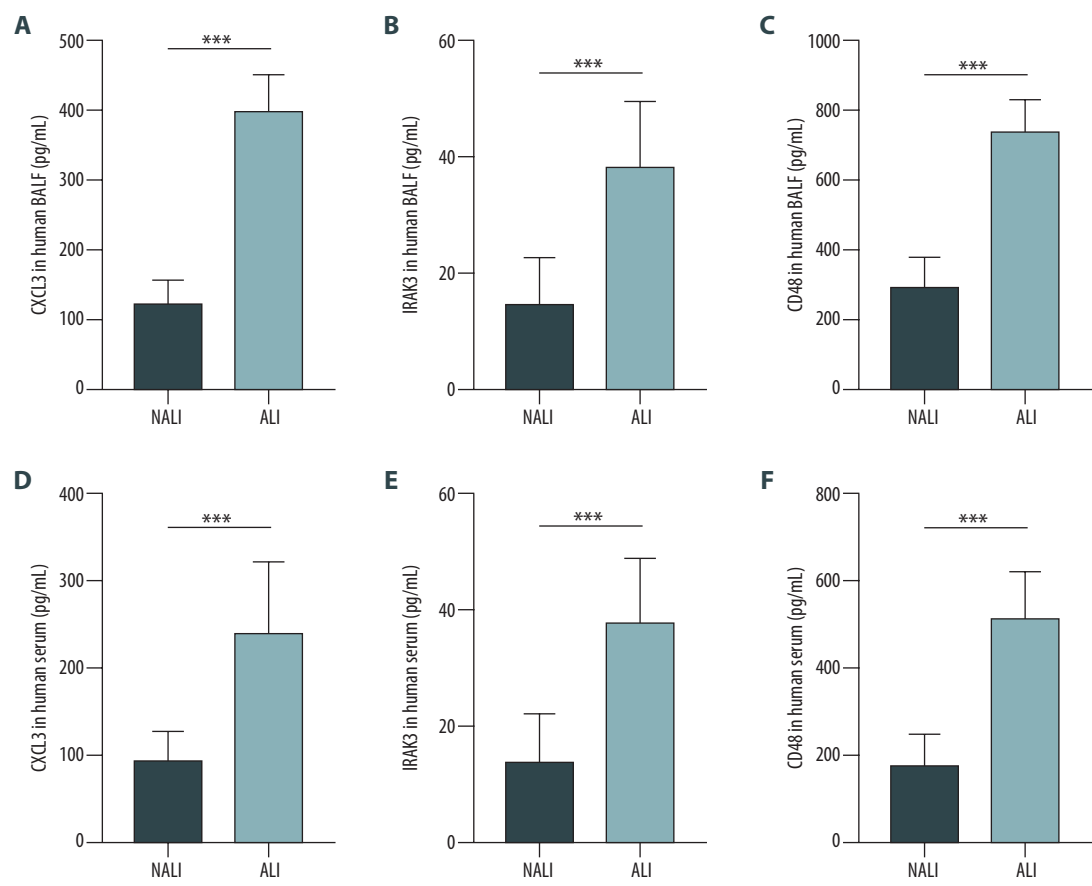


Figure 9. ELISA test of clinical samples. (A-C) The CXCL3, CD48, and IRAK3 in BALF was detected in ALI and NALI groups using ELISA (n=5 per group). (D-F) The CXCL3, CD48, and IRAK3 in serum was detected in ALI and NALI groups using ELISA (n=5 per group). BALF – bronchoalveolar lavage fluid.

moderate positive correlation (Figure 6E). IRAK3 exhibited a distinct expression pattern compared with CXCL3 and CD48 in the single-cell analysis (Figure 6E), suggesting context- and cell-state-dependent regulation (Figure 6E). Further investigation demonstrated that all 3 genes were predominantly expressed in monocytes, with minimal or no significant changes in expression observed in other cell types (Figure 6F). These findings provide high-resolution cellular evidence that monocytes are the principal source of these biomarkers and play a central role in ALI pathogenesis.

Regulatory Network Analysis: TF–Hub Gene and ceRNA Networks

To explore the regulatory mechanisms underlying ALI pathogenesis, a TF–hub gene regulatory network was constructed, identifying 16 transcription factors (TFs) predicted to regulate CXCL3, CD48, and IRAK3 (Figure 7A). Additionally, 15 miRNAs were predicted to interact with these hub genes, forming a miRNA–hub gene interaction network. A competing endogenous

RNA (ceRNA) network was subsequently established, integrating predicted TFs, miRNAs, and hub genes (Figure 7B). These regulatory networks highlight the complexity of transcriptional and post-transcriptional control governing the 3 biomarkers and offer multiple potential nodes for therapeutic intervention.

External Validation of Hub Genes Using an Independent GEO Dataset

Validation of CXCL3, CD48, and IRAK3 was performed using an external GEO dataset (GSE255726). Consistent with initial predictions, the mRNA expression levels of the 3 hub genes were significantly upregulated in ALI samples compared to non-ALI samples ($P<0.01$) (Figure 8A–8C). The diagnostic performance of these genes was assessed using ROC curve analysis, with all 3 hub genes showing very high AUC values in this small external validation set (Figure 8D–8F). These independent validation results further confirm the strong diagnostic potential of the 3 biomarkers and support their robustness across datasets.

Validation of Hub Genes in Clinical Samples

To further confirm the findings, protein expression levels of CXCL3, CD48, and IRAK3 were evaluated in bronchoalveolar lavage fluid (BALF) and blood serum samples from post-LT ALI patients. ELISA results revealed significantly ($P < 0.05$) higher protein levels of CXCL3 and CD48 in BALF and serum from ALI patients compared with controls, and IRAK3 also showed a significant between-group difference (Figure 9A-9F). Moreover, Spearman's rank correlation analysis demonstrated a strong positive association between ALI and hub gene expression, aligning with bioinformatics predictions. These clinical results provide compelling translational evidence that CXCL3, CD48, and IRAK3 are promising biomarkers for early identification of post-LT ALI, supporting their potential integration into postoperative monitoring strategies.

Discussion

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) represent a continuum of inflammatory lung damage arising from diverse etiologies and remain major causes of morbidity and mortality. Clinically, these conditions present as acute hypoxemic respiratory failure accompanied by bilateral pulmonary infiltrates unrelated to left heart dysfunction. Early prediction, timely detection, and effective treatment are crucial for improving patient outcomes [1-3]. However, the precise mechanisms underlying ALI/ARDS remain incompletely understood, and traditional diagnostic criteria are still based largely on clinical manifestations. Post-liver transplantation (post-LT) ALI poses an even greater diagnostic and therapeutic challenge [7], highlighting the urgent need for reliable biomarkers that can facilitate early detection and guide targeted management. In the present study, machine-learning techniques and publicly available gene expression data were utilized to identify biomarkers significantly associated with ALI/ARDS. DEGs were identified from 5 GEO datasets (GSE172222, GSE2322, GSE3037, GSE68610, GSE10361). Subsequently, functional enrichment analyses, including GO, KEGG, and ssGSEA, were performed based on these DEGs. The results of GO and KEGG analyses revealed that the identified DEGs participate in various inflammatory and immune-related biological processes, cellular components, molecular functions, and signaling pathways. In addition to its vital role in gas exchange, the lung also serves as a hub immune organ, protecting against inhaled pathogens, allergens, and xenobiotics [35]. ssGSEA demonstrated significantly higher levels of immune cell infiltration in the ALI group, particularly in neutrophils, macrophages, and activated CD4⁺ T cells. Numerous studies have reported that neutrophils play an essential role in initiating and amplifying lung injury through recruitment, migration, and the release of NETs [11,36]. Similarly, aberrant

macrophage activation and mitochondrial dysfunction have been implicated in ALI pathogenesis [37]. Macrophage polarization imbalance between pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes may exacerbate sepsis-related ALI [38]. Activated CD4⁺ T cells have also been shown to intensify ALI by promoting hyper-NETosis induced by antigen-presenting aged neutrophils [39]. These findings align with our immune infiltration analysis, reinforcing the central contribution of innate and adaptive immune dysregulation to the development of post-LT ALI.

Using 3 complementary machine-learning algorithms (LASSO, SVM-RFE, and RF), this study identified CXCL3, CD48, and IRAK3 as the most robust biomarkers associated with ALI. The diagnostic nomogram constructed from these genes demonstrated strong predictive performance and clinical utility across multiple datasets. Importantly, scRNA-seq data confirmed that these biomarkers are predominantly expressed in monocytes, a hub immune population involved in ALI pathogenesis. ELISA results from BALF and serum samples of LT recipients further validated the upregulation of these biomarkers in post-LT ALI. By integrating bioinformatics, machine-learning, single-cell transcriptomics, and clinical sample validation, our study provides strong evidence supporting the diagnostic potential of these biomarkers and offers new mechanistic insights into post-LT ALI. CXCL3, also known as CINC-2 α (Cytokine-Induced Neutrophil Chemoattractant), activates the CXCR2 receptor and participates in several hub signaling cascades, including NF- κ B, JAK/STAT3, and ERK1/2 MAPK pathways [40]. Substantial evidence highlights that leukocytes and their chemokines are critical contributors to ALI progression. Polymorphonuclear neutrophils (PMNs) generate NETs that contribute to alveolar damage [41-44]. CXCL3 is significantly upregulated in a wide range of inflammatory and pulmonary disorders, including ALI/ARDS, infections, asthma, and lung cancer [45-48]. It has been shown to induce robust neutrophil infiltration and exacerbate lung inflammation [45], while the CXCL3/CXCR2 axis is involved in ventilator-induced lung injury [49]. Conversely, maresin-1 has been shown to mitigate septic lung injury by suppressing CXCL3-associated neutrophil subpopulations and regulating inflammatory transcriptional programs [50]. Consistent with these data, our study demonstrated a strong association between elevated CXCL3 expression and post-LT ALI, suggesting that CXCL3 can amplify inflammatory responses in the early postoperative period. Collectively, these findings highlight CXCL3 as a potential biomarker for disease severity and a promising target for therapeutic intervention.

CD48 is a glycosylphosphatidylinositol (GPI)-anchored protein belonging to the CD2 subfamily and is primarily expressed on hematopoietic cells in both membrane-bound and soluble forms [51,52]. As a low-affinity ligand for CD2, CD48 functions as a co-stimulatory molecule essential for lymphocyte

activation [53]. Multiple studies have reported upregulation of membrane-bound CD48 (mCD48) in asthma, COPD, and pulmonary inflammation [54-58]. CD48 promotes B-cell proliferation and differentiation [59], elevates intracellular calcium levels in neutrophils [60], and exerts co-stimulatory effects on T cells [61]. Additionally, CD48 facilitates leukocyte adhesion and can mediate eosinophil recruitment to the lungs [62], and studies suggest its potential as a biomarker for lung inflammation [63]. Elevated CD48 expression on circulating leukocytes has also been observed in SARS-CoV-2 infection, further indicating its role as a marker of lung injury [64,65]. In line with these findings, our study showed marked upregulation of CD48 in both BALF and serum of post-LT ALI patients, reinforcing its role as an immune activation marker and its potential utility in early postoperative monitoring. IRAK-3 (also known as IRAK-M) is an inactive kinase of the IRAK family predominantly expressed in monocytes and macrophages [66]. Increasing evidence indicates that IRAK-3 negatively regulates Toll-like receptor (TLR) signaling, maintaining innate immune homeostasis by inhibiting NF- κ B activation and downstream cytokine production [66-68]. However, IRAK-3 exhibits context-dependent functions. Its deficiency has been shown to enhance bacterial clearance and preserve macrophage effector activity during sepsis by promoting leukocyte-active cytokine production and increased neutrophil infiltration [67]. Similarly, reduced IRAK-3 expression exacerbates pulmonary neutrophil infiltration in influenza pneumonia [69]. Conversely, chronic exposure to LPS can upregulate IRAK-3, intensifying inflammatory mediator production through TLR4 signaling [66,70]. IRAK-3 has been implicated in several pulmonary conditions, including influenza pneumonia, COVID-19 cytokine storms, bleomycin-induced lung injury, and mechanical ventilation-associated lung injury [71-74]. Our study revealed that IRAK-3 expression is significantly elevated in post-LT ALI/ARDS, which differs from findings in classical sepsis or viral pneumonia. This discrepancy may reflect the unique immunologic context of liver transplantation, graft ischemia-reperfusion injury, and postoperative systemic inflammation. Given the small cohort size, further mechanistic studies are required to clarify the precise role of IRAK-3 in post-LT ALI.

This study has several unavoidable limitations. Firstly, the bioinformatic analyses relied on datasets derived from ALI/ARDS and normal lung or blood samples, while lung tissue data specific to post-LT ALI were unavailable. Validation was therefore limited to BALF and blood samples. Secondly, the sample size of the clinical cohort was small (n=10), reflecting the limited availability of post-LT ALI cases; larger, multicenter cohorts are

needed to confirm the generalizability of CXCL3, CD48, and IRAK3. Thirdly, although we validated the transcriptional and protein-level expression of the hub genes, their downstream biological functions were not investigated in vivo. Future studies should include mechanistic animal experiments and longitudinal patient monitoring to establish temporal biomarker dynamics and therapeutic applicability. Overall, this study integrated multi-dataset bioinformatics, machine-learning, single-cell transcriptomics, and clinical validation to identify CXCL3, CD48, and IRAK3 as novel biomarkers for post-LT ALI. These findings not only advance the current understanding of ALI immunopathogenesis but also provide promising avenues for early diagnosis, risk stratification, and targeted intervention in liver transplant recipients.

Conclusions

This study integrates bioinformatics, machine learning, scRNA-seq, and clinical validation to identify CXCL3, IRAK3, and CD48 as reliable biomarkers associated with post-liver transplantation acute lung injury. These genes, together with the NF- κ B, TNF, and IL-17 pathways, appear to be central to the inflammatory processes driving post-LT ALI. Monocytes were identified as the primary cell type linked to these biomarkers, underscoring their hub role in disease progression. The TF and ceRNA regulatory networks further highlight potential molecular mechanisms controlling their expression. Overall, these findings enhance current understanding of post-LT ALI and provide promising candidates for early diagnosis and future therapeutic targeting.

Department and Institution Where Work Was Done

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Patient Permission/Consent Declarations

Written informed consent was obtained from all patients or their legal guardians prior to inclusion in the study.

Declaration of Figures' Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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