

Cell Death Index Predicts Sepsis Outcomes and Highlights Necroptosis as a Therapeutic Target

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Background: Excessive immunogenic cell death (ICD) drives early mortality in sepsis, but its dominant subtype and clinical relevance remain poorly defined.

Methods: We systematically screened 3,421 genes involved in 15 forms of cell death across multiple sepsis transcriptomic datasets. Machine learning and COX regression identified two ICD-associated genes (PDZD8 and ADRB2) and were used to build a Cell Death Index (CDI). CDI performance was verified in terms of discrimination, risk stratification and clinical relevance. Gene set scoring was used to assess the association of CDI with necroptosis. Single-cell RNA sequencing was performed to investigate PDZD8 and ADRB2 expression and function. Treatment with the necroptosis inhibitor Necrostatin-1 (Nec-1) was evaluated in a cecal ligation and puncture (CLP) mouse model.

Results: The CDI robustly predicted sepsis prognosis across multiple datasets (AUC: 0.628–0.70). Multivariate analysis identified CDI as an independent risk factor for 28-day mortality (OR = 1.63–2.91, $p < 0.001$). CDI levels were significantly elevated in sepsis patients compared to pneumonia and upper respiratory infection patients. Meanwhile, serum high-mobility group box 1 (HMGB1) was markedly elevated in sepsis cases, whereas caspase-8 (CASP8) remained unchanged, supporting an active necroptotic process. Single-cell RNA sequencing revealed that PDZD8 was highly expressed in neutrophils and associated with oxidative stress and necroptosis, while ADRB2 was enriched in NK cells and linked to suppressed inflammatory signaling. Treatment with Nec-1 in the CLP model significantly improved survival (from 0% to 90%), reduced inflammatory cytokine release, and alleviated organ damage.

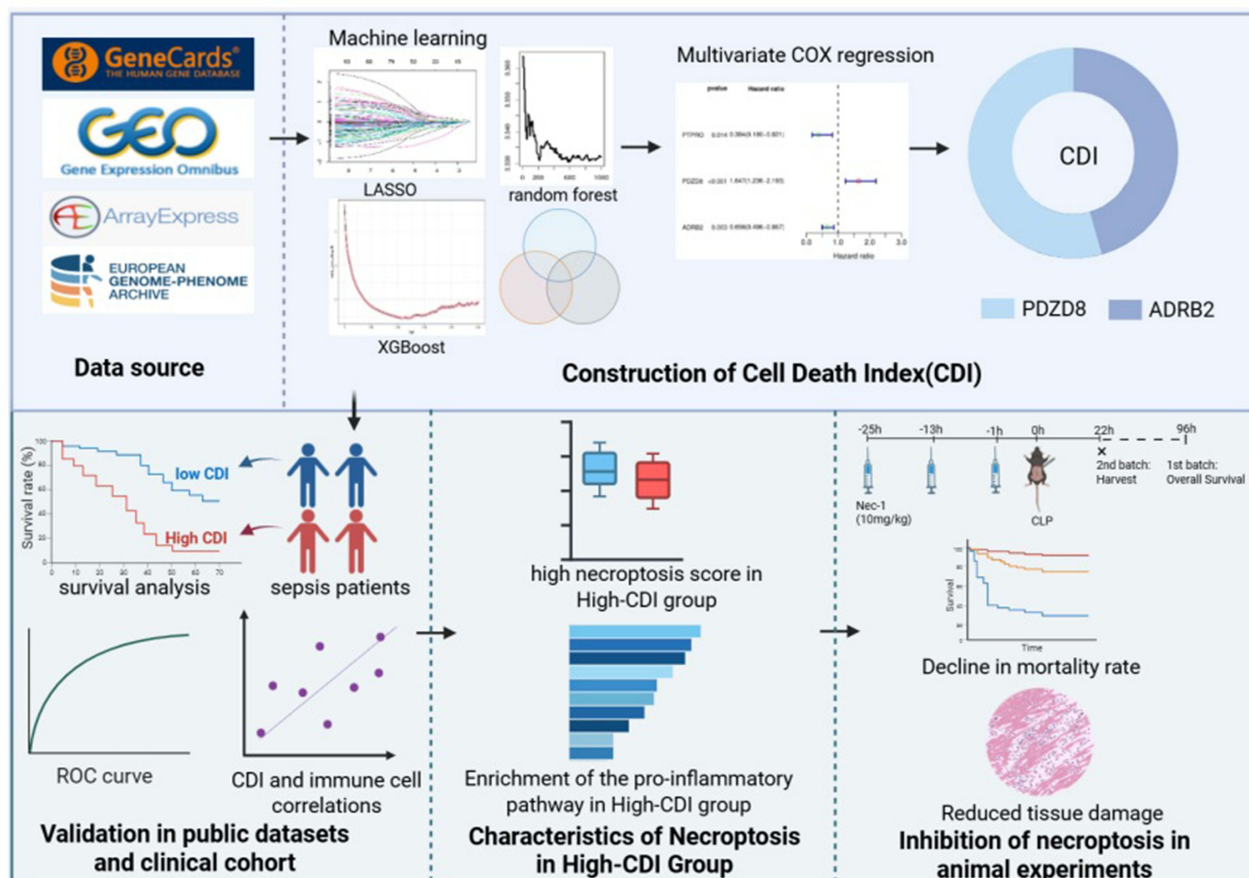
Conclusion: This study identifies necroptosis as the dominant ICD subtype driving immune imbalance in sepsis and establishes CDI as a clinically relevant prognostic biomarker, thereby providing mechanistic insight into sepsis pathogenesis and lay the groundwork for precision therapeutic interventions targeting necroptosis.

Keywords: sepsis, immunogenic cell death, necroptosis, prognosis, cell death index

Introduction

Sepsis is a life-threatening condition characterized by organ dysfunction resulting from a dysregulated host response to infection.¹ It is associated with high morbidity and mortality and remains a significant global public health challenge.² During sepsis, systemic inflammation occurs as different cells release pro-inflammatory mediators like cytokines, proteases, and reactive oxygen species. This response activates the complement and coagulation systems, resulting in tissue inflammation and multi-organ damage.³ Although advances in antibiotics, fluid resuscitation, and intensive life support have improved patient outcomes, deaths due to persistent inflammation and immune dysregulation remain common.^{3,4} Therefore, a deeper understanding of the mechanisms driving the inflammatory response is essential,

Graphical Abstract



particularly the role of cell death in immune imbalance. Moreover, identifying early-warning biomarkers is crucial for effective risk stratification and developing individualized intervention strategies in sepsis.

During sepsis, various cell death pathways are activated either by the septic inflammatory response itself or through direct pathogen interactions.⁵ Immunogenic cell death (ICD) is a highly inflammatory, regulated cell death that stimulates innate immunity, characterized by the release of DAMPs such as ATP, HMGB1, and calreticulin. ICD also promotes antigen presentation, bridging innate and adaptive immune responses.⁶ Recent studies have demonstrated that necroptosis, pyroptosis, and ferroptosis, three forms of regulated cell death that trigger damage-associated molecular pattern (DAMP) release, constitute key subroutines of ICD.⁷ These processes commonly induce the release of DAMPs, activate type I interferon responses, and generate pathogen-associated chemokines that enhance the immunogenicity of dying cells. The ICD cascade in sepsis can be initiated by exogenous pathogen-associated molecular patterns (PAMPs), including LPS and dsRNA. This leads to immunogenic stress and cell death, contributing to tissue inflammation and organ injury.^{8,9}

By serving as a crucial immune-regulating mechanism, ICD has been widely studied in tumor immunotherapy, guiding both therapeutic and biomarker development. Various antitumor strategies, including chemotherapy, radiotherapy, oncolytic viruses, and photodynamic therapy (PDT), can induce ICD to varying extents.¹⁰ By inducing ICD, these therapies potentiate immune cell activity and tumor surveillance, thus countering immune escape.¹¹ In contrast, relatively little attention has been paid to ICD-mediated hyperinflammatory responses in the context of sepsis. While several studies have investigated biomarkers related to cell death in sepsis,^{12–14} the specific contribution of ICD has

largely been overlooked. Notably, sepsis provides a favorable environment for ICD, the excessive inflammation from which may critically worsen patient outcomes. Therefore, ICD and its key subroutines may hold promise as clinically relevant biomarkers for prognostic stratification in sepsis. Current sepsis immunotherapies primarily target the subsequent immunosuppressive phase, overlooking the initial hyperinflammatory state driven by pro-inflammatory cell death.¹⁵ These strategies often fail to consider that DAMPs released during ICD are central to sustained immune activation.

Previous studies using transcriptomics for sepsis subtyping have aimed to advance precision therapies. The Molecular Diagnosis and Risk Stratification of Sepsis (MARS) endotype system categorizes sepsis patients into four subgroups based on 140 differentially expressed genes. Notably, this classification system identifies BPGM and TAP2 as key biomarkers for rapid identification of Mars1 patients.¹⁶ Clinical observations reveal that the Mars1 endotype demonstrates the most unfavorable prognosis, exhibiting characteristic features of immunoparalysis. In parallel research, the Sepsis Response Signatures (SRS) classification system dichotomizes septic shock patients into SRS1 (immunosuppressive subtype) and SRS2 (immunoactivated subtype) through evaluation of seven critical biomarkers. Intriguingly, cohort studies demonstrate paradoxical therapeutic outcomes where SRS2 patients administered corticosteroid therapy exhibit significantly higher mortality rates compared to placebo controls.¹⁷ These sepsis classifications focus on immunosuppression-linked outcomes but neglect the mechanisms of early hyperinflammatory death.

This study utilized machine learning to develop a Cell Death Index (CDI) from two immunogenic cell death (ICD)-related genes, ADRB2 and PDZD8, demonstrating its superior ability to predict sepsis prognosis. We further validated the clinical relevance of CDI and revealed a strong association between CDI and necroptosis. Importantly, *in vivo* experiments showed that necroptosis inhibition significantly improved survival in a sepsis model, highlighting CDI as a promising tool for personalized therapeutic strategies in sepsis.

Materials and Methods

Data Acquisition

Cell death-related genes ($n = 3421$) were collected from the GeneCards database (<https://www.genecards.org>), including apoptosis, autophagy, lysosome-dependent cell death, necrosis, netotic cell death, oxeiptosis, panoptosis, parthanatos, entotic cell death, extrinsic cell death, ferroptosis, necroptosis, pyroptosis, anoikis, immunogenic cell death.

The dataset GSE65682 (platform file GPL16791) was downloaded from the GEO public database (<http://www.ncbi.nlm.nih.gov/geo>), containing whole blood gene expression data of 40 healthy subjects and 479 septic patients with prognostic information. The datasets E-MTAB-4421 and E-MTAB-7581 were downloaded from Array Express (<https://www.ebi.ac.uk/biostudies/arrayexpress>), containing 265 and 176 septic patients with prognostic information, respectively. Single-cell transcriptome dataset Kwok-2023 (EGAS00001006283) was downloaded from the European Genome-phenome Archive (EGA) (<https://ega-archive.org>), containing six healthy volunteers, 26 septic patients, and 9 septic controls. GSE167363 was also down from the GEO public database, containing PBMC from 2 Healthy volunteers, 3 sepsis survivors, and 2 non-survivors.

Data Processing

Batch Transcriptome Data

Preprocessing of transcriptome data includes the following steps: After removing duplicate values, filtering out low-expression genes, and deleting missing values, we conducted gene re-annotation of the dataset probes through platform files. All data were log-transformed and normalized. Surrogate variable analysis (SVA) was used to merge transcriptomic datasets and correct for batch effects.

Single-Cell RNA-Seq Data

Quality control criteria for single-cell data included retention of cells with `nFeature_RNA` in the range of 100 to 4000 and mitochondrial gene expression below 10%. Cell cluster annotation standards were performed according to the uploader's

instructions. The R package “Seurat” was used to process 10× scRNA data, including data filtering, normalization, PCA, uniform manifold approximation, and projection (UMAP) analysis.

Identification of Prognostic Genes via Machine Learning

Univariate Cox regression analysis with overall survival as the endpoint was performed in the GSE65682 and E-MTAB-4421 cohorts ($P < 0.01$), identifying programmatic death-related genes that were significantly enriched in both datasets as candidate genes (Table S1). Three machine learning methods were used to screen candidate genes in GSE65682.

LASSO-Cox regression analysis determines the penalty coefficient λ value via 10-fold cross-validation, retaining genes with non-zero coefficients.

The Random Forest model was trained with 10-fold cross-validation, and the top 15 genes with the highest relative importance were selected.

The XGBoost algorithm selected the top 20 most contributory feature variables via 500 iterations and 5-fold cross-validation.

Finally, co-identified genes by machine learning were taken to do multivariate COX regression analysis. Genes with a P value of less than 0.01 were identified as target genes.

Predictive Capability Validation

ROC curves were analyzed for each CDI and the area under the curve (AUC) with 95% confidence intervals (CIs) was calculated. The significance of the CDIs was judged by the AUC value, with values closer to 1 indicating greater predictive accuracy.

The CDI was calculated using the multivariate Cox regression coefficients and gene expression levels as follows: $CDI = 0.4987 \times (PDZD8 \text{ expression}) - 0.4216 \times (ADRB2 \text{ expression})$, and the detailed model coefficients were presented in Figure 1i. Patients were categorized into high and low CDI groups based on the median value of CDI. Survival curves were created, and the Log rank test was used to compare significant differences in survival data between the two groups.

Transcriptome Functional and Enrichment Analysis

Following the identification of differentially expressed genes (DEGs) among distinct CDI subtypes, Gene Ontology (GO) enrichment analysis was performed. Pathways with corrected P-values and false discovery rates (FDR) below 0.05 were deemed statistically significant. Transcriptome scoring was conducted using the “ssgsea” method. The immune cell composition of high- and low-CDI groups was assessed via the “CIBERSORT” algorithm.

Single-Cell Sequencing Analysis

Single-cell clusters were manually annotated using classical immune cell marker genes. Annotation accuracy was validated by visualizing marker gene expression in dot plots. The CDI for individual cells was calculated with the “AddModuleScore” function and visualized on UMAP projections and violin plots. Gene set scores were derived using the same method. Heatmaps and violin plots were used to display the expression of key genes.

Based on the median CDI value and the median expression of key genes, cells from septic patients were stratified into high and low CDI groups. Functional enrichment was assessed through Gene Ontology (GO) analysis. Gene Set Enrichment Analysis (GSEA) was performed to evaluate different modes of cell death.

Clinical Sample Collection

The cohort included 8 sepsis patients, 11 pneumonia patients (community-acquired pneumonia), and 6 patients with acute upper respiratory tract infections at Xiamen University Xiang'an Hospital from January 3, 2024, to November 27, 2024. All included sepsis patients met the diagnostic criteria of the Sepsis 3.0 Consensus. Among them, 5 were diagnosed with sepsis and 3 with septic shock. Their ages ranged from 55 to 92 years old, comprising 4 females and 4 males. Four patients improved, while four died. All included sepsis patients met the diagnostic criteria of the Sepsis 3.0 consensus. Exclusion criteria included age <18 years old, hospitalization <72h, hematologic malignant diseases, autoimmune diseases, chronic infectious diseases, and immunosuppressive therapy within the past two weeks. Blood samples were

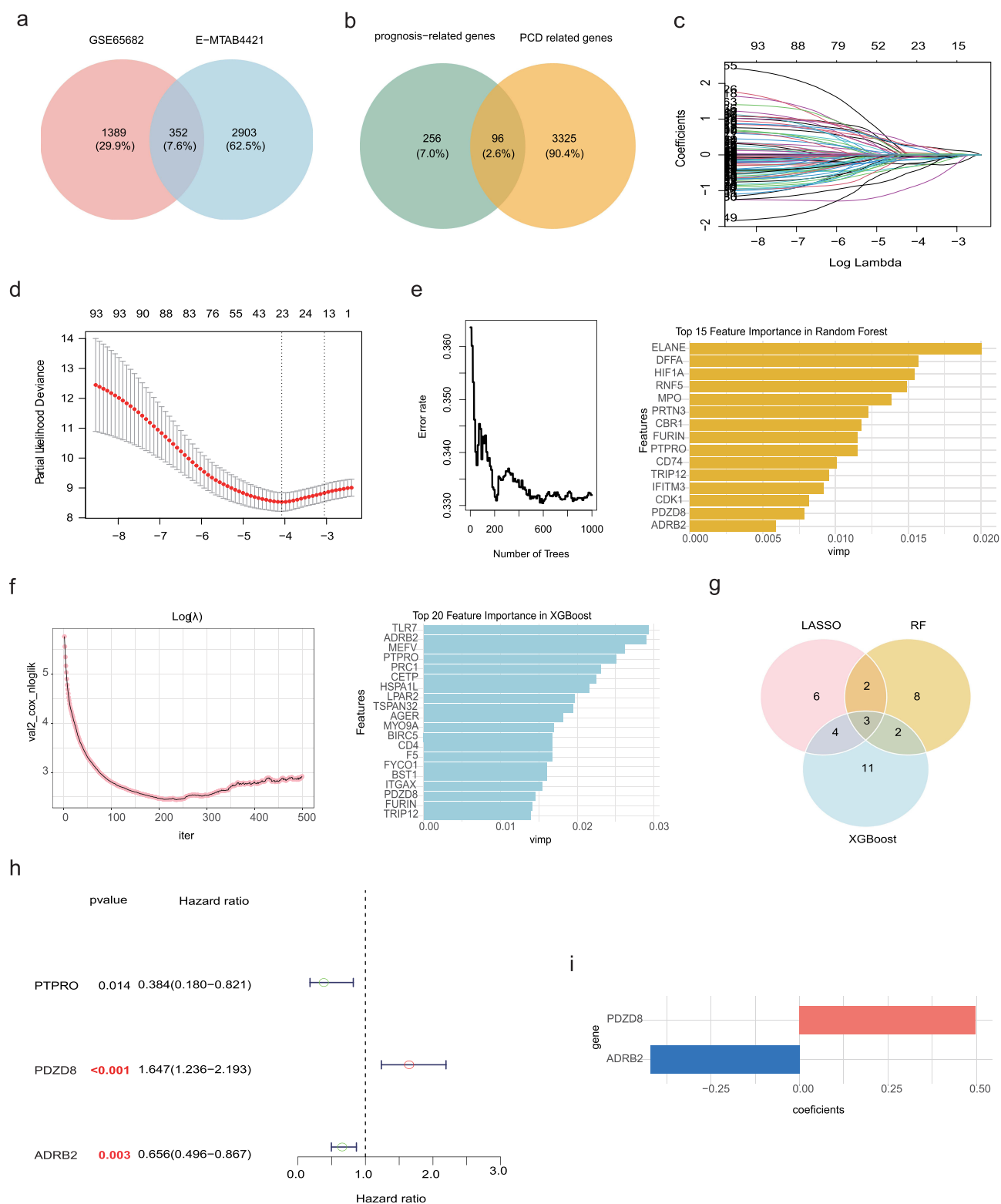


Figure 1 Construction of the cell death index (CDI). **(a)** Prognostic genes common to the GSE65682 and the E-MTAB-4421 dataset. **(b)** Venn diagram of the PCD genes and prognostic genes. **(c and d)** LASSO regression was conducted to identify signature genes between sepsis survivors and non-survivors. **(e)** Random forest identified the top 15 genes in terms of relative importance to sepsis prognosis. **(f)** XGBoost identifies the top 20 genes in terms of relative significance to sepsis prognosis. **(g)** Venn diagram showed the intersection of genes obtained by three algorithms. **(h)** Multifactorial COX regression identified independent prognostic genes ($P < 0.01$). Genes with P-values less than 0.01 were highlighted in red. **(i)** The coefficients of the 2 key genes to calculate CDI.

EDTA-anticoagulated and stored at -80°C until analysis. Basic clinical information of patients in this study, including CDI were shown in [Table S2](#).

The study was approved by the Ethics Committee of the School of Medicine, Xiamen University (ethics number: XDYX202302K06), and the study itself was conducted in accordance with the Declaration of Helsinki and followed RECORD guidelines. All participants or their guardians provided written informed consent before sample collection.

Cecal Ligation and Puncture (CLP) Mouse Model

Specific pathogen-free (SPF) grade male C57BL/6 mice (6–8 weeks old, approximately 20 g) were purchased from the Xiamen University Laboratory Animal Center, Xiamen, China. All mice were housed in a specific SPF environment (temperature: $22 \pm 2^{\circ}\text{C}$, humidity: $45 \pm 5\%$, 12 h light/dark cycle) and provided unrestricted food and water. The animal experiment followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, and all the experimental protocols were approved by the Ethics Committee of Laboratory Animals of Xiamen University (Permit number: XMULAC20210021).

Mice were randomly assigned to a sham operation group, a CLP group, and a Nec-1 group. Mice were anesthetized with tribromoethanol (250 mg/kg) (MACKLIN 75–80-9, Shanghai, China) by intraperitoneal injection. Then the CLP surgery was performed according to standard methods to establish a sepsis model.¹⁸ Necrostatin-1 (TOPSCIENCE, 64419–92-7, Shanghai, China) was administered via intraperitoneal injection at 25, 13, and 1 hours before surgery. Administer ibuprofen (5 mg/kg) subcutaneously 30 minutes prior to surgery as an analgesic, followed by continuous administration for 3 days postoperatively.

Survival was monitored for 96 hours postoperatively. Mice were euthanized when they met the criteria for humane endpoints, including labored breathing with gasps, a core body temperature below 30°C , or severe lethargy (defined as unresponsiveness to gentle stimuli).¹⁹ Tissue samples were collected at 22 hours for histological analysis via hematoxylin and eosin (HE) staining.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

The RT-PCR experiment validated the PDZD8 and ADRB2 expression levels. Peripheral blood samples were collected using anticoagulant tubes and RNA was extracted from WBCs using the Trizol method, and cDNA was synthesized from 500ng of total RNA using the Accurate Biotechnology Reverse Transcription Kit (Accurate Biotechnology AG11701). Each cDNA sample was diluted 10-fold and a 5 μL aliquot was used in a 12 μL PCR reaction mixture, and qPCR reactions were performed using the PCR System Kit 2 $\times$ SYBR Green qPCR Master Mix (Aikerui Bioengineering, SYBR Green Premix Pro Taq HS qPCR Kit, Changsha, China). The relative expression levels were calculated using the $2^{-\Delta\Delta\text{CT}}$ method. The sequence of the primers and qRT-PCR results are shown in [Table S3-5](#)

Enzyme-Linked Immunosorbent Assay (ELISA)

According to the manufacturer's protocols, concentrations of HMGB1 (Elabscience, E-EL-H1554, Wuhan, China), CASP8 (Elabscience, E-EL-H0659, Wuhan, China), IL-6 (Solarbio, SEKM-0034, Beijing China), and TNF- α (ABclonal, RK04595, Wuhan, China) were measured using ELISA kits with standard curves ([Table S6](#) and [7](#)). Absorbance was measured at 450 nm using a Thermo Scientific Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and cytokine concentrations were calculated using a four-parameter logistic (4-PL) fitting curve.

Statistical Analysis

Statistical analyses and visualization of datasets were achieved via R software (Version 4.3.2), R packages used were listed in [Table S8](#). Data results are expressed as the mean $\pm$ standard error of the mean (SEM) in vivo validation of the clinical samples and animal experimentation of the key genes, including at least three independent experiments with replicated samples. For two-group comparisons, the Student's *t* test was applied for normal distribution, and the Wilcoxon test was applied for normal distribution with GraphPad Prism software (Version 8.0). The chi-square test was used for categorical variable data between the two groups. The level of statistical significance was set at $p < 0.05$.

Results

Construction of a Cell Death Index for Prognostic Prediction in Sepsis

We utilized a sepsis transcriptomic dataset GSE65682 from 479 patients with complete prognostic data as the derivation cohort. To identify genes significantly associated with sepsis outcomes, we first performed univariate Cox proportional hazards regression analysis, revealing 1,389 genes with prognostic significance ($HR > 1$ or $HR < 1$, $P < 0.05$). To enhance robustness, we cross-validated the results using the independent E-MTAB-4421 dataset, identifying 352 overlapping prognostic genes (Figure 1a and Table S1). Among these, 96 genes were identified as being related to programmed cell death (PCD) and remained significantly associated with OS ($P < 0.05$) (Figure 1b).

To further refine the candidate genes, we applied three machine learning algorithms to the GSE65682 derivation set, including Least Absolute Shrinkage and Selection Operator (LASSO) regression, Random Forest, and Extreme Gradient Boosting (XGBoost). For LASSO-Cox regression, the optimal penalty parameter λ was selected via 10-fold cross-validation, resulting in a final model retaining 15 survival-associated genes (Figure 1c and d). The Random Forest model ranked genes ranked by feature importance, from which the top 15 genes were selected (Figure 1e). XGBoost identified the top 20 genes contributing most significantly to sepsis prognosis (Figure 1f). The three genes, PTPRO, ADRB2, and PDZD8, were consistently selected across all three algorithms (Figure 1g). To verify their independent prognostic value, we performed a multivariate Cox regression analysis. Clinical prediction models commonly use $P < 0.01$ for variable screening.²⁰ To prioritize high-confidence variables and construct parsimonious models, we set $P < 0.01$ as an inclusion criterion. Of the three, only PDZD8 and ADRB2 remained statistically significant ($P < 0.01$) and were subsequently used to construct the Cell Death Index (CDI) for prognostic prediction in sepsis (Figure 1h).

CDI Serves as a Prognostic Marker for Sepsis

The derivation cohort GSE65682 ($n = 479$) was used for internal validation of the Cell Death Index (CDI). Receiver operating characteristic (ROC) curve analysis demonstrated that CDI exhibited significant discriminatory power for mortality prediction, with area under the curve (AUC) values of 0.65, 0.68, and 0.70 at days 7, 14, and 28, respectively (Figure 2a). Patients were stratified into high-CDI and low-CDI groups based on the median CDI value. Mortality was significantly higher in the high-CDI group compared to the low-CDI group ($\chi^2 = 11.611$, $p < 0.001$) (Figure 2b). Consistent with the index construction, PDZD8 expression was elevated in the high-CDI group, while ADRB2 expression was higher in the low-CDI group (Figure 2c). Kaplan–Meier survival analysis confirmed the prognostic value of the CDI, showing significantly longer overall survival in the low-CDI group compared to the high-CDI group ($p < 0.05$) (Figure 2d). At the gene level, low PDZD8 expression and high ADRB2 expression were both associated with improved survival (Figure 2e and f). Both univariate and multivariate logistic regression analyses identified high CDI as an independent risk factor for 28-day mortality, after adjusting for age, sex, and MARS endotype (Univariate: OR = 1.82 (1.19–2.80), $p = 0.006$; Multivariate: OR = 1.63 (1.05–2.54), $p = 0.03$) (Figure 2g and h). Furthermore, decision Curve Analysis (DCA) showed that CDI provided a greater net clinical benefit than the MARS endotype for predicting 28-day mortality (Figure 2i), highlighting the superior prognostic utility of CDI.

The sepsis datasets E-MTAB-4421 ($n = 265$) and E-MTAB-7851 ($n = 176$) were further utilized to assess the predictive performance of CDI. ROC curve analysis demonstrated that CDI achieved AUC values of 0.655 and 0.628 for predicting 28-day mortality in the respective cohorts (Figure 3a and d). These values were significantly higher than those of the SRS endotypes (AUC = 0.542 and 0.466, respectively), confirming CDI's superior predictive performance ($p < 0.05$, CDI vs. SRS) (Figure 3a and d). Compared to the low CDI group, more decrease cases were observed in the high CDI group (chi-squared = 11.601, $p < 0.001$; chi-squared = 1.8331, $p = 0.176$) (Figure 3b and e). Expression patterns of PDZD8 and ADRB2 were consistent with those observed in the derivation cohort, further supporting the stability of the index (Figure 3c and f). Multivariate logistic regression confirmed high CDI as an independent risk factor for 28-day mortality after adjusting for age, sex, and SRS endotype (OR = 2.91 (1.55–5.46), $p < 0.001$) (Figure 3i). Moreover, DCA demonstrated a greater net clinical benefit of CDI compared to SRS endotypes in both validation cohorts (Figure 3g and h), reinforcing its potential clinical value for risk stratification in sepsis.

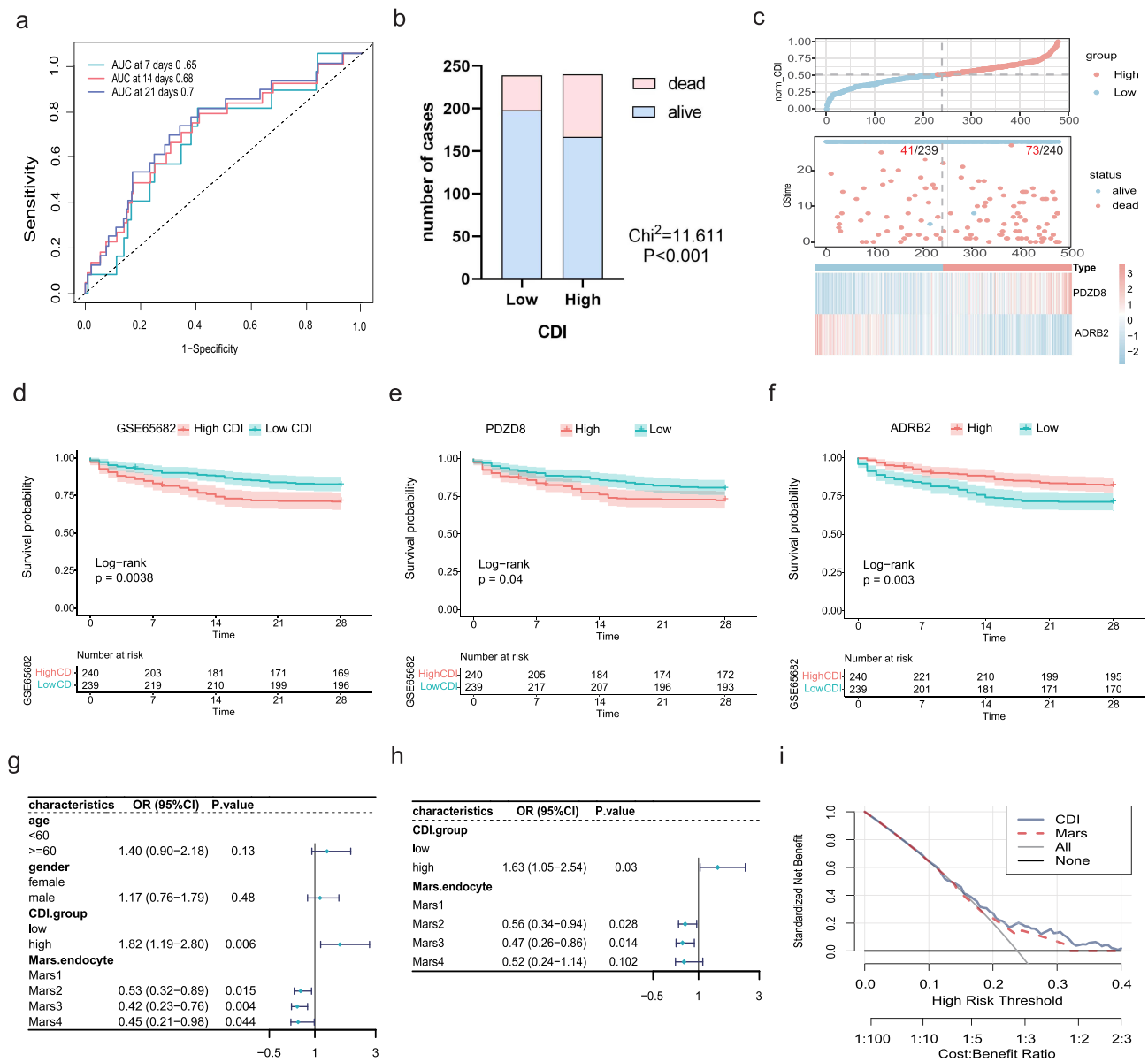


Figure 2 Internal validation of CDI in GSE65682. **(a)** ROC curve for CDI. **(b)** The survival of patients in low- and high-CDI groups, divided by the median value of CDI. **(c)** The survival status chart of Low-CDI and High-CDI subgroups. **(d)** Kaplan–Meier curves of OS of patients in the Low-CDI and High-CDI subgroups. **(e and f)** Kaplan–Meier curves of OS of patients in PDZD8/ADRB2 high- and low- expression groups. **(g and h)** Univariate and Multivariate logistic regression analysis performed. **(i)** Comparison of clinical utility of CDI and Mars endotypes by decision curve analysis (DCA).

To validate CDI in a clinical setting, mRNA expression levels of PDZD8 and ADRB2 were assessed in patient samples. Peripheral blood white blood cells (WBCs) were collected from 25 individuals, including 6 with acute upper respiratory tract infections (URTIs), 11 with pneumonia, and 8 with sepsis. As expected, ADRB2 expression was significantly lower in sepsis patients compared to both pneumonia and URTI patients (Figure 3j). In contrast, PDZD8 expression was significantly higher in sepsis patients than in URTI patients, although not significantly different from pneumonia patients. CDI levels were also significantly elevated in sepsis patients compared to non-sepsis patients (Figure 3k). Correlation analyses revealed that CDI was significantly positively correlated with neutrophil percentages of peripheral blood, and negatively correlated with lymphocyte percentages ($p < 0.05$) (Figure 3m and Figure S1). ADRB2 expression was significantly positively correlated with lymphocyte percentages ($R = 0.57$, $p < 0.05$) (Figure 3l) and negatively correlated with neutrophil percentages ($R = -0.64$, $p < 0.05$) (Figure 3n). Conversely, PDZD8 expression showed a positive correlation with neutrophil percentages ($R = 0.46$, $p < 0.05$) (Figure 3o), and a negative correlation

with lymphocyte percentages ($R = -0.50$, $p < 0.05$) (Figure 3p). Collectively, these findings indicated that CDI is significantly associated with immune cell composition and serves as a reliable prognostic biomarker for mortality in sepsis.

Transcriptome Explores the Association of CDI with Necroptosis

The two genes, ADRB2 and PDZD8, are both implicated in immunogenic cell death (ICD). ADRB2 (adrenoceptor beta 2) participates in antigen presentation and has been shown to convert immunogenic dendritic cells (DCs) into tolerogenic DCs.²¹ PDZD8 (PDZ domain-containing protein 8) functions as a tether between the endoplasmic reticulum (ER) and mitochondria, facilitating ER-mitochondria contact sites that coordinate DAMP sensing and promote inflammation.²² ICD encompasses subroutines such as necroptosis, pyroptosis, and ferroptosis. Thus, we further investigated the relationship between CDI and these cell death pathways.

Representative gene sets for each ICD pathway were obtained from the Gene Set Enrichment Analysis (GSEA) database, and the corresponding pathway scores were calculated using single-sample gene set enrichment analysis (ssGSEA) via the Gene Set Variation Analysis (GSVA) package. In the E-MTAB-4421 cohort, necroptosis and

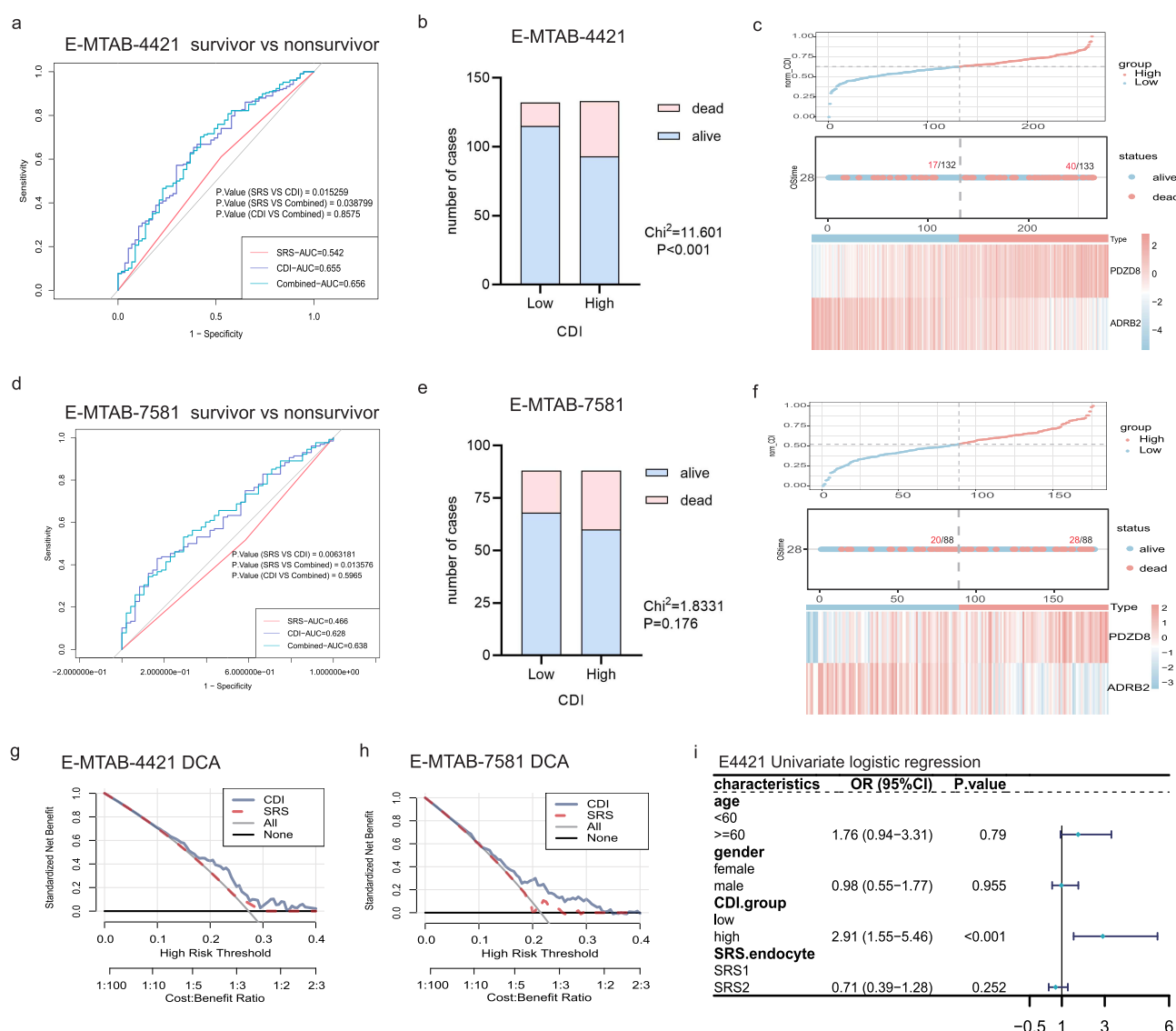


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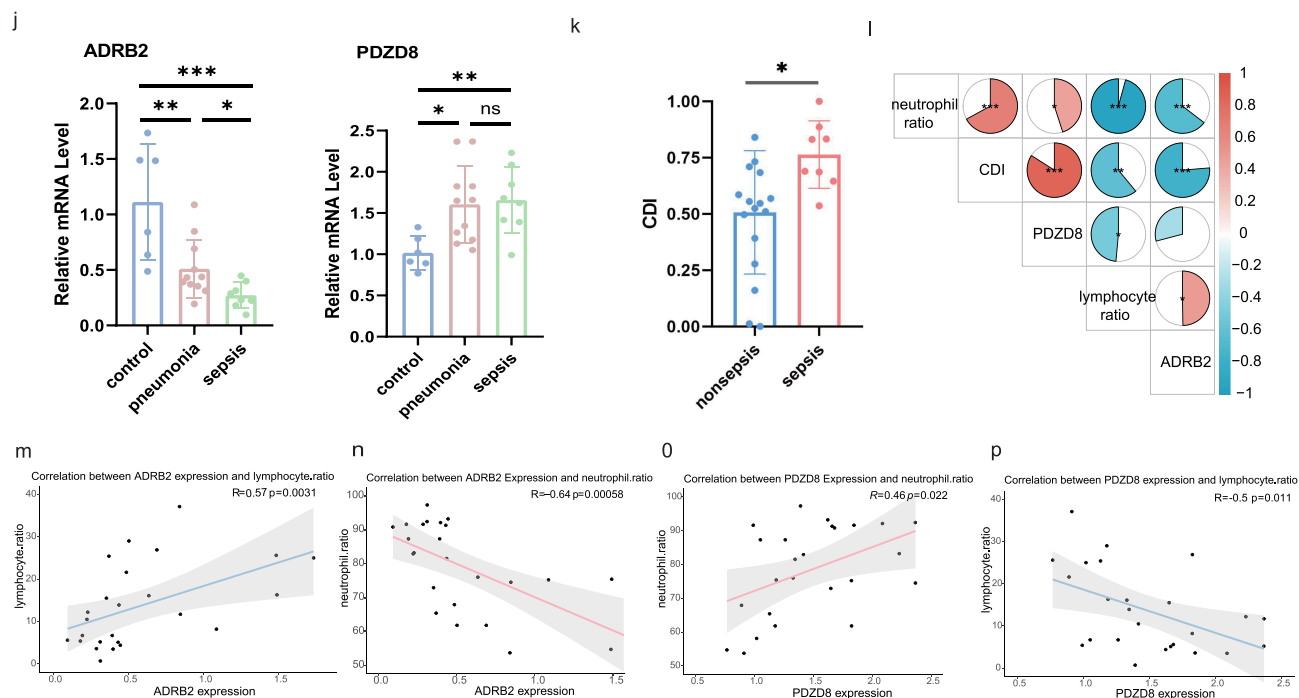


Figure 3 External validation of CDI. (a) Prognostic accuracy in comparing CDI with SRS endpoints by ROC curves in the E-MTAB-4421 cohort. (b) The survival of patients in Low- and High-CDI groups in the E-MTAB-4421 cohort. (c) CDI Distribution Map and Expression Heatmap in the E-4221 Dataset. (d) Prognostic accuracy in comparing CDI with SRS endpoints by ROC curves in the E-MTAB-7581 cohort. (e) The survival status chart of Low-CDI and High-CDI subgroups in the E-MTAB-7581 cohort. (f) CDI Distribution Map and Expression Heatmap in the E-7581 Dataset. (g and h) Comparison of clinical utility of CDI and SRS endpoints by decision curve analysis (DCA) in the E-MTAB-4421 and E-MTAB-7581 cohort. (i) Univariate logistic regression analysis performed on the E-MTAB-4421 dataset. (j) RT-PCR results of the key genes PDZD8 and ADRB2 in clinical samples, as calculated by one-way ANOVA with nonparametric test. Results were represented as means with error bars representing the standard error of the mean (SEM). (k) The CDI level between non-sepsis and sepsis in clinical samples. Results were represented as means with error bars representing the standard error of the mean (SEM). (l) Heatmap of lymphocyte and neutrophil correlations with CDI and key genes in clinical samples. (m-p) Scatter plot of correlation between key genes and lymphocyte percentage and neutrophil percentage. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ferroptosis scores were significantly higher in the high-CDI group compared to the low-CDI group, whereas pyroptosis scores showed no significant difference (Figure 4a). Due to the absence of non-sepsis controls in this dataset, we repeated the analysis the GSE65682 derivation cohort, comparing sepsis with non-sepsis patients. In this setting, necroptosis and pyroptosis scores were significantly elevated in sepsis patients, whereas ferroptosis scores remained unchanged (Figure 4b). Importantly, necroptosis was the only pathway consistently upregulated in both high CDI and sepsis patients, suggesting a strong association between CDI and necroptotic activity.

Pearson correlation analysis further supported this observation. PDZD8 expression was significantly positively correlated with necroptosis scores, whereas ADRB2 expression was negatively correlated ($p < 0.05$), consistent with their respective weights in the CDI formula. In contrast, correlations between these genes and pyroptosis or ferroptosis scores did not mirror CDI trends (Figure 4c), reinforcing the predominance of necroptosis in CDI-associated transcriptional alterations.

To explore necroptosis-related transcriptional features, we merged the GSE65682 and E-MTAB-4421 datasets and applied batch effect correction (Figure S2a). DEGs between high- and low-CDI groups were identified, followed by subsequent Gene Ontology (GO) enrichment analysis. The results revealed significant enrichment in pathways related to inflammatory responses typical of sepsis (Figure S2b). Likewise, GSEA showed that many of the top 15 upregulated pathways in the high CDI group were involved in septic inflammation (Figure S2c and d). Notably, GO enrichment also highlighted pathways related to TNF signaling, necroptosis, and pattern recognition receptor activation (Figure 4d). Canonical TNF pathway genes, including TNFRSF1A, TNFRSF1B, FADD, and NF- κ B, were significantly upregulated in the high CDI group (Figure 4e), as well as classical pattern recognition receptors (TLRs) and downstream mediators in the MAPK pathway (Figure 4f). To investigate the association between CDI and immune cell composition, immune cell

profiling was performed in high- and low-CDI groups within the merged dataset using the CIBERSORT algorithm. The high-CDI group showed a significant enrichment of neutrophils, whereas the low-CDI group exhibited increased levels of activated NK cells, consistent with findings from clinical samples (Figure 4g). Together, these results highlighted necroptosis as the dominant immunogenic cell death subroutine associated with CDI.

Single-Cell Transcriptome Delves Into Cell Death Characteristics of High-CDI Populations

Building upon the bulk transcriptomic findings, we next sought to dissect the cell-type-specific characteristics of CDI at single-cell resolution. To this end, we analyzed a publicly available whole blood single-cell RNA sequencing dataset (EGAS0001006283), which included 181,514 cells derived from 6 healthy volunteers (HV), 26 sepsis patients (SEP), and 9 sepsis convalescents (SEP-CON). Using canonical immune marker genes (Figure S3a), cells were clustered into five major immune populations: neutrophils, T/natural killer (T/NK) cells, monocytes, B/plasma cells, and megakaryocytes (Figure 5a). CDI was calculated for each individual cell based on the predefined formula (Figure 5b). As expected, CDI levels were significantly higher in sepsis patients compared to both sepsis convalescents and healthy volunteers (Figure 5c and d), reinforcing the clinical relevance of CDI at the single-cell level.

We then profiled the expression profiles of ADRB2 and PDZD8 across cell types. ADRB2 was predominantly expressed in T/NK cells, while PDZD8 was most abundant in neutrophils (Figure S3b). As expected, ADRB2 expression

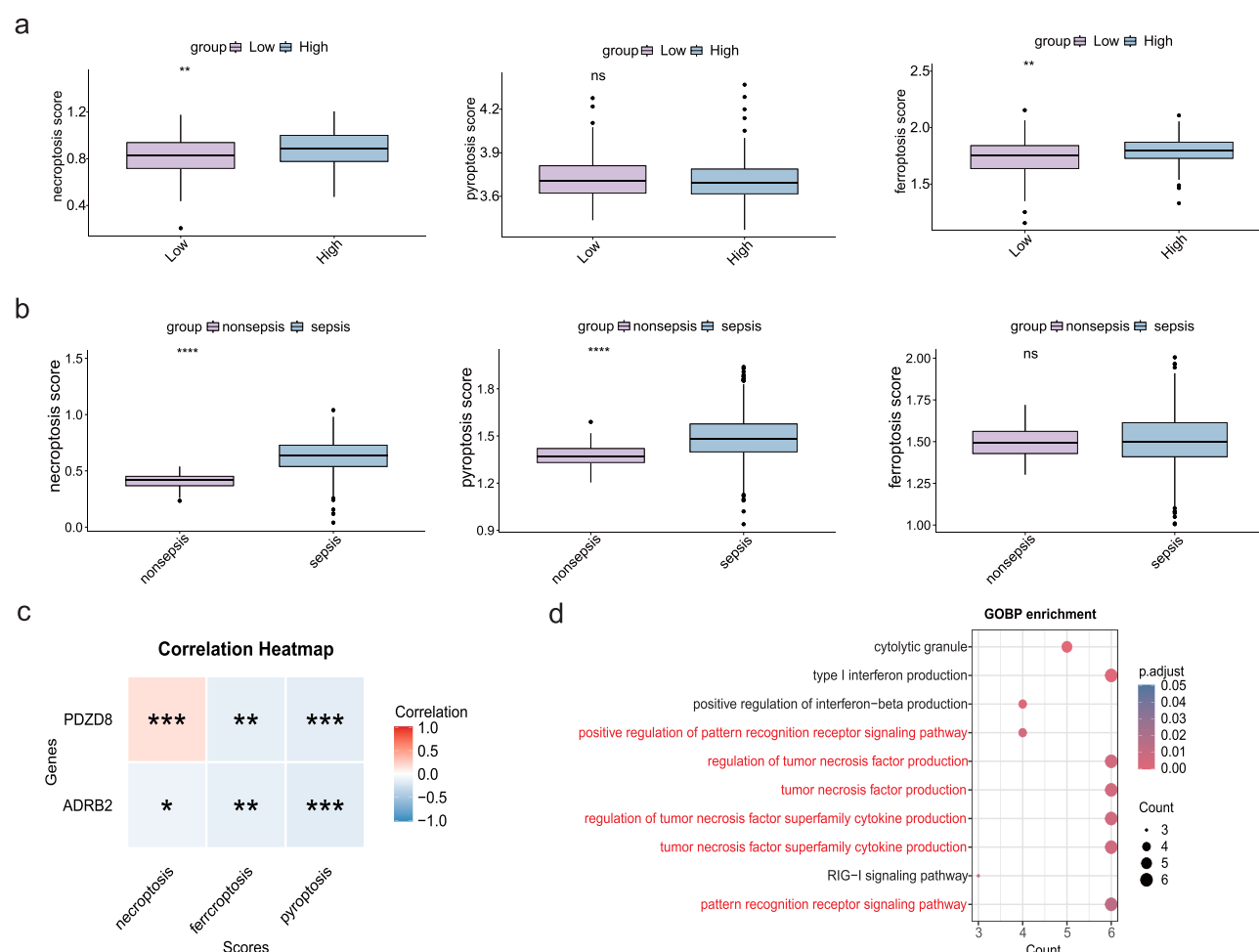


Figure 4 continued.

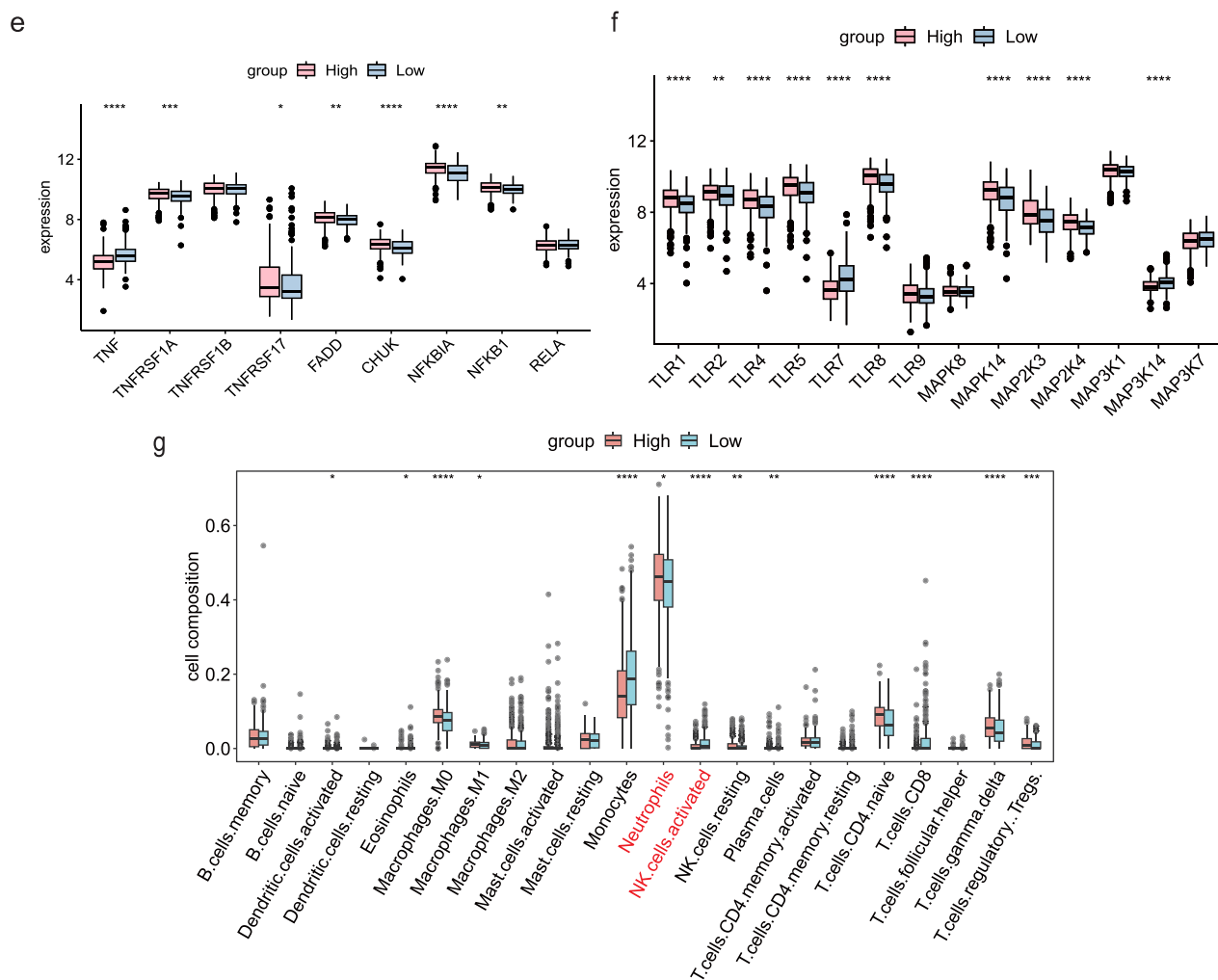


Figure 4 Association of CDI with necroptosis in transcriptome datasets. (a and b) Comparisons of necroptosis, pyroptosis, and ferroptosis scores between Low- and High-CDI groups presented by boxplots in E-MTAB-4421 and GSE65682 dataset. (c) The correlation between two key genes and three cell death scores above shown by heatmap. (d) The enrichment analysis identified the GOBP terms enriched in the transcriptome presented by bubble plot in the combined dataset. Red text highlighted pathways highly associated with necroptosis. (e) Comparisons of gene expression of TNF signaling pathway between high- and low-CDI groups presented by boxplot in the combined dataset. (f) Comparisons of gene expression of TLR and MAPK pathway between high- and low-CDI groups presented by boxplot in the combined dataset. (g) Comparison of immune cell composition between the high- and low-CDI groups in the combined dataset. Red text highlighted neutrophils and activated NK cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

was significantly reduced in sepsis patients compared to both control groups, whereas PDZD8 expression was significantly elevated ($p < 0.05$ for all comparisons) (Figure 5e and f).

To elucidate the functional implications of these expression patterns, we conducted pathway enrichment analysis within specific cell types. Neutrophils were stratified by median PDZD8 expression, and GSEA revealed enrichment for oxidative stress and cell killing pathways in the PDZD8-high group (Figure 5i), both of which are known components of necroptotic signaling. Due to limited numbers of T/NK cells in the whole blood dataset, ADRB2-associated pathways were further explored using a PBMC single-cell dataset GSE167363 from 2 Healthy volunteers, 3 sepsis survivors, and 2 non-survivors (Figure 5g and Figure S3c). This analysis confirmed that ADRB2 expression was highest in NK cells rather than T cells (Figure 5h). Moreover, as shown in Figure 5j, ADRB2^{high} T cells exhibited suppressed activities in pathways related to acute inflammation, antigen processing and presentation, pattern recognition receptors, and cytokine production (eg., TNF- α , IL-6), suggesting an anti-inflammatory or immunoregulatory role for ADRB2 in these cells.

To further characterize CDI-associated immune responses across cell types, we stratified neutrophils and T/NK cells by CDI score and performed GO enrichment analyses. Both cell types demonstrated enrichment in necroptosis-related

and proinflammatory signaling pathways in the high-CDI group (Figure S3d and e), mirroring the trends observed in bulk transcriptomic data. Finally, to compare the involvement of different forms of immunogenic cell death at the single-cell level, we calculated necroptosis, pyroptosis, and ferroptosis scores for each cell using hallmark gene sets from the GSEA database. Necroptosis scores were significantly higher in sepsis patients and showed strong concordance with CDI (Figure 5k). In contrast, pyroptosis and ferroptosis scores were highest in healthy volunteers and were not elevated in sepsis (Figure 5l and m).

Collectively, these findings indicated the link between CDI and necroptosis, extending the association to the single-cell level. The data suggest that CDI reflects a transcriptional program centered on necroptosis-driven immunopathology.

Necrostatin-I Improved Survival in Sepsis by Targeting Necroptosis

Building on transcriptomic and single-cell analyses that identified necroptosis as the dominant form of immunogenic cell death in high-CDI populations, we next sought to validate its presence and therapeutic relevance in sepsis. In the clinical samples, serum levels of HMGB1, a canonical DAMP released during necroptosis, were significantly elevated in sepsis patients compared to both pneumonia patients and UTRIs (Figure 6a). In contrast, levels of CASP8,²³ a key apoptotic regulator that inhibits necroptosis when activated, showed no significant difference between sepsis and pneumonia patients. These findings suggest that necroptosis is not actively restrained during the progression from pneumonia to sepsis, supporting its pathological involvement and therapeutic potential.

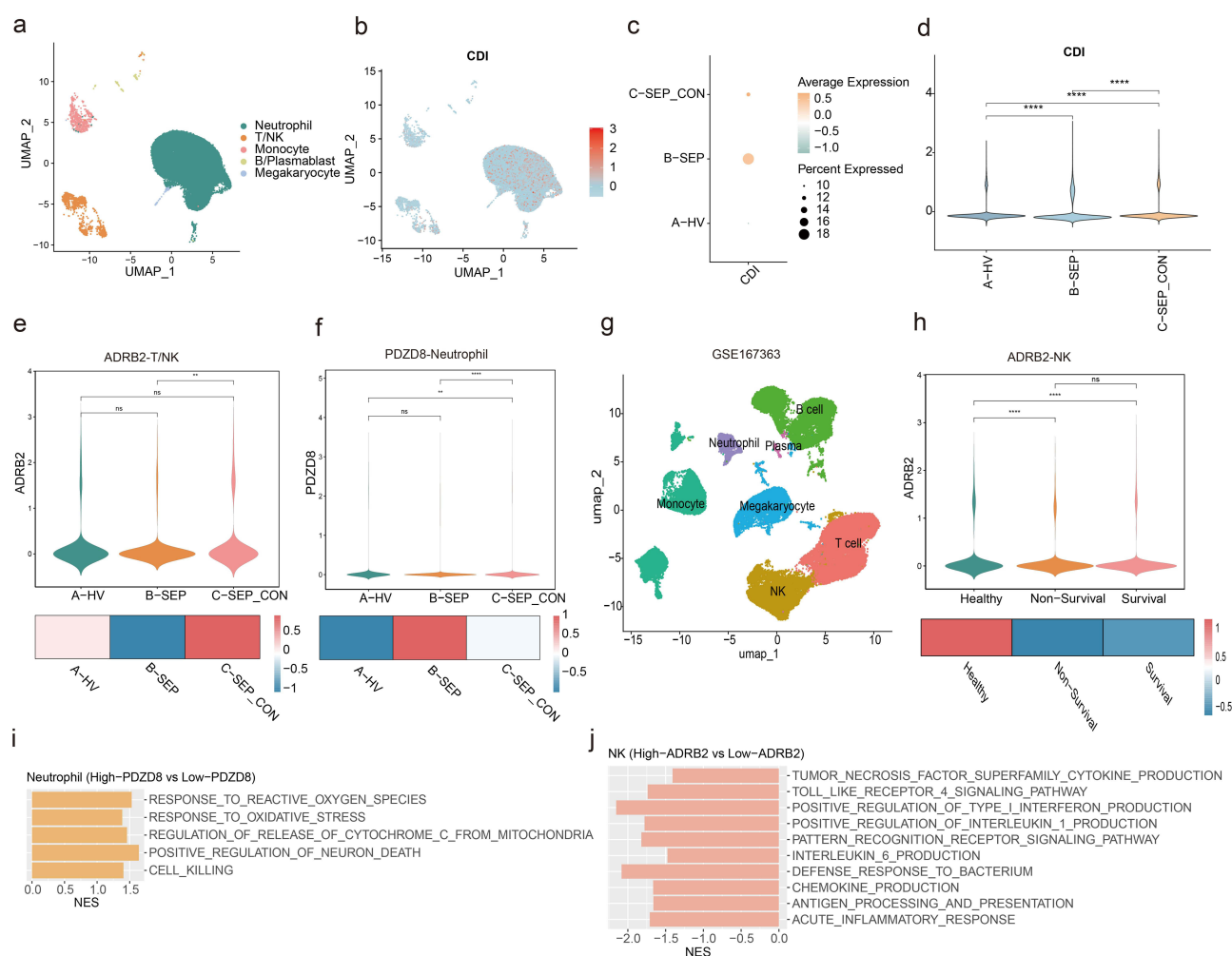


Figure 5 continued.

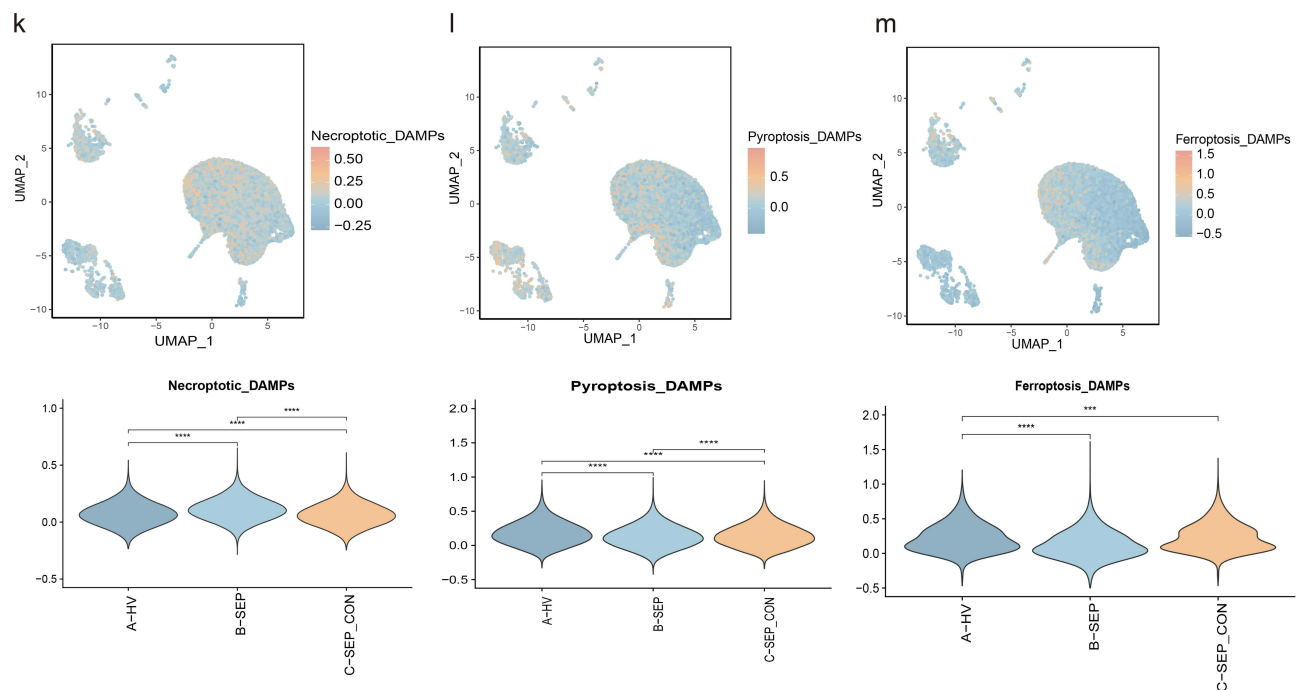


Figure 5 Single-cell transcriptome exploration of cell death-related features of CDI. (a) The cell type annotation of EGAS0001006283 scRNA-seq data using uniform manifold approximation and projection (UMAP) plots. (b) Enrichment of CDI in each cell type shown by UMAP plot. (c) Enrichment of CDI in each disease group shown by bubble plot. (d) Comparisons of CDI in disease groups shown by violin plot. (e) Comparisons of ADRB2 expression in the most relevant enriched cluster among disease groups. (f) Comparisons of PDZD8 expression in the most relevant enriched cluster among disease groups. (g) The cell type annotation of GSE167363 scRNA-seq data using UMAP plot. (h) Comparisons of ADRB2 expression in the most relevant enriched cluster among disease groups in GSE167363. (i) Biological processes enriched in neutrophils with high PDZD8 transcripts, as analyzed by GSEA separately. (j) Biological processes enriched in neutrophils with high ADRB2 transcripts, as analyzed by GSEA separately. (k–m) Enrichment scores of necroptotic genes, pyroptosis genes and ferroptosis genes in each cell type are shown by UMAP plots, and comparisons of scores among disease groups. All statistical significance was calculated using the Wilcoxon test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To test whether pharmacological inhibition of necroptosis could improve sepsis outcomes, we conducted animal experiments using the necroptosis inhibitor Nec-1, a selective RIPK1 inhibitor, in the CLP mouse model of sepsis (Figure 6b). Nec-1 was administered intraperitoneally at a dose of 10 mg/kg at 25 h, 13 h, and 1 h prior to CLP procedure. In a survival study ($n = 10$), mice subjected to CLP exhibited 0% survival at 96 hours, whereas Nec-1 administration dramatically improved survival to 90% ($p < 0.0001$) (Figure 6c). In a separate mechanistic study ($n = 3$), blood and tissue samples collected 22 hours post-CLP were analyzed. Gene expression analysis in peripheral WBCs revealed that PDZD8 was significantly upregulated, and ADRB2 was significantly downregulated in CLP mice relative to the sham group ($p < 0.05$) (Figure 6d). In the Nec-1 treatment group, PDZD8 expression was significantly decreased ($p < 0.05$), while ADRB2 showed a non-significant downward trend. Plasma concentrations of inflammatory cytokines TNF- α and IL-6 were significantly reduced following Nec-1 treatment ($p < 0.05$) (Figure 6e). Histological examination further confirmed that Nec-1 mitigated multi-organ injury typical of sepsis (Figure 6f).

Together, these *in vivo* results provided compelling functional evidence that necroptosis contributes to the immunopathogenesis of sepsis. The consistent upregulation of PDZD8 in both clinical and animal models, and its downregulation following Nec-1 treatment, underscored its potential as a therapeutic target.

Discussion

In this study, we provide a systematic characterization of cell death pathway expression patterns in sepsis, highlighting the significant role of immunogenic cell death (ICD) in driving excessive immune activation. We identified two key ICD-associated genes, PDZD8 and ADRB2, and developed the CDI, which effectively predicts sepsis prognosis and implicates necroptosis as a key contributor to hyperinflammation. Notably, high CDI was associated with neutrophilia and lymphopenia in clinical peripheral blood samples. Single-cell transcriptome analysis further revealed that PDZD8

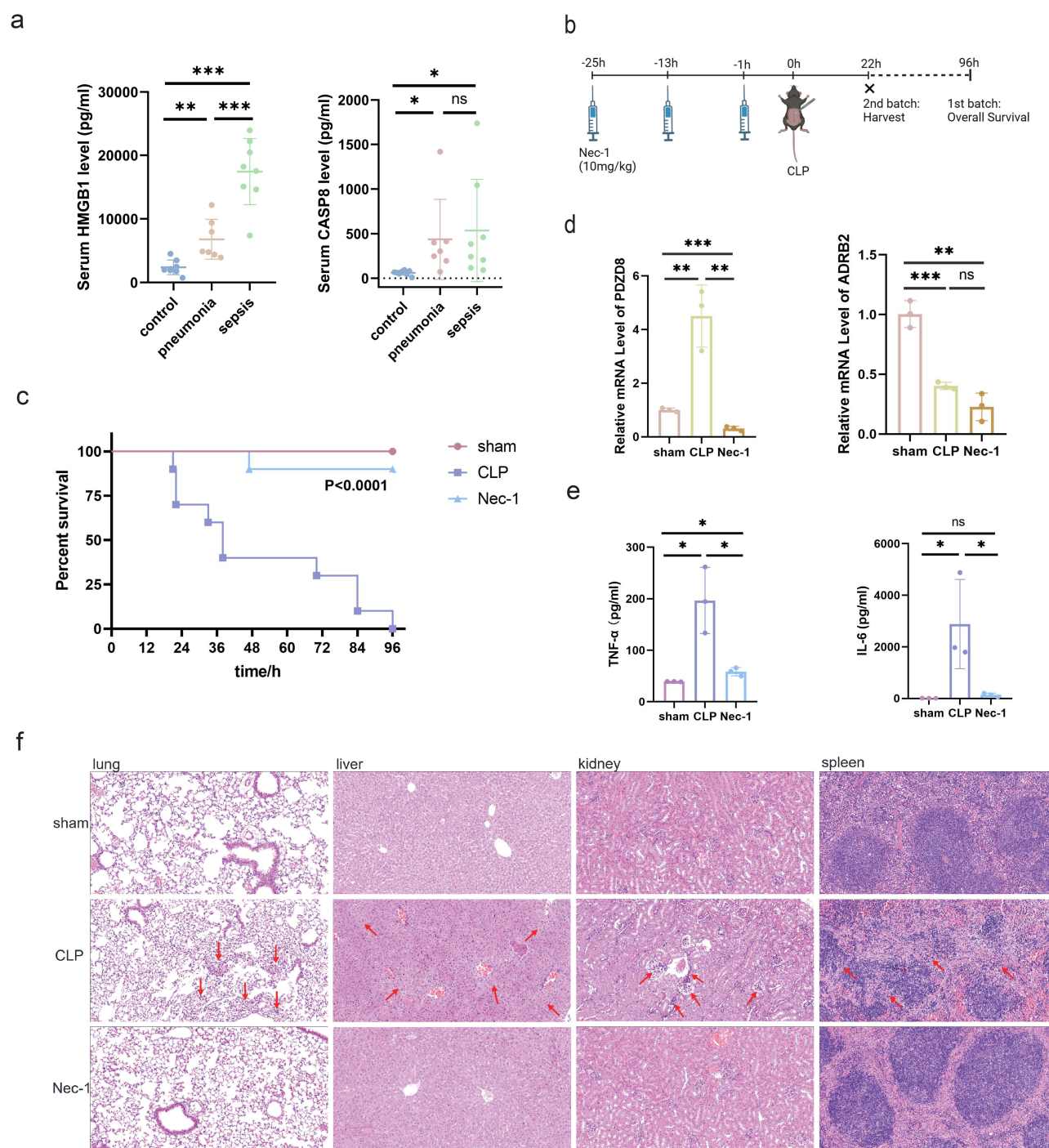


Figure 6 Necrostatin-1 significantly improved sepsis outcomes in CLP models. **(a)** Serum levels of HMGB1 and CASP8 in clinical patients. **(b)** Schematic of the animal experimental design. **(c)** Survival rates of CLP mice with or without Nec-1 (10 mg/kg). **(d)** The mRNA levels of PDZD8 and ADRB2 were analyzed by RT-PCR in the peripheral blood of mice. **(e)** At the 22h post-CLP, the levels of inflammatory cytokines (TNF- α and IL-6) in plasma were detected by ELISA tests. **(f)** Hematoxylin-eosin staining of lung, liver, kidney and spleen sections, with red arrows indicating pathological areas. Scale bar = 50 μ m. All data were expressed as mean $\pm$ SEM (n=3). *P< 0.05, **P< 0.01, ***P< 0.001.

was enriched in neutrophils and ADRB2 in NK cells. Our findings offer a refined understanding of ICD's role in sepsis, suggesting that targeting necroptosis may hold therapeutic potential.

ADRB2 encodes the β 2-adrenergic receptor (β 2-AR), which is expressed on various immune cell types.²¹ Previous studies have shown that ADRB2 modulates immune responses through multiple mechanisms. In macrophages, β 2-AR

signaling suppresses lipopolysaccharide (LPS)-induced systemic inflammation by inducing the expression of T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3), thereby promoting an anti-inflammatory phenotype.²⁴ Similarly, in LPS-stimulated mouse bone marrow-derived dendritic cells (BMDCs), β 2-AR agonist treatment inhibits antigen cross-presentation to CD8⁺ T cells by impairing phagosomal degradation, effectively converting immunogenic dendritic cells into tolerogenic ones.²⁵ In a mouse model of colitis, adrenaline acts through ADRB2 to respond to Toll-like receptor signaling, promoting IL-10 secretion and suppressing the release of pro-inflammatory cytokines such as IL-6 and TNF- α .²⁶ Together, these findings suggest that ADRB2 primarily exerts anti-inflammatory effects. In our study, NK cell subsets with high ADRB2 expression showed downregulation of immune activation pathways, including those related to antigen processing and presentation, pattern recognition receptors, and pro-inflammatory cytokines. These observations are consistent with previous findings and support the hypothesis that, in sepsis, ADRB2 may participate in ICD by modulating antigen presentation via β 2-AR signaling, thereby influencing the immunogenicity of dying cells.

PDZD8 is an endoplasmic reticulum (ER) protein located at ER-mitochondria contact sites and plays a critical role in maintaining mitochondrial homeostasis.²⁷ In our study, PDZD8 was highly expressed in neutrophils and associated with cell killing and oxidative stress pathways. Previous studies have shown that activated neutrophils produce reactive oxygen species (ROS) during the “respiratory burst” observed in sepsis.²⁸ That mitochondria-derived ROS can trigger necroptosis through TNF/TNFR1 signaling pathways.²⁹ Whether high PDZD8 expression in sepsis promotes ROS generation via TNF signaling, thereby inducing necroptosis and exacerbating immune overactivation, remains to be experimentally confirmed. In vitro experiments demonstrated that macrophage-specific PDZD8 knockout mice exhibited significantly lower serum levels of pro-inflammatory cytokines, such as TNF- α and IL-6, following LPS stimulation compared to wild-type controls. Further analysis revealed that PDZD8 promoted pro-inflammatory responses by enhancing the glutamate catabolic pathway.³⁰ Collectively, these findings suggest that PDZD8 contributes to inflammatory activation and may serve as a potential therapeutic target for modulating the cytokine storm in sepsis.

Necroptosis has been described as a “storm of cell death” due to its potent pro-inflammatory nature, which can lead to acute inflammation and prolonged immune activation.³¹ In our study, GSEA analysis revealed that the high CDI group showed strong concordance with necroptosis signatures. Further validation in animal models demonstrated that treatment with Nec-1—a specific inhibitor of RIPK1, a critical kinase in the necroptotic pathway³²—improved survival in CLP-induced septic mice by 90%, reduced serum levels of pro-inflammatory cytokines, and alleviated tissue damage. The CLP model closely mimics the clinical features of human sepsis caused by bacterial infection.¹⁸ Consistent with previous findings, Nec-1 also inhibited the NF- κ B pathway, significantly prolonged survival time, suppressed inflammation, and mitigated lung injury in mice with LPS-induced acute lung injury.³³ In a separate TNF- α -induced systemic inflammatory response syndrome (SIRS) model, Nec-1 conferred complete protection against hypothermia and death, achieving a 100% survival rate.³⁴ Collectively, these results indicate that targeting necroptosis via Nec-1 improves outcomes across multiple sepsis models, highlighting it as a promising therapeutic strategy. Notably, expression of PDZD8 was significantly downregulated following Nec-1 treatment, suggesting that neutrophils with high PDZD8 expression may play a pivotal role in necroptosis-driven inflammation. This potential mechanism warrants further investigation in future studies.

This study provides a foundation for understanding the role of ICD and necroptosis in sepsis, but several limitations point to important areas for future research. First, the clinical validation of CDI would benefit from larger, more diverse patient cohorts to ensure broad applicability. Mechanistically, further investigation is needed to fully define how PDZD8 and ADRB2 influence necroptosis and immune activation, including the role of ROS and TNF signaling. Additionally, future studies should adopt a systems biology approach to integrate the contributions of other cell death pathways (eg., pyroptosis, ferroptosis) and immune mediators in sepsis pathogenesis. This will enable the establishment of more comprehensive models and the development of more refined therapeutic strategies to improve clinical outcomes for sepsis patients.

Conclusion

This study established a CDI based on PDZD8 and ADRB2 that effectively stratifies sepsis prognosis by capturing immunogenic cell death patterns, particularly necroptosis. Our findings provide new insights into the inflammatory

pathogenesis of sepsis and highlight necroptosis as a promising target for precision therapy, laying the groundwork for novel therapeutic strategies that could significantly improve clinical outcomes for sepsis patients.

Abbreviations

ICD, Immunogenic Cell Death; CDI, Cell Death Index; ADRB2, β 2-Adrenergic Receptor; PDZD8, PDZ Domain Containing 8; COX, Cox Proportional Hazards Regression; AUC, Area Under the Curve; MARS, Molecular Diagnosis and Risk Stratification of Sepsis; SRS, Sepsis Response Signatures; HMGB1, High Mobility Group Box 1; CASP8, Caspase 8; CLP, Cecal Ligation and Puncture; DAMP, Damage-Associated Molecular Pattern; PAMP, Pathogen-Associated Molecular Pattern; ER, Endoplasmic Reticulum; GO, Gene Ontology; GO-BP, Gene Ontology Biological Process; TNF, Tumor Necrosis Factor; NF- κ B, Nuclear Factor kappa-light-chain-enhancer of activated B cells; TLR, Toll-Like Receptor; MAPK, Mitogen-Activated Protein Kinase; HV, Healthy Volunteer; SEP, Sepsis Patient; SEP-CON, Sepsis Convalescent; PBMC, Peripheral Blood Mononuclear Cell; GSEA, Gene Set Enrichment Analysis; ssGSEA, Single-Sample Gene Set Enrichment Analysis; GSVA, Gene Set Variation Analysis; DEG, Differentially Expressed Gene; RT-qPCR, Real-Time Quantitative Polymerase Chain Reaction; SVA, Surrogate Variable Analysis; RF, Random Forest; XGBoost, Extreme Gradient Boosting; ROC, Receiver Operating Characteristic; CI, Confidence Interval; DCA, Decision Curve Analysis; FDR, False Discovery Rate; UMAP, Uniform Manifold Approximation and Projection; PCA, Principal Component Analysis; SEM, Standard Error of the Mean; SPF, Specific Pathogen-Free; ARRIVE, Animal Research: Reporting of In Vivo Experiments; TNFRSF1A / TNFRSF1B, Tumor Necrosis Factor Receptor Superfamily Member1A / 1B; FADD, Fas-Associated Protein with Death Domain; ROS, Reactive Oxygen Species; RIPK1, Receptor-Interacting Protein Kinase 1; SIRS, Systemic Inflammatory Response Syndrome; β 2-AR, β 2-Adrenergic Receptor; Tim-3, T cell immunoglobulin and mucin domain-containing protein 3; RNA-seq, RNA Sequencing; scRNA-seq, Single-Cell RNA Sequencing; 4-PL, Four-Parameter Logistic; AMPK, AMP-Activated Protein Kinase; OS, Overall Survival.

Data Sharing Statement

The datasets analyzed for this study can be found in the GEO website (<https://www.ncbi.nlm.nih.gov/geo/>), the Array Express (<https://www.ebi.ac.uk/biostudies/arrayexpress>), and European Genome-phenome Archive platforms (<https://ega-archive.org/datasets/>). The analysis methods and used packages are illustrated in the “Materials and methods” section. All other R codes and analyses are available from the corresponding author upon request.

Ethics Approval and Consent to Participate

All procedures involving human participants were approved by the Ethics Committee of the School of Medicine, Xiamen University (approval no. XDYX202302K06) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants or their legal guardians prior to sample collection.

The animal experiment of this study followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and was in strict accordance with the guidelines of the Xiamen University Laboratory Animal Welfare and Ethics Committee (2019.01.01) to ensure ethical treatment of the mice involved. Euthanasia procedures were also in accordance with these guidelines. And the project has been reviewed and approved by the Ethics Committee of Laboratory Animals of Xiamen University (Permit number: XMULAC20210021).

The human datasets used in this study fall under the circumstances specified in Article 32, Paragraphs 1 and 2 of the “Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects” promulgated by China, and are therefore exempt from ethical review: (1) Data derived from publicly available sources obtained legally, or from observational studies that do not interfere with public activities; (2) Research conducted using anonymized information data.

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Author Contributions

JL Gan: Formal analysis(lead), Investigation(lead), Writing – original draft and revise(lead), Data curation(supporting), Software(supporting); QY Long: Methodology(lead), Formal analysis (supporting), Writing – review & editing (equal); SQ Fang: Visualization(lead), Data curation (supporting), Writing – original draft(supporting); SX Song: Resources(lead), Investigation (supporting), Writing – original draft(supporting); J Wu: Validation (lead), Writing – review & editing (equal); XM Li: Data curation (lead), Writing – original draft(supporting); HL Ye: Software(lead), Writing – original draft(supporting); ZC Gao: Supervision(lead), Methodology(supporting), Writing – review & editing-(equal); YL Zheng: Conceptualization(lead), Project administration(lead), Funding acquisition(lead), Writing – review & editing(equal). All authors took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest to report regarding the present study.

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