

Role of Cellular Senescence Genes and Immune Infiltration in Sepsis and Sepsis-Induced ARDS Based on Bioinformatics Analysis

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Introduction: Sepsis is the leading cause of death in critically ill patients; it results in multiorgan dysfunction, including acute respiratory distress syndrome (ARDS). Our study was conducted to determine the role of cellular senescence genes and immune infiltration in sepsis and sepsis-induced ARDS via bioinformatic analyses.

Experimental Procedures: Datasets GSE66890 and GSE145227 were obtained from the Gene Expression Omnibus (GEO) database and utilized for bioinformatics analyses. Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of the differentially expressed genes (DEGs) were performed to identify the key functional modules. Two machine learning algorithms, least absolute shrinkage and selection operator (LASSO) and support vector machine recursive feature elimination (SVM-RFE), were used to screen for characteristic genes in sepsis and sepsis-induced ARDS. ROC curves were generated to evaluate the predictive ability of gene hubs. Differences in immune infiltration levels between the disease and control groups were compared via ssGSEA. The diagnostic value of the hub genes was verified via quantitative PCR (qPCR) in hospitalized patients.

Results: Four characteristic genes (ATM, CCNB1, CCNA1, and E2F2) were identified as biomarkers for the progression of sepsis-induced ARDS. E2F2 showed the highest predictive ability for the occurrence of ARDS in patients with sepsis. CD56bright and plasmacytoid dendritic cells exhibited high infiltration in the sepsis-induced ARDS group, whereas eosinophils, MDSCs, macrophages, and neutrophils exhibited low infiltration. In addition, ATM expression was lower in patients with sepsis than in those without sepsis ($n = 6$).

Conclusion: Sepsis-induced ARDS is correlated with circulating immune responses, and the expression of ATM, CCNB1, CCNA1, and E2F2 may serve as potential diagnostic biomarkers and therapeutic targets in sepsis-induced ARDS.

Keywords: sepsis, cellular senescence genes, immune infiltration, acute respiratory distress syndrome, bioinformatics analysis

Introduction

Sepsis is a life-threatening condition that results in organ dysfunction caused by a dysregulated host response to infection. Sepsis and septic shock are leading causes of death worldwide; they are fatal in approximately 25% of diagnosed patients.¹ Patients with acute respiratory distress syndrome (ARDS) are at high risk of death in intensive care units (ICUs).^{2,3} Among the factors that influence the development of ARDS, sepsis is a major contributor. ARDS caused by sepsis is more severe than ARDS due to nonsepsis causes. Specifically, recovery from lung injury is worse, extubation success rates are lower, and mortality is greater.⁴ Sepsis-related ARDS has been demonstrated to be more severe than nonsepsis-related ARDS, as sepsis-induced ARDS causes more severe lung injuries, and its mortality is greater.⁵ Thus, key molecules involved in sepsis-related ARDS must be identified to treat sepsis more effectively.

Numerous studies have been conducted in recent years to determine whether genetic factors contribute to ARDS development.^{6,7} Clinical and biological markers are useful for predicting ARDS and its outcomes in patients with sepsis.

Demand for biomarker-targeted therapies for ARDS is currently increasing,^{8,9} and previous studies have shown that specific markers may play important roles in detecting and preventing sepsis-induced ARDS. Specifically, cyclin B1 (CCNB1), cyclin B2 (CCNB2), DNA topoisomerase II alpha (TOP2A) and the transcription factor fork head box protein M1 (FOXM1) are potential targets for gene therapy.⁶ In this context, the analysis of gene expression profiles in relation to ARDS may yield breakthroughs to elucidate the pathological mechanisms underlying ARDS. Thus, cellular senescence may be involved in the development and onset of ARDS. Notably, inflammation, proliferation, and fibrosis may be causes or consequences of senescence. However, studies establishing a link between ARDS and cellular senescence are limited. Naturally occurring senescence in the early stages of acute injury in other tissues has been shown to be beneficial by limiting apoptosis and favouring tissue repair.¹⁰

In this study, we comprehensively evaluated the expression profiles of senescence genes in sepsis and sepsis-induced ARDS. Using the GEO database, the mRNA expression profiles of patients with sepsis and sepsis-related ARDS were analysed via differential expression, immune infiltration, gene expression, immune infiltration, and functional enrichment analyses. To effectively diagnose sepsis or sepsis-induced ARDS, we used two machine learning algorithms to predict outcomes. This study aimed to provide useful biological information for identifying the key cellular senescence genes involved in sepsis-induced ARDS.

Experimental Procedures

Data Source

RNA-seq profiles and clinical features of the GSE66890 and GSE145227 datasets were accessed from the GEO website (<https://www.ncbi.nlm.nih.gov/gds/?term=>). GSE66890 includes 29 sepsis-induced ARDS samples and 28 sepsis samples collected on the platform GPL6244. GSE145227 includes 10 sepsis samples and 12 normal samples collected on the platform GPL23178. Senescence genes were obtained from the “cellular senescence” terms in the KEGG database (<https://www.kegg.jp/entry/map04218>).

Identification of DEGs

The R (version 4.1.1) package “preprocessCore” was used to homogenize the data prior to differential expression analysis. After homogenization, DEGs were screened with a threshold of $p < 0.05$ using the “limma” package. Genes that were significantly upregulated or downregulated in both GEO datasets were identified as DEGs.

Functional Enrichment Analysis

The Gene Ontology (GO) resource (<http://geneontology.org>) provides structured, computable knowledge regarding the functions of genes and gene products.¹¹ Gene Ontology function enrichment includes three categories: molecular function (MF), biological process (BP) and cellular component (CC). The Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.ad.jp/kegg/>)¹² is a database of biological systems that collects genomic, chemical and systemic functional information. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed and visualized via the R package “clusterProfiler.”¹³ Statistical significance was set at $p < 0.05$. Correlation analysis of the hub genes with all transcripts was conducted for gene set enrichment analysis (GSEA) of the hub genes. The R package “clusterProfiler” was used to perform GSEA via correlation analysis to explore the functions of the hub genes.

Machine Learning Analysis

To effectively diagnose sepsis or sepsis-induced ARDS, we used two machine learning algorithms to predict outcomes. The LASSO logistic regression¹⁴ and SVM-RFE¹⁵ algorithms are highly important for identifying key biomarkers. In recent years, these two algorithms have been widely used in research to identify diagnostic or prognostic factors. The LASSO regression algorithm was applied via the R package “glmnet” to determine genes significantly related to the screening ability of the disease group and the control group. A support vector machine (SVM) is a monitoring technology used for classification and regression analysis. To avoid overfitting, the RFE algorithm was used to screen hub genes

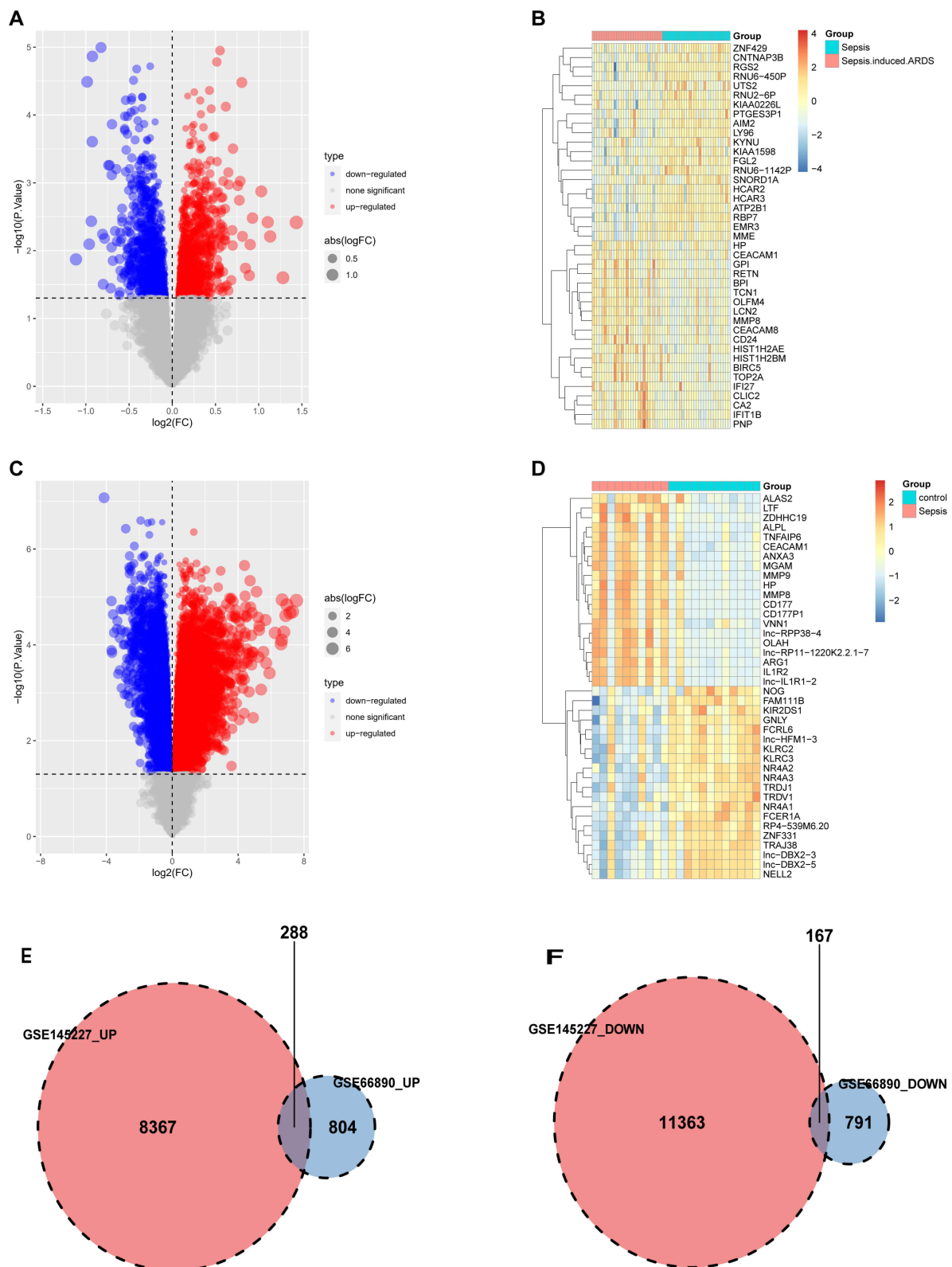


Figure 1 Identification of DEGs. A total of 8655 upregulated DEGs and 11530 downregulated DEGs were identified in GSE145227 (A-B), whereas 1092 upregulated DEGs and 958 downregulated DEGs were identified in GSE66890 (C-D), and 288 upregulated genes (E) and 167 downregulated genes (F) were identified in GSE66890 and GSE145227.

from the metadata. Thus, we used SVM-RFE technology to select hub genes. The ROC curve was used to evaluate the predictive ability of the hub genes via the R package “pROC.”

Immune Infiltration Assessment

Single Sample Gene Set Enrichment Analysis (ssGSEA), an extension of the Gene Set Enrichment Analysis (GSEA) methodology, is extensively utilized in bioinformatics research, particularly in the context of immune infiltration studies.¹⁶ The ssGSEA algorithm within the R package “GSVA” was used to evaluate the infiltration levels of the 23 immune cells. The differences in immune infiltration levels between the disease and control groups were compared. The Spearman rank correlation coefficient was used to evaluate the correlation between the infiltration scores of immune cells and the expression levels of the hub genes.

qRT-PCR

This study was approved by the Medical Ethics Committee of the Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology (WHZXKYL2022-007). Whole blood samples from six patients with sepsis and six patients without sepsis were collected from the intensive care unit of the Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, from October 20, 2023 to January 30, 2024. Sepsis was diagnosed on the basis of Sepsis version 3.0. Patients with neoplastic diseases or autoimmune disorders, as well as those taking oral immunosuppressants, were excluded from the study. Quantitative real-time polymerase chain reaction (qPCR) was performed to measure the expression levels of four characteristic genes (ATM, CCNB1, CCNA1, and E2F2).

Statistical Analysis

Student’s *t* test was used to compare the differences between two groups. Statistical analysis was performed via R software 4.1.1. Differences were considered statistically significant at **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

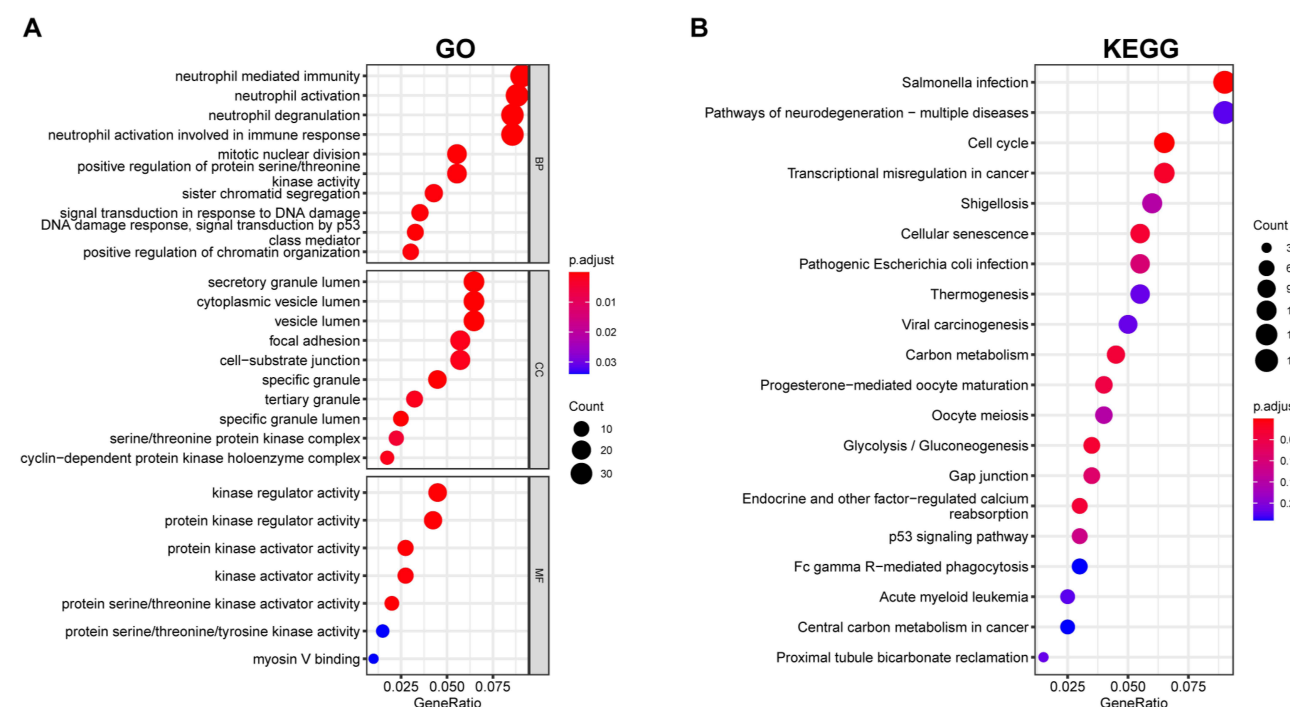


Figure 2 Functional enrichment of DEGs. GO analysis (A); KEGG analysis (B).

Results

Identification of DEGs

We first homogenized the mRNA expression data from the GSE66890 and GSE145227 datasets before performing differential expression analysis (Sup-Figure 1A–D). We identified 8655 upregulated DEGs and 11530 downregulated DEGs in GSE145227 (Figure 1A–B) and 1092 upregulated DEGs and 958 downregulated DEGs in GSE66890 (Figure 1C–D). DEGs that were simultaneously upregulated or downregulated in both GSE66890 and GSE145227 were selected as differentially expressed genes for subsequent analysis, which yielded 288 upregulated (Figure 1E) and 167 downregulated genes (Figure 1F).

Functional Enrichment Analysis

The functional enrichment of the DEGs was performed to identify possible biological functions. GO analysis revealed that the DEGs were related to neutrophil-mediated immunity in the biological process (BP), secretory granule lumen in

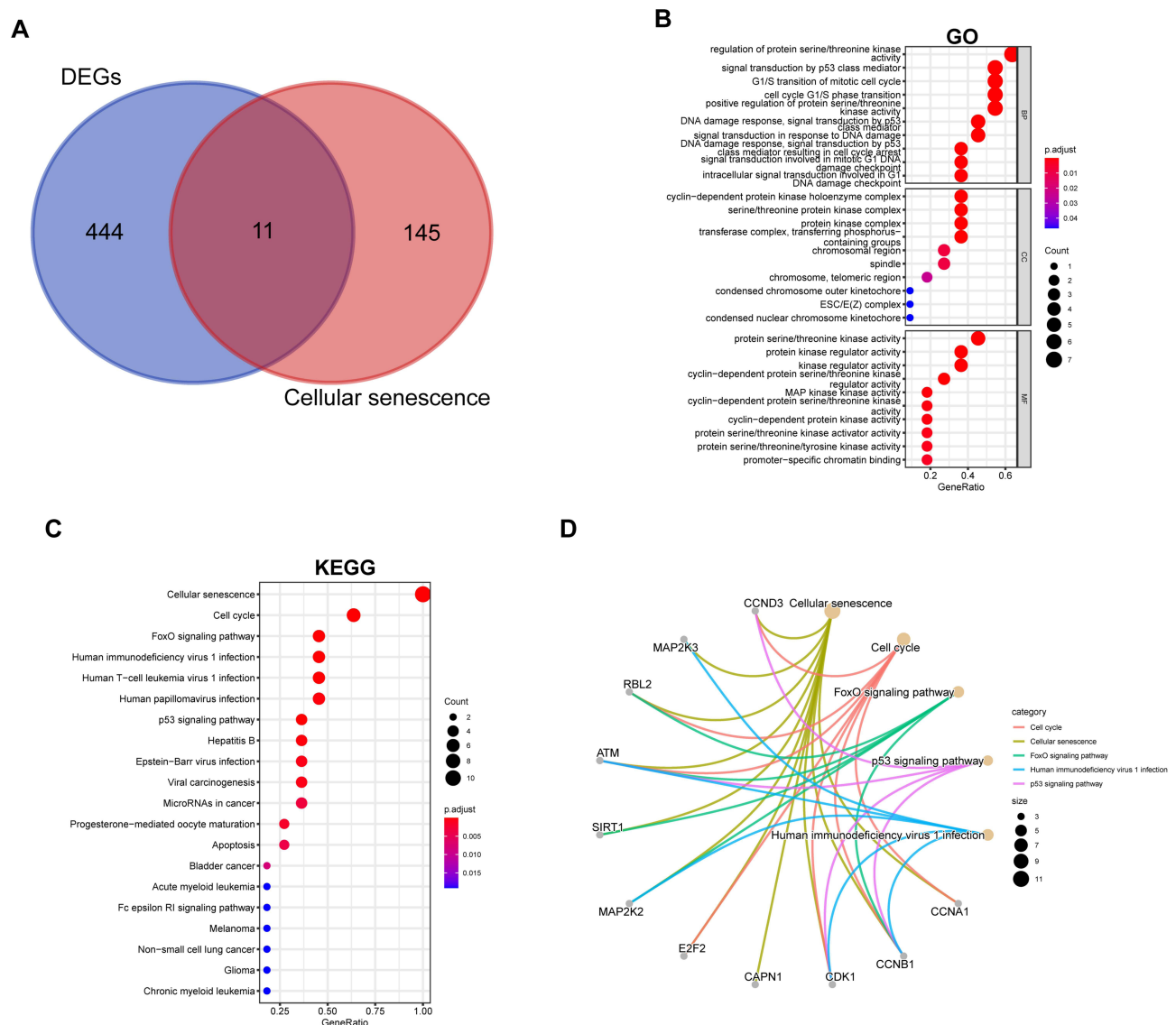


Figure 3 Identification of DEGs involved in cellular senescence. Eleven genes were identified (A). The enriched Gene Ontology (GO) terms in the molecular function (MF), biological process (BP) and cellular component (CC) categories (B). The results of the KEGG pathway enrichment analysis (C); the top 5 KEGG results and their relationships with the identified genes (D).

the cellular component (CC), and kinase regulatory activity in the molecular function (MF) (Figure 2A). KEGG analysis revealed that genes related to Salmonella infection, the cell cycle, cellular senescence, and carbon metabolism were enriched (Figure 2B). These results suggest that cellular senescence-related functions and pathways play vital roles in sepsis-induced ARDS.

Identification of Cellular Senescence Genes Among the DEGs

Next, we intersected the DEGs with genes related to cellular senescence. Eleven cellular senescence genes were identified (Figure 3A). We further explored the functions and pathways of these genes. GO analysis revealed that these 11 genes were involved primarily in the regulation of protein serine/threonine kinase activity (BP and MF) and the cyclin-dependent protein kinase holoenzyme complex (CC) (Figure 3B). KEGG analysis revealed that the cellular senescence, cell cycle, and FoxO signalling pathways were enriched (Figure 3C–D).

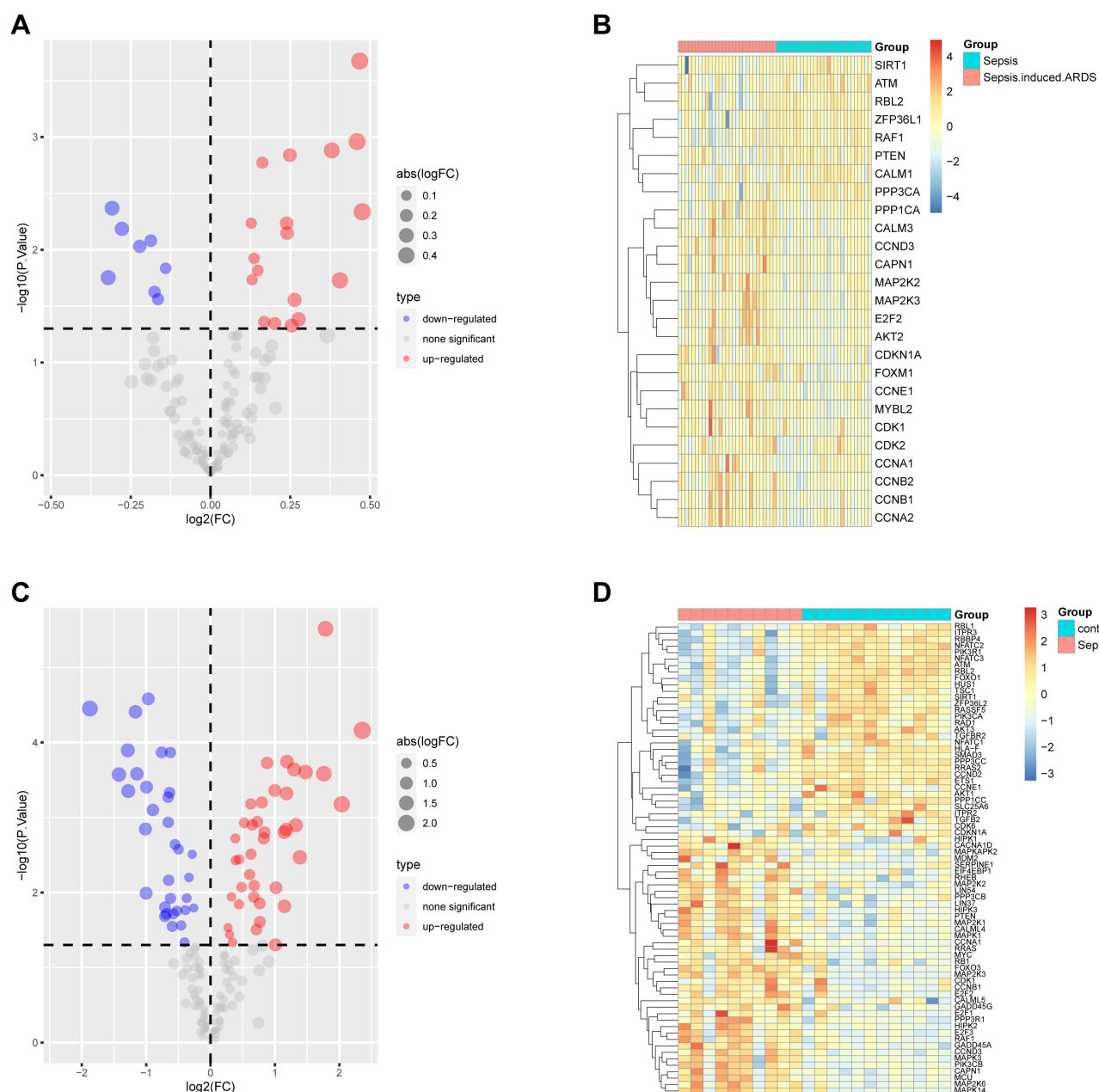


Figure 4 Volcano map and heatmap of cell senescence genes in the GSE66890 (A–B) and GSE145227 (C–D) datasets.

Volcano plots and heatmaps were generated to depict the differences in the expression of cell senescence genes between the GSE66890 (Figure 4A–B) and GSE145227 datasets (Figure 4C–D). The differential expression of the 11 intersecting genes is displayed via data from the GSE66890 (Figure 5A) and GSE145227 datasets (Figure 5B). Eight genes (CCNA1, CCNB1, CDK1, CAPN1, E2F2, MAP2K2, MAP2K3, and CCND3) were upregulated in the sepsis-induced ARDS group, whereas three genes (SIRT1, ATM, and RBL2) were downregulated.

Identification of Hub Genes

Two machine learning algorithms were used to select biomarker genes. Using the LASSO regression algorithm, we screened for four hub genes as diagnostic markers (Figure 6A). Using the SVM-RFE algorithm, we identified 11 hub genes (Figure 6B). Finally, we selected four overlapping features (ATM, CCNB1, CCNA1, and E2F2) between the two algorithms (Figure 6C). These four genes may be involved in the progression of sepsis-induced ARDS.

Predictive Ability of the Hub Genes

Figure 7A shows the log₂-fold change values of the four hub genes in both datasets. The correlations between the hub genes based on the GSE66890 dataset are shown in Figure 7B. Notably, CCNA1 is positively correlated with CCNB1 and E2F2 and negatively correlated with ATM. The AUC value of the ROC curve indicates that E2F2 has the highest predictive ability (AUC = 0.749) for the occurrence of ARDS in patients with sepsis (Figure 7C).

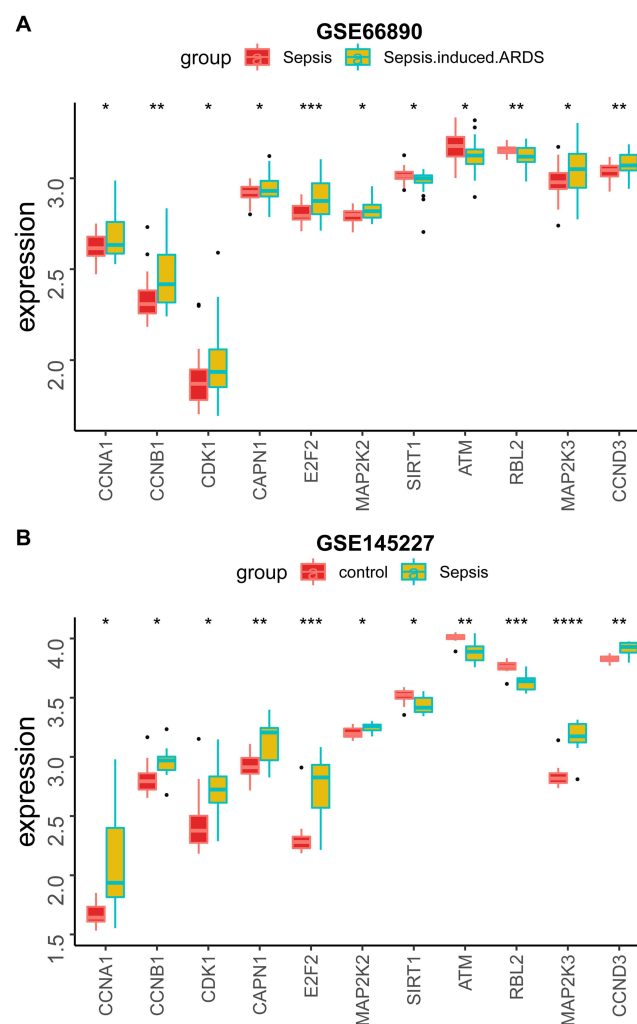


Figure 5 Differential expression of 11 cellular senescence genes in GSE66890 (A) and GSE145227 (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

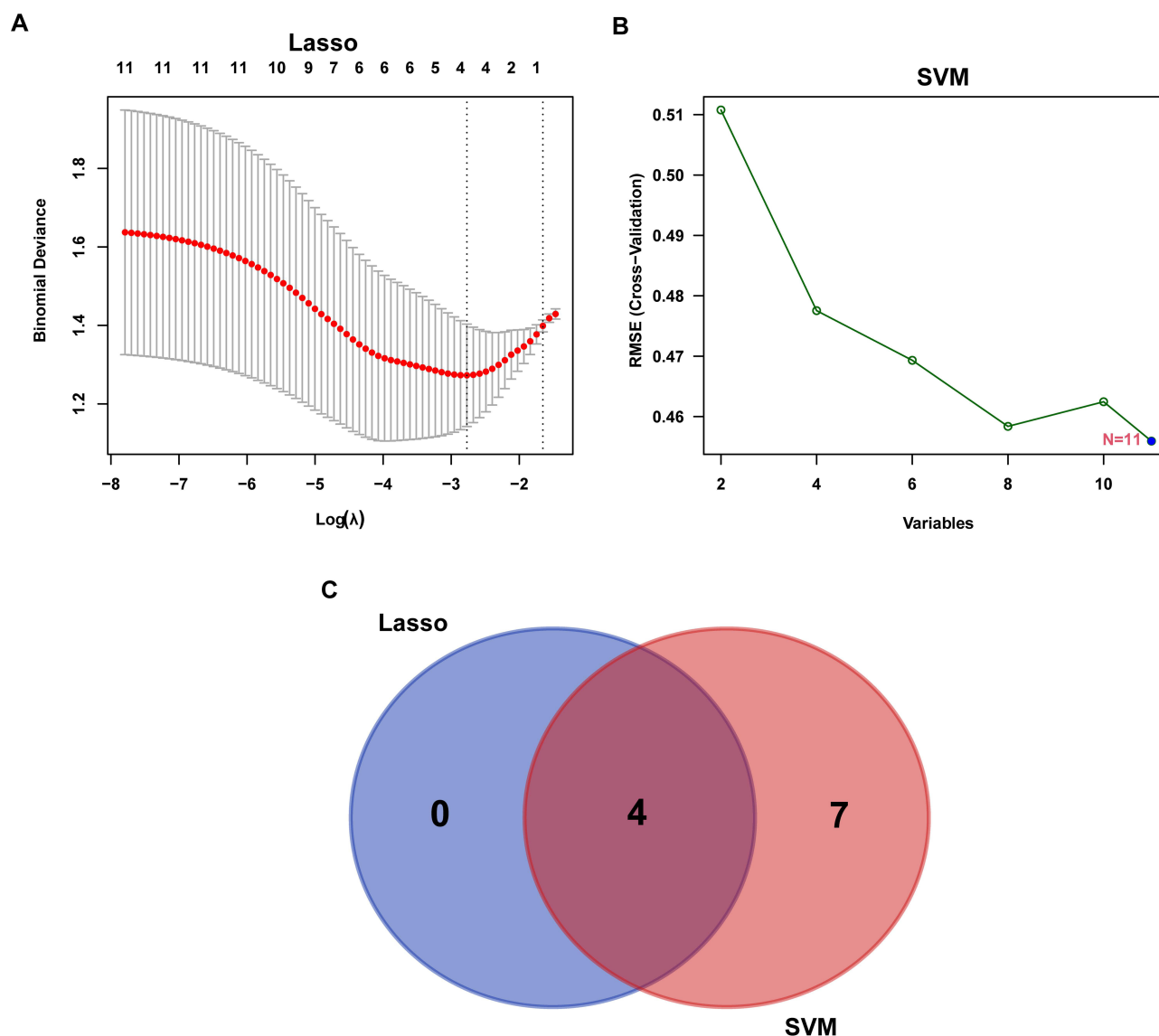


Figure 6 Machine learning algorithms. LASSO (A); SVM-RFE (B); overlapping features between these two algorithms (C).

Immune Infiltration Analysis

Because the immune system plays a crucial role in sepsis-induced ARDS, we assessed the infiltration levels of 23 immune cells in each patient. [Figure 8A](#) shows the relationship between each type of immune cell. We found that CD56bright structural killer cells and plasmacytoid dendritic cells were highly infiltrated in the sepsis-induced ARDS group, whereas eosinophils, MDSCs, macrophages, and neutrophils were poorly infiltrated ([Figure 8B](#)). Immune cell infiltration was correlated with the four hub genes ([Figure 8C](#)).

Exploration of the Hub Genes

Correlation analysis between the hub genes and all transcripts was performed based on data from GSE66890. The expression levels of the 50 genes most positively correlated with each hub gene are shown in [Figure 9](#). GSEA was conducted on the basis of the correlation results. The 20 terms most enriched for the 50 genes correlated with each of the four hub genes are shown in [Figure 10](#). For example, genes positively correlated with CCNA1 were enriched for functions involved in membrane trafficking, neutrophil degranulation, and vesicle-mediated transport terms and depleted for functions related to signal transduction and

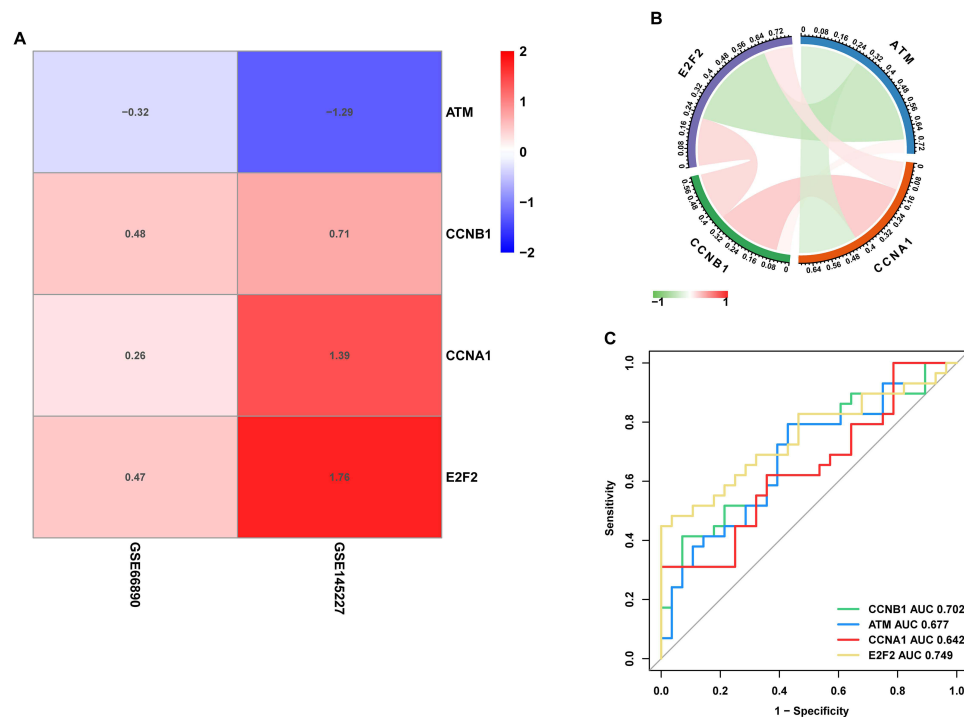


Figure 7 Predictive ability of the four hub genes. The log2(FC) values of 4 genes in GSE66890 and GSE145227 (A). Correlations between the hub genes in GSE66890 (B). The AUC value of the ROC curve for predicting the occurrence of ARDS in sepsis patients (C).

postmitotic nuclear pore complex (NPC) reformation. These results may facilitate in-depth investigations of the involvement of hub genes in sepsis-induced ARDS.

Preliminary Validation Analysis of Hub Genes

We performed RT-PCR analysis of peripheral blood mononuclear cells (PBMCs) from 6 sepsis patients and 6 nonsepsis patients to verify the expression of the four hub genes. The results revealed that only ATM expression significantly differed between the two groups (Figure 11), which was consistent with the results of the bioinformatics analysis. Further exploration of ATM genes in sepsis is therefore important.

Discussion

Owing to the lack of effective risk prediction, accurate genetic diagnosis, and molecular interventions, sepsis-induced acute respiratory distress syndrome adds to the global impact of sepsis, a life-threatening condition. The prognosis of patients with sepsis-induced ARDS is poor due to limited effective prevention and treatments.¹⁷ Because appropriate treatment for sepsis-induced ARDS relies heavily on early prediction, identification, and diagnosis, bioinformatic analysis can provide a useful tool for exploring the underlying mechanisms of sepsis-induced ARDS via large-scale gene expression data.¹⁸ To investigate the transcriptomic consequences of sepsis-induced ARDS, we conducted a bioinformatics analysis of human sepsis-related ARDS samples and sepsis samples. In total, 288 DEGs were upregulated, and 167 DEGs were downregulated. The differentially expressed transcripts were primarily involved in the cell cycle and cellular senescence, suggesting that cellular senescence-related functions and pathways play a vital role in sepsis-induced ARDS.

Pulmonary diseases may benefit from cellular senescence or may have detrimental effects. The senescence process can limit DNA damage during acute injury, preserve senescent cells, and prevent apoptosis. Following the removal of senescent cells, proliferation occurs to regenerate the injured tissue. Senescent cells may prevent recovery from lung injury if cell turnover slows because they decrease the proliferation rate and prolong proinflammatory states. Abundant evidence supports that senescence plays a role in chronic and age-related lung diseases, but less evidence is available for ALI.¹⁰ In recent years,

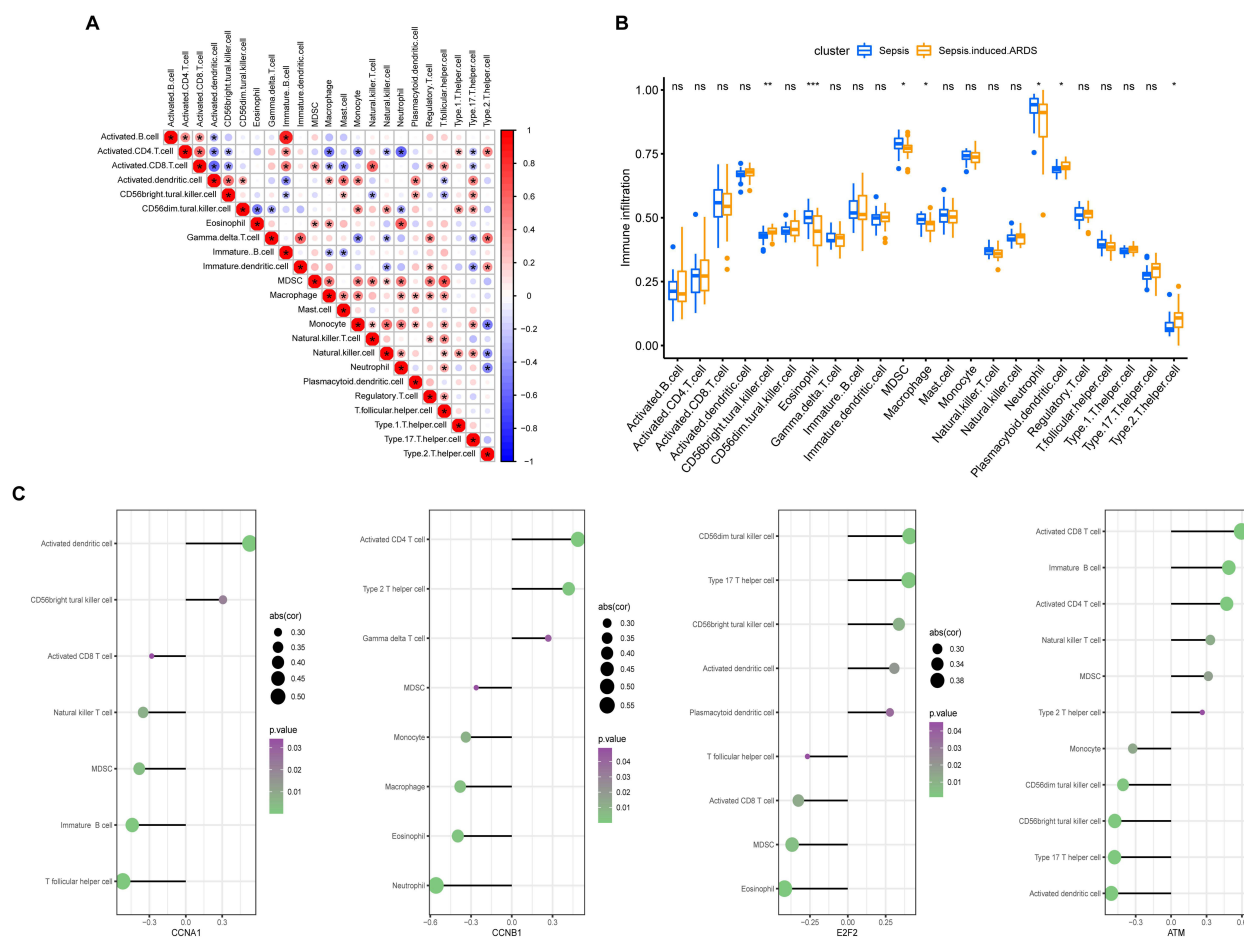


Figure 8 Immune infiltration analysis. Relationships between each immune cell type (A). Infiltrating immune cells in the sepsis group and sepsis-induced ARDS group (B). Correlations of the 4 hub genes with immune cell types (C). *p < 0.05, **p < 0.01 and ***p < 0.001.

animal and cell experiments have demonstrated that cellular senescence plays an important role in sepsis-induced lung injury. In a two-hit mouse model generated via caecal ligation and puncture (CLP) followed by sublethal *Pseudomonas aeruginosa* (PA) lung infection, a relatively high mortality rate was observed because of increased lung inflammation and cellular senescence.¹⁹ LPS can induce cellular senescence in alveolar epithelial cells, suggesting that LPS-induced cellular senescence plays a critical role in limiting tissue repair when sepsis occurs.²⁰ Sepsis-induced ARDS has a multifactorial pathogenesis, among which cellular senescence is believed to play a crucial role.

In the present study, 11 cellular senescence genes were identified. The differential expression of these 11 genes was investigated via data from the GSE66890 and GSE145227 datasets. Eight genes, namely, CCNA1, CCNB1, CDK1, CAPN1, E2F2, MAP2K2, MAP2K3, and CCND3, were upregulated in the sepsis-induced ARDS group, whereas three genes, SIRT1, ATM, and RBL2, were downregulated. KEGG analysis revealed that the functions and pathways of these genes were enriched in the cellular senescence, cell cycle, and FoxO signalling pathways. These findings increase our understanding of sepsis-induced acute respiratory distress syndrome. To effectively diagnose sepsis or sepsis-induced ARDS, we used the LASSO regression algorithm and SVM-RFE algorithm to predict disease outcomes and identified four biomarkers (ATM, CCNB1, CCNA1, and E2F2) across both algorithms. These four genes may be involved in the progression of sepsis-induced ARDS. The AUC value of the ROC curve indicated that E2F2 had the highest predictive ability (AUC 0.749) for the occurrence of ARDS in patients with sepsis.

Targeted E2F2 knockdown causes disproportionate entry into the cell cycle upon T-cell stimulation in mice. This study suggests that E2F2 plays a critical role in regulating apoptosis and may help in the design of new approaches for treating immune-mediated diseases. Both in vitro and in vivo, E2F2 provides genomic stability to activated

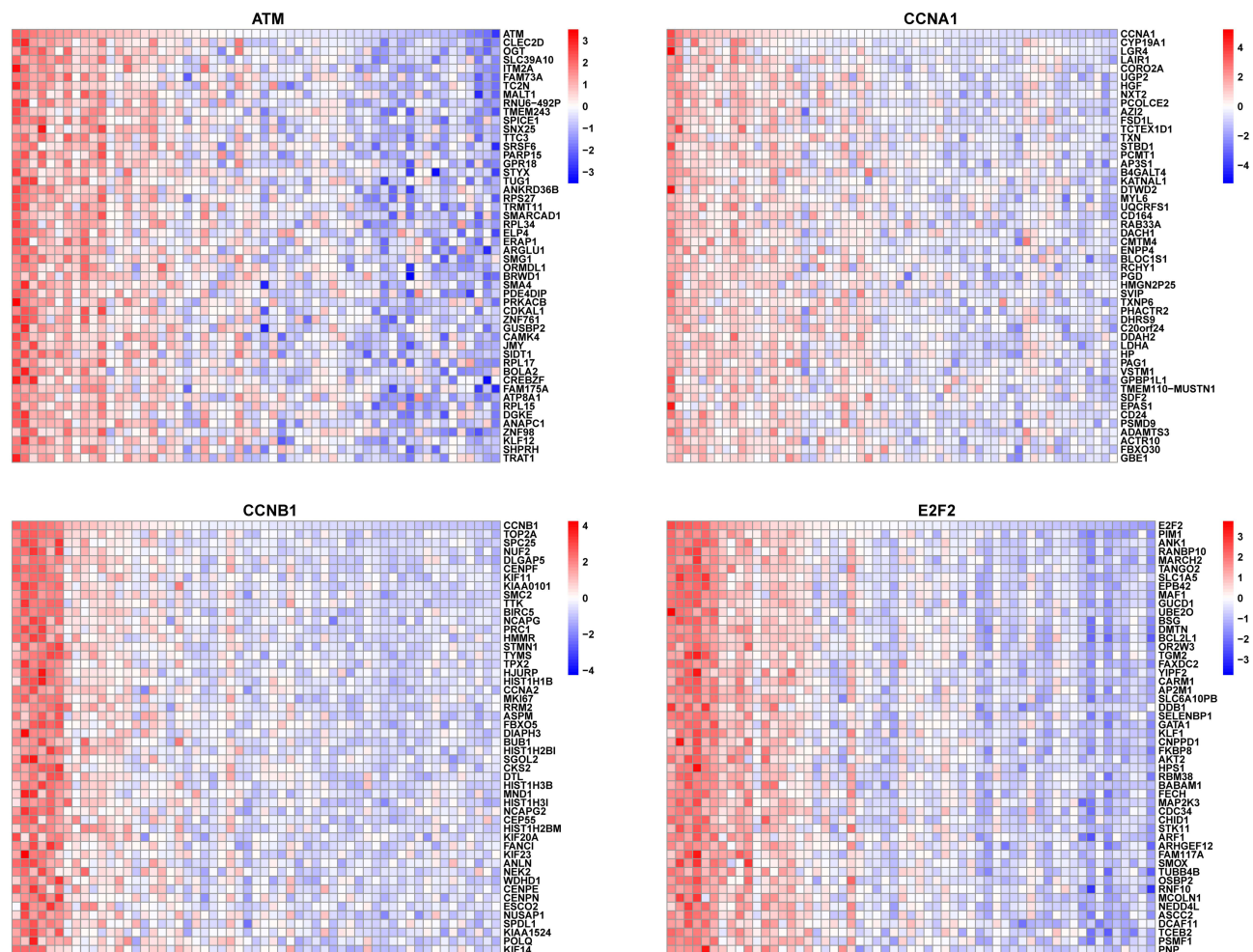


Figure 9 The expression of the top 50 genes positively correlated with each hub gene.

T lymphocytes and plays a specific antiapoptotic role.²¹ In LPS-induced acute lung injury, the processes of resolving lung injury and regulatory T (Treg) cells appear to be important mediators.²² Although E2F2 has not been studied previously, we speculate that E2F2 may be involved in the progression of ARDS by regulating T cells or antiapoptotic properties.

Both CCNA1 and CCNB1 are cell cycle regulatory proteins that are involved in mitosis. A study reported that errors in mitosis can either kill the cell through apoptosis or cause potentially pathogenic mutations.²³ GO functional analysis revealed that DEGs associated with the mitotic cell cycle and cellular senescence, such as CCNA1 and CCNB2, were the most significantly enriched biological processes among the upregulated genes. The abnormal cytoplasmic expression of cyclin B1 and T-cell cells is associated with immune responses specific to cyclin B1.²⁴ A growing body of evidence suggests that the immune system plays a key role in recovery from lung diseases and acute lung injury.²⁵ ARDS is driven by the activation of innate immunity via the binding of cell injury-associated endogenous molecules to pattern recognition receptors.²⁶ However, neither CCNA1 nor CCNB2 are directly implicated in ARDS; therefore, we speculated that CCNA1 and CCNB1 might be closely related to ARDS development.

The ataxia-telangiectasia mutated (ATM) gene causes a rare autosomal recessive disorder that results in a defective repair of double-strand breaks in DNA.²⁷ Ataxia-telangiectasia (A-T) is an autosomal recessive, progressive, multisystem disease caused by mutations in the ATM gene (11q22.3). This gene, which encodes ATM kinase, a kinase involved in cellular stress response signalling, is widely expressed. More than one hundred proteins related to the DNA damage response, cell cycle regulation, and other pathways are correlated with ATM expression. ATM plays several important roles in neuroprotection, innate immunity, inflammation, metabolism (eg, insulin signalling), longevity, and fertility.²⁸

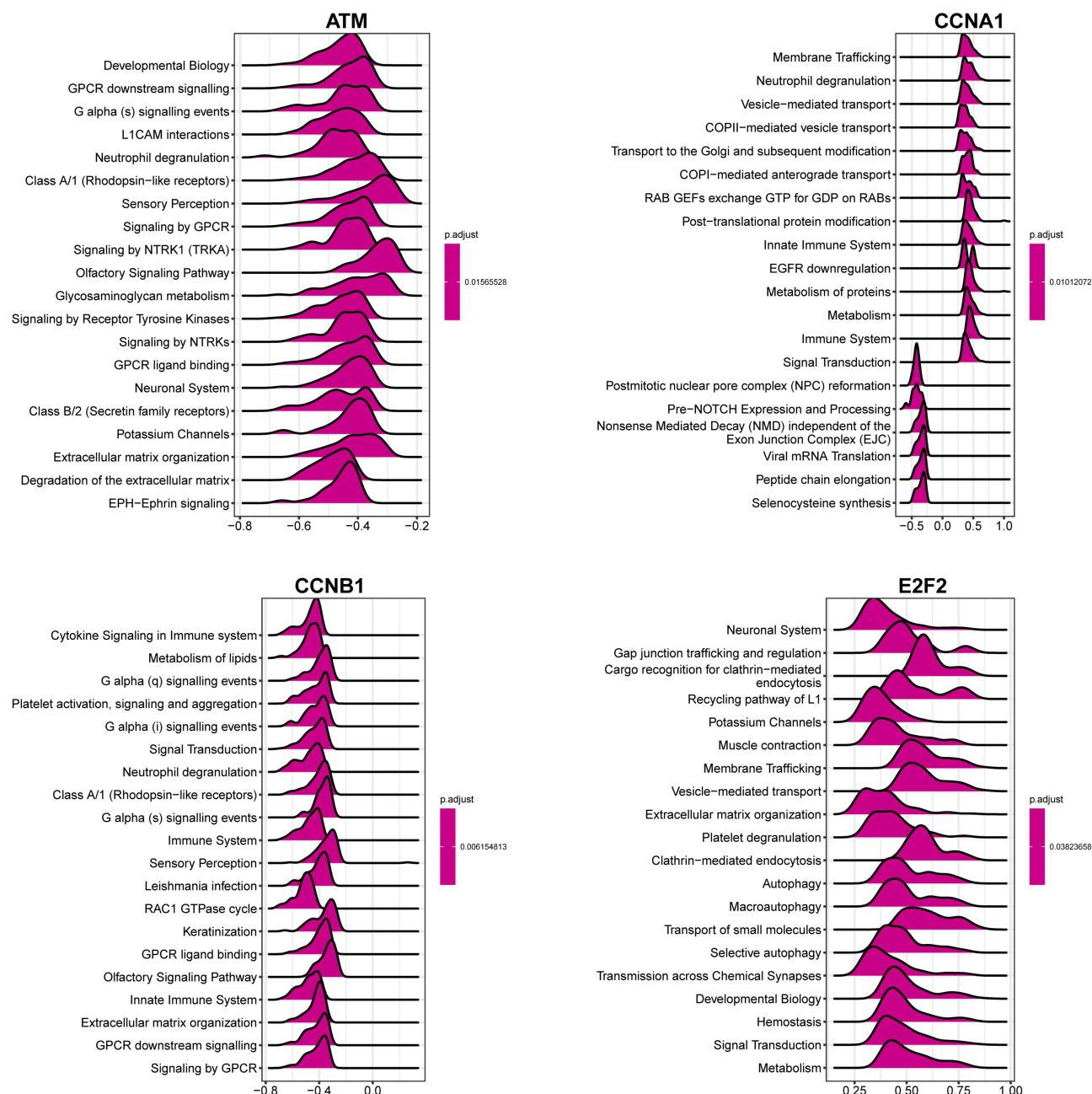


Figure 10 The top 20 terms of the 4 hub genes identified via GSEA.

Several factors can contribute to the development of ataxia-telangiectasia (A-T), including recurrent sinus infections, immunodeficiency, and aspiration due to impaired swallowing caused by neurodegenerative progression.^{27,29} The immune deficiency in A-T is highly variable and affects both B and T cells. Therefore, we speculated that ATM downregulation might be related to sepsis-induced ARDS. In addition, ATM gene expression was significantly decreased in patients with sepsis ($p = 0.0327$).

Overall, our study revealed that the four identified cellular senescence genes exhibited marked differences and significant correlations with each other and with immune cell infiltration during the development of sepsis-induced ARDS in human transcriptomic studies. Recent studies have shown that LPS-induced ALI activates alveolar macrophages rather than causing systemic activation and that aged mice exhibit impaired adaptive immunity.³⁰ Ageing and immune regulation are also inextricably linked. Immune regulation plays a crucial role in sepsis-induced ARDS.^{31,32} Multiple immune cells, including

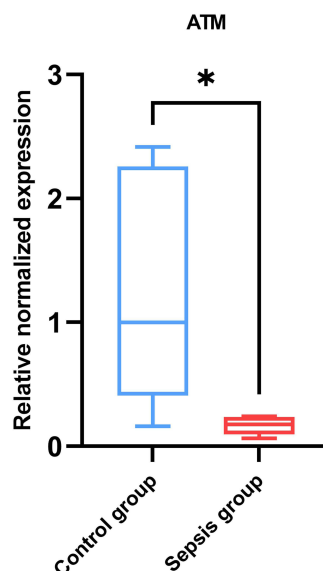


Figure 11 Expression of the ATM gene in sepsis patients and nonsepsis patients. * $p < 0.05$, $P = 0.0327$.

neutrophils and monocytes, are considered closely involved.³³ Therefore, to explore the underlying immune mechanisms, we performed GSEA and immune infiltration assays. According to the GSEA results, the high-risk groups presented dysregulated expression of genes that were significantly enriched in neutrophil degranulation and innate immune pathways. We found that CD56bright structural killer cells and plasmacytoid dendritic cells were highly infiltrated in the sepsis-induced ARDS group, whereas eosinophils, MDSCs, macrophages, and neutrophils were poorly infiltrated.

To further explore the pathogenesis of ARDS, correlation analysis between the four hub genes and all transcripts was performed on the GSE66890 dataset. These analyses revealed that CCNA1 was positively correlated with membrane trafficking, neutrophil degranulation, and vesicle-mediated transport terms and negatively associated with signal transduction and postmitotic nuclear pore complex (NPC) reformation terms. These results may facilitate in-depth investigations of the hub genes involved in sepsis-induced ARDS.

Although this study produced some positive results, it also has several limitations. First, our findings were not verified in cells or tissues; we did not combine expression profiles to confirm our findings, and the sample size was limited. Furthermore, mRNA expression profiles were obtained from blood samples in this study and not from other important cells in ARDS, such as the epithelial and endothelial cells of the lungs. Hence, further studies involving other ARDS cell types with experimental verification and diverse samples are necessary.

Conclusions

In conclusion, the results of the present study revealed that ATM, CCNB1, CCNA1, and E2F2 can predict the progression of ARDS in sepsis patients. This study provides a comprehensive understanding of the genes involved in sepsis-induced ARDS pathogenesis.

Data Sharing Statement

The data used to support the findings of this study are available from the GEO website (<https://www.ncbi.nlm.nih.gov/gds/?term=>) and KEGG database (<https://www.kegg.jp/entry/map04218>), and the code is supplied by the corresponding author upon request. This paper has been uploaded to ResearchGate as a preprint: <https://www.researchsquare.com/article/rs-4476919/v1>.

Ethics Approval and Consent to Participate

Our study adhered to the principles outlined in the Declaration of Helsinki concerning ethical considerations in medical research involving human subjects. All procedures performed in this study involving human participants were conducted in

accordance with the ethical standards of the institutional and/or national research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all participants included in this study. The participants were informed of the nature of the study and its objectives, procedures, potential risks, and benefits. They were assured that their participation was voluntary and that they could withdraw from the study at any time without any consequences. The participants were also informed of the confidentiality of their data and how the data would be used and shared. Written consent was obtained from each participant before inclusion in the study. This study was approved by the Ethics Committee of the Central Hospital of Wuhan (Ethics approval NO: WHZXXYL2022-007).

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Author Contributions

All authors made a significant contribution to the work reported, including the conception, study design, execution, acquisition of data, analysis and interpretation; participated in drafting, revising or critically reviewing the article; gave final approval of the version to be published; agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this study.

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