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Inhibition of Interleukin-40 prevents multi-organ damage during sepsis by blocking NETosis

Shijie Cai^{1,2†}, Xiao Li^{1,2†}, Chen Zhang¹, Yuqian Jiang³, Yonghui Liu¹, Zhi He¹, Shuo Ma^{1,2}, Yuming Yao^{1,2}, Chun-Kwok Wong^{4,5,6}, Guoqiu Wu^{1,2*} and Xun Gao^{1,2*}

Abstract

Despite intensive clinical and scientific efforts, the mortality rate of sepsis remains high due to the lack of precise biomarkers for patient stratification and therapeutic guidance. Interleukin 40 (IL-40), a novel cytokine with immune regulatory functions in human diseases, was elevated at admission in two independent cohorts of patients with sepsis. High levels of secreted IL-40 in septic patients were positively correlated with PCT, CRP, lactate (LDH), and Sequential Organ Failure Assessment (SOFA) scores, in which IL-40 levels were used to stratify the early death of critically ill patients with sepsis. Moreover, genetic knockout of IL-40 (IL-40^{-/-}) improved outcomes in mice with experimental sepsis, as evidenced by attenuated cytokine storm, multiple-organ failure, and early mortality, compared with those of wild-type (WT) mice. Mechanistically, single-cell RNA sequencing (scRNA-seq) and bulk RNA sequencing (RNA-seq) have revealed that S100A8/9^{hi} neutrophil influx into the peritoneal cavity along with neutrophil extracellular trap (NETs) formation accounts predominantly for the IL-40-mediated worsening of sepsis outcomes. Clinically, the IL-40 level was positively correlated with the NET-related MPO/dsDNA ratio in septic patients. Finally, with antibiotics (gentamycin), genetic knockout of IL-40 prevented polymicrobial sepsis fatalities more efficiently than without gentamycin treatment. In summary, these data reveal a novel prognostic strategy for sepsis and that IL-40 may serve as a novel therapeutic target for sepsis.

Keywords IL-40, NETs, Prognostic, Theranostic, Sepsis

[†]Shijie Cai and Xiao Li have been contributed equally to the work.

*Correspondence:

Guoqiu Wu

zdyynjwf@126.com

Xun Gao

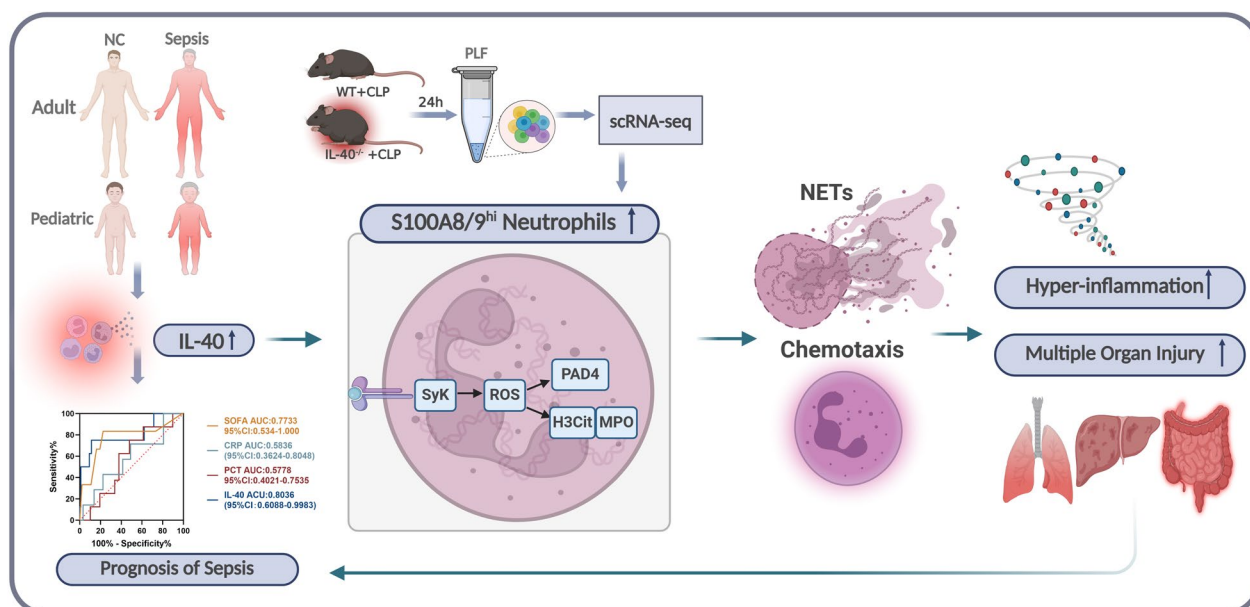
gaoxun1021@163.com

Full list of author information is available at the end of the article



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Graphical abstract



Introduction

Sepsis, a life-threatening multi-organ dysfunction syndrome caused by dysregulation of host immunity against infection, is currently one of the leading causes of death from infection worldwide [1, 2]. Globally, approximately 48.9 million septic patients are newly diagnosed each year, which ultimately leads to the death of more than 11 million patients, and patients have a lengthy course of treatment and poor prognosis because no breakthroughs have been made in its pharmacological treatment [3, 4]. The development of sepsis involves complex imbalances in the host inflammatory response, manifesting as excessive inflammation and immune suppression [2, 5, 6]. In recent decades, host immune regulatory factors have proven crucial in modulating the innate immune response and serve as vital immune targets for clinical diagnosis and host-directed therapies for sepsis [7]. However, despite decades of research aimed at identifying biological agents that alter immune responses to sepsis, no significant positive effects or reductions in mortality have been demonstrated [8]. A potential reason for this failure is that single-molecule antagonists are incapable of mitigating severe systemic inflammation, which is physiologically intricate; the other factor contributing to the lack of efficacy is the heterogeneity of sepsis [9, 10]. Hence, a novel theranostic biomarker that can identify patients on the basis of their underlying immunopathologic

condition while simultaneously serving as a therapeutic target from the perspective of host regulation is needed for sepsis precision immunotherapy.

IL-40 is a secreted protein (27 kDa) synthesized by C17orf99, first discovered by Albert Zlotnik in 2017 [11]. IL-40 is expressed mainly in the bone marrow, fetal liver, and activated B cells after being stimulated with anti-CD40 monoclonal antibodies (mAbs), anti-IgM, IL-4, and TGF- β [12]. Since C17orf99^{-/-} mice presented significantly lower levels of IgA expression in the serum, intestine, feces, and mammary glands, IL-40 may be a key effector protein in the humoral immune system [11]. Subsequent studies have also confirmed the upregulation of IL-40 expression in rheumatoid arthritis [13–15], systemic lupus erythematosus [16], obligate spondylitis [17], and primary desiccation syndrome [18], with positive correlations with disease activity and the production of autoantibodies [14]. Biological studies further confirmed that IL-40 exerts powerful proinflammatory effects, which not only promote the release of IFN- γ from B cells and TNF- α and IL-17A from CD4⁺/CD8⁺ cells [18], but also increase the release of matrix metalloproteinases (MMPs) from synovial fibroblasts [14]. Previously, we reported that after treatment with the anti-inflammatory cytokine IL-38, C17orf99 was significantly down-regulated in a coculture of human respiratory epithelial cells with macrophages upon LPS stimulation, indicating

possible regulation of IL-40 in infections [19]. Recent studies have revealed that neutrophils are important sources of IL-40 [15, 18], but a regulatory role for IL-40 in sepsis has not yet been established. Here, we demonstrated that IL-40 is a candidate prognostic biomarker in sepsis. Furthermore, we investigated and highlighted the application of the IL-40 knockout strategy, which simultaneously suppresses multiple proinflammatory mediators and neutrophil extracellular traps (NETs), as a promising therapy for preventing death in polymicrobial sepsis.

Results

Overly secreted IL-40 levels may stratify patients with sepsis

To investigate the potential role of IL-40 as a stratification biomarker for sepsis, a total of 116 participants diagnosed with either sepsis ($n=53$) or septic shock ($n=63$), as well as healthy controls ($n=50$), were included. Furthermore, 50 patient control ($n=50$) in critical conditions with no infection were recruited as we previously described [20]. The baseline characteristics of patients with sepsis, patient control as well as healthy controls are shown in Table S1. There were no differences in sex, age, white blood cell (WBC) count, neutrophil count, C-reactive protein (CRP) level, or procalcitonin (PCT) concentration between survivors and non-survivors. IL-40 levels at disease onset were significantly higher in patients with sepsis than in patient controls ($P<0.01$, Fig. 1A) and healthy controls ($P<0.0001$, Fig. 1A). They persisted at high levels until day 3 ($P<0.05$, Fig. 1B). Moreover, IL-40 levels were higher in septic shock patients than in non-shock patients ($P<0.01$, Fig. 1C), and were significantly higher in non-survivors than in survivors ($P<0.01$, Fig. 1D). Additionally, IL-40 levels in adults with sepsis were positively correlated with PCT, CRP, lactate (LDH), and SOFA scores (Fig. 1E). Since IL-40 has been shown to promote NETosis and that neutrophils are important sources of IL-40 [15, 18], in this project, we also investigated and found that IL-40 correlated positively with the absolute WBC and neutrophil counts (Fig. S1A and B). To evaluate the predictive value of these markers in sepsis, receiver operating characteristic (ROC) curves were generated for the ability of the IL-40 level, SOFA score, CRP level, and PCT level to predict sepsis-related 28-day mortality. The highest area under the curve (AUC) was observed for IL-40 [0.7266 (95% CI: 0.6265–0.8268)], followed by SOFA scores [0.6157 (95% CI: 0.4904–0.7410)], PCT [0.5438 (95% CI: 0.4272–0.6604)] and CRP [0.5522 (95% CI: 0.4294–0.6749)] (Fig. S1C) (Table S2). Together, the results reveal a marked increase in IL-40 in septic patients and its value as a novel surrogate prognostic marker for sepsis outcomes.

We then sought to validate the above results in a cohort of septic children. The demographic, clinical, and laboratory characteristics of the 106 pediatric patients, 50 patient controls, and 90 healthy controls are shown in Table S3. Similar to findings from adult patients with sepsis, serum IL-40 levels were also significantly higher in pediatric septic patients at admission than in healthy children ($P<0.0001$, Fig. 1F) as well as disease controls ($P<0.0001$, Fig. 1F). Dynamic monitoring revealed that high IL-40 concentrations may be sustained as long as day 3 ($P<0.01$, Fig. 1G). Additionally, septic shock patients presented substantially higher IL-40 levels than those without shock ($P<0.01$, Fig. 1H), and those who did not survive had significantly higher IL-40 levels than the survivors ($P<0.01$, Fig. 1I). Consistent with those in adult patients, serum IL-40 concentrations in children were positively correlated with PCT, CRP, LDH and SOFA scores (Fig. 1J). Additionally, there was a positive correlation of IL-40 with WBCs and neutrophils (Fig. S1D and E). In our further investigations, the ROC curve for IL-40 yielded an AUC of 0.8036 (95% CI: 0.6088–0.9983) for the prediction of 28-day mortality in pediatric patients with sepsis, followed by SOFA [AUC: 0.7733 (95% CI: 0.5341–1.000)], CRP [AUC: 0.5836 (95% CI: 0.3624–0.8048)] and PCT [AUC: 0.5778 (95% CI: 0.4021–0.7535)] (Fig. S1F) (Table S4). Taken together, these data indicate that excessive secretion of IL-40 may stratify pediatric septic patients by the risk of death.

IL-40 is an upstream exacerbating factor of polymicrobial sepsis

To investigate whether IL-40 is involved in the pathogenesis of sepsis, we established a polymicrobial sepsis mouse model via cecal ligation and puncture (CLP) as previously described [21] (Fig. 2A). Following CLP, plasma IL-40 levels continuously increased and were significantly higher than those in naïve mice (Fig. 2B). Moreover, IL-40 concentrations were also elevated in the peritoneal lavage fluid (PLF), liver and kidney compared with those in naïve mice (Fig. 2B). We then treated the mice that had undergone CLP with recombinant murine IL-40 (rmIL-40) (Fig. 2C). Compared with vehicle treatment, early treatment with rmIL-40 at the time of CLP decreased mouse survival (Fig. 2D). Histological analysis revealed morphological evidence of organ injury, including lung, spleen, liver and kidney injury (Fig. 2E). Moreover, compared with vehicle-treated mice, rmIL-40-treated mice showed increased circulating parameters, including hepatocellular injury associated alanine aminotransferase (ALT) and aspartate aminotransferase (AST), general cellular injury associated LDH, as well as kidney injury-related urea (Fig. 2F). We also examined the effects of IL-40 treatment on bacterial burdens. We

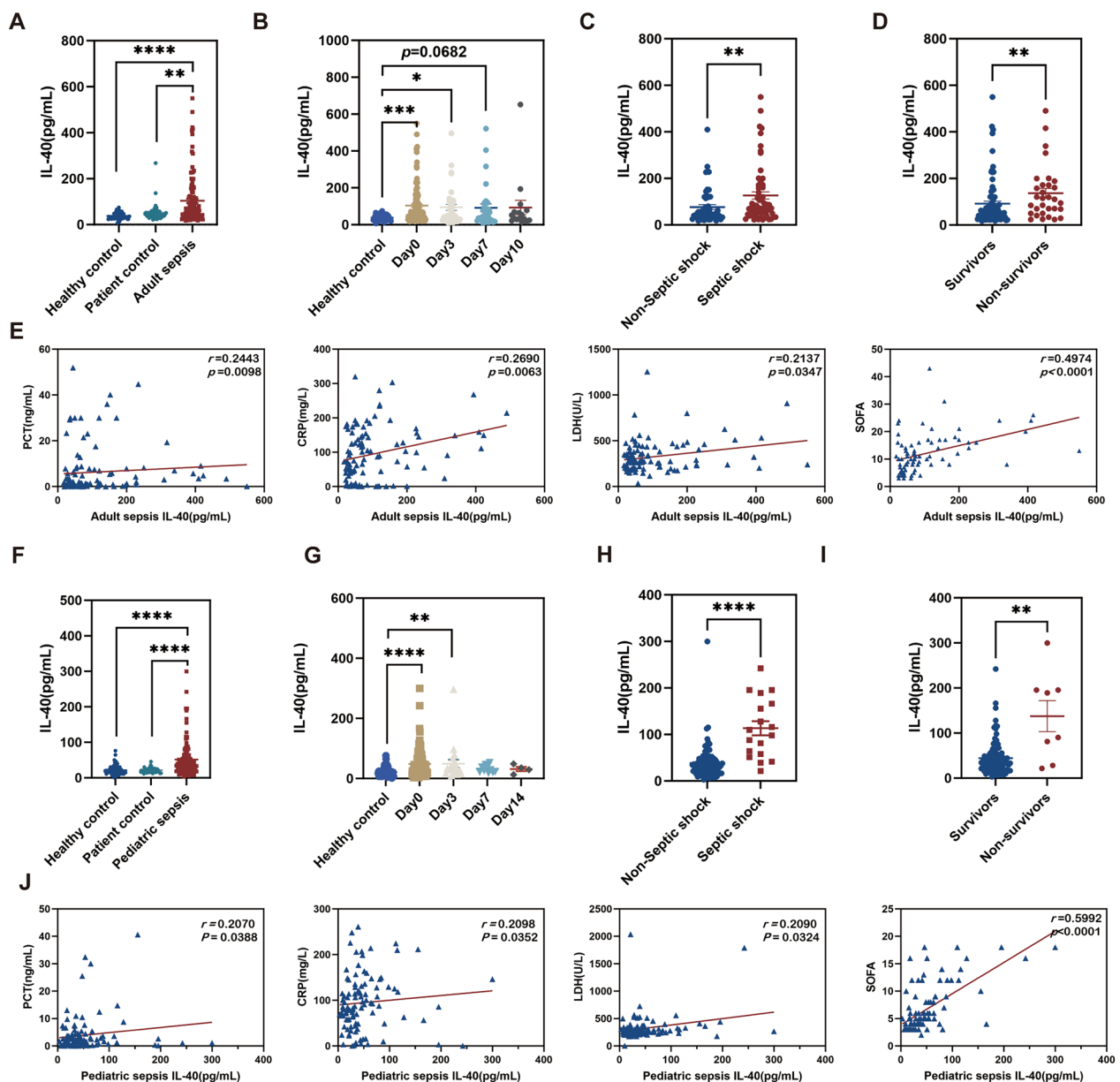


Fig. 1 Elevated IL-40 levels correlate with multiple organ damage and mortality in critically ill patients with sepsis. **A** Plasma levels of IL-40 in healthy controls (n=50), patient controls (n=50) as well as patients with sepsis (n=116). **B** Dynamic changes in plasma IL-40 levels in adult sepsis patients at days 0, 3, 7, or 10. **C** Plasma levels of IL-40 in septic shock patients (n=63) and non-shock patients (n=53). **D** Plasma levels of IL-40 in survivors (n=85) and non-survivors (n=31). **E** Correlations between IL-40 levels and PCT (n=111), CRP (n=102) and LDH (n=98) levels as well as SOFA score (n=80) in adult septic patients. **F** Plasma levels of IL-40 in healthy controls (n=90), patient controls (n=50) and pediatric patients with sepsis (n=106). **G** Dynamic changes in plasma IL-40 levels at admission and 3, 7, and 10 days after admission in pediatric patients with sepsis, when appropriate. **H** Plasma levels of IL-40 in septic shock pediatric patients (n=18) and non-shock pediatric septic patients (n=88). **I** Plasma levels of IL-40 in survivors (n=98) and non-survivors of pediatric septic patients (n=8). **J** Correlations between IL-40 levels and PCT levels (n=100), CRP levels (n=101), LDH (n=105) and the SOFA score (n=99) in pediatric patients with sepsis. The data are expressed as the means with 95% confidence intervals (means $\pm$ SEMs). Statistical analysis was performed with the Mann–Whitney U test (**A**, **C**, **D**, **F**, **H**, **I**), nonparametric Kruskal–Wallis test (**B**, **G**) and Spearman's correlation coefficient test (**E**, **J**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$

found that, compared with vehicle-treated septic mice, rmIL-40-treated septic mice had greater bacterial loads in the PLF, blood and spleen after CLP (Fig. S2A). These

data suggest that IL-40 is an upstream exacerbating factor that modifies the progression of sepsis and can be used as a therapeutic target for polymicrobial sepsis.

IL-40 treatment induces a hyperinflammatory response during sepsis

To evaluate the effects of rmIL-40 on CLP-induced general inflammation, the levels of inflammatory cells and their corresponding cytokines and chemokines were subsequently determined. Cell counts and flow cytometry analysis revealed a substantial increase in the total number of inflammatory cells and corresponding neutrophils infiltrating the peritoneum after treatment with rmIL-40 (Figs. 2G and S2B). Among the cytokines, rmIL-40 treatment significantly increased CCL2, IL-6, and TNF- α in the serum and PLF (Fig. 2H), while there were no differences in INF- γ or IL-10 expression between the IL-40-treated and vehicle-treated mice 24 h after CLP (Fig. S2C). Additionally, we found that rmIL-40 replenishment led to increased apoptosis in the lungs and spleen (Fig. 2I), while it was reported that the cytokine storm may induce cell dysfunction and apoptosis, causing organ dysfunction [22], indicating that IL-40 had direct effects on the induction of hyperinflammation to promote the pathology of polymicrobial sepsis.

Deficiency in IL-40 prevents hyperinflammation and organ dysfunction in polymicrobial sepsis

We then wondered whether IL-40 deficiency may provide therapeutic targets for sepsis. In this context, we generated IL-40^{-/-} septic mice as a therapeutic model, and we used WT and IL-40^{-/-} mice to establish a polymicrobial model (Fig. 3A). Compared with WT mice, genetic knockout of IL-40 increased septic mouse survival from 20 to 60% (Fig. 3B). The decreased mortality of IL-40^{-/-} septic mice was associated with reduced evidence of organ injury, as AST, ALT, LDH, and urea were decreased in IL-40^{-/-} mice (Fig. 3C). Pathological changes and apoptosis in the tissues were detected via H&E and TUNEL assays. Similarly, the incidence of hepatic hemorrhage, observable damage to the kidney parenchyma, enlarged and fused white pulp in the

spleen, pulmonary destruction, and interstitial edema were significantly lower in the IL-40^{-/-} septic mice than in the WT septic mice 24 h after CLP (Fig. 3D). Moreover, apoptosis of lung cells and splenocytes was lower in the IL-40^{-/-} mice than in the WT mice (Fig. 3E). Longitudinal cytokines profiles revealed that IL-40 deficiency decreased the expression of IL-6, CCL2, and TNF- α in both the serum and peritoneum when compared to the WT mice (Fig. 3F), whereas no significant differences in INF- γ and IL-10 expression were detected (Fig. S3A). Additionally, we examined the effects of IL-40 deficiency on bacterial burdens and found that genetic knockout of IL-40 decreased bacterial loads in the PLF, blood and spleen 24 h after CLP (Fig. S3B). Finally, to mimic human clinical scenarios, we injected gentamicin into the mice 2 h before CLP, and resistance to CLP-induced mortality was observed in the IL-40^{-/-} mice in the presence of gentamicin (Fig. 3G). These results suggest that the inhibition of IL-40 protects septic mice from lethality by mitigating the overwhelming production of proinflammatory mediators and reducing organ destruction during polymicrobial sepsis.

IL-40 contributes to sepsis progression by promoting S100A8/9^{hi} neutrophil recruitment and activation

To clarify the mechanisms underlying the IL-40-mediated progression of polymicrobial sepsis, we performed single-cell RNA sequencing (scRNA-seq) of PLF cells from WT and IL-40^{-/-} septic mice (Fig. 4A). After removing doublets and filtering out low-quality cells, 42,896 cells were eligible for subsequent analysis. By UMAP analysis, 23 cell clusters (clusters 0–22) were identified, revealing highly disease-specific clusters after separate visualization of cells from septic or sham mice in the WT and IL-40^{-/-} groups (Fig. 4B and C). For each cluster, we identified the top ten DEGs (Fig. S4A). By annotating each cluster according to the marker genes, 8 cell types, including neutrophils,

(See figure on next page.)

Fig. 2 IL-40 promotes hyperinflammation and organ dysfunction in polymicrobial sepsis. **A** Experimental design for establishing a polymicrobial sepsis model via CLP. **B** Circulating IL-40 concentrations in the serum, PLF, liver, and kidney at 6 h and 24 h after CLP, as measured via ELISA (n = 4–5). **C** Study design for establishing a polymicrobial sepsis model in WT mice treated with rmIL-40. rmIL-40 (800 ng/100 μ L) or vehicle control was intraperitoneally (i.p.) administered to C57BL/6 mice at the time of CLP and were sacrificed 24 h after CLP for analysis, or rmIL-40 (800 ng/100 μ L) was injected i.p. into WT mice immediately, 24 h and 48 h after CLP, and survival was monitored for 14 consecutive days. **D** Monitoring of the 14-day survival of septic mice treated with sterile PBS (100 μ L) or rmIL-40 (800 ng/100 μ L) (n = 5). **E** Representative images of hematoxylin and eosin (H&E)-stained lung, liver, spleen, and kidney tissues from vehicle- and rmIL-40-treated septic mice at 24 h after CLP (n = 5). Scale bars, 50 μ m at X400 magnification. **F** Serological parameters of organ damage, including ALT, AST, LDH, and urea, in vehicle- and rmIL-40-treated septic mice at 24 h after CLP (n = 3–6). **G** Bar chart showing the total number of inflammatory cells in the PLF and corresponding CD11b⁺Ly6G⁺ neutrophils determined by flow cytometry in vehicle- and rmIL-40-treated septic mice 24 h after CLP (n = 4). **H** Concentrations of the proinflammatory mediators IL-6, TNF- α , and CCL2 in the blood and PLF of vehicle- and rmIL-40-treated septic mice 24 h after CLP, as detected via the Milliplex system (n = 5). **I** Representative images of apoptotic lung and spleen cells measured by TUNEL in vehicle- and rmIL-40-treated septic mice at 24 h after CLP (n = 5). The red arrow indicates apoptotic cells. The values are expressed as the means $\pm$ SEMs. Statistical analysis was performed with the Mann–Whitney U test (**B**, **F–H**) and log-rank test (**D**). * P < 0.05 and ** P < 0.01

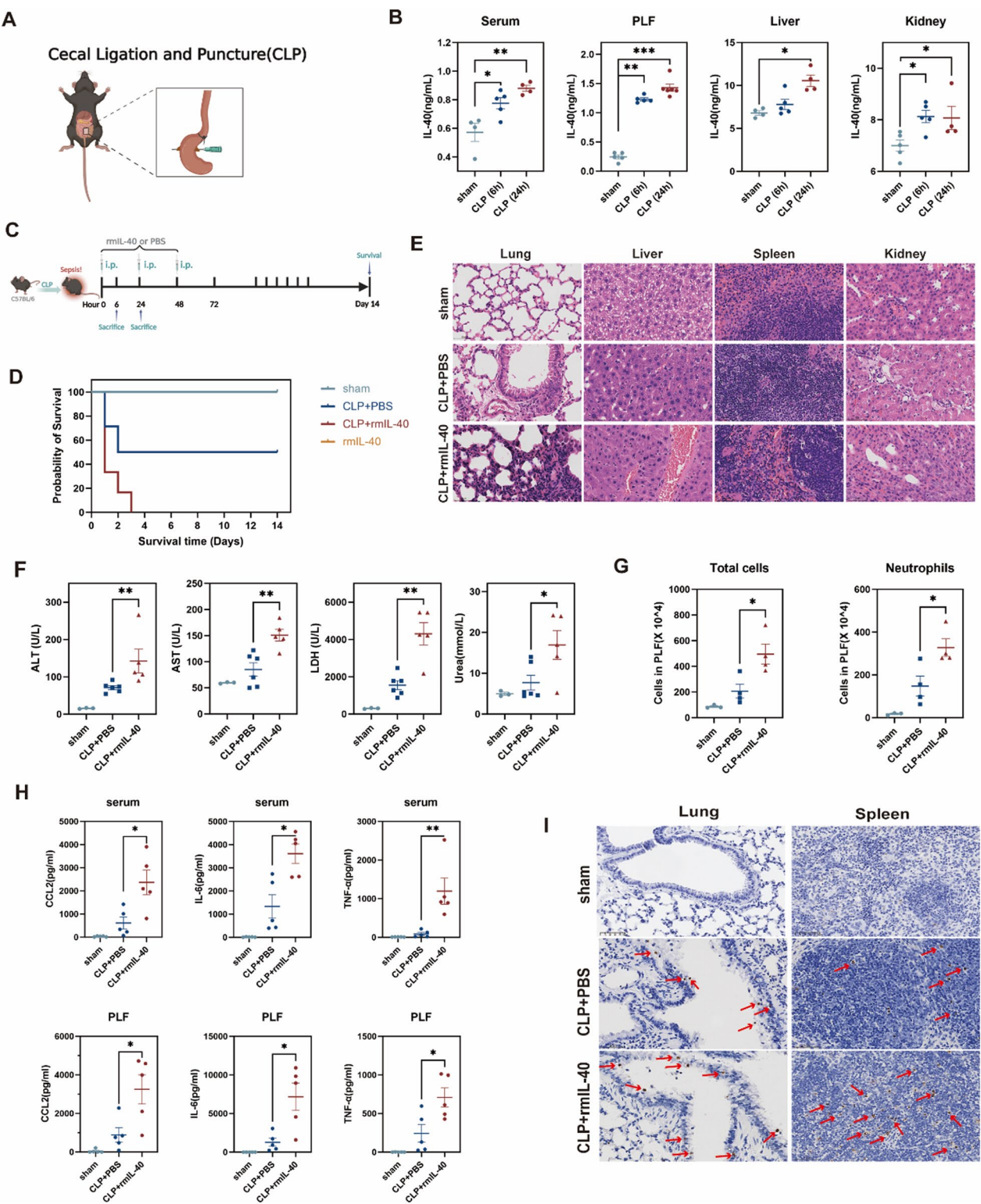


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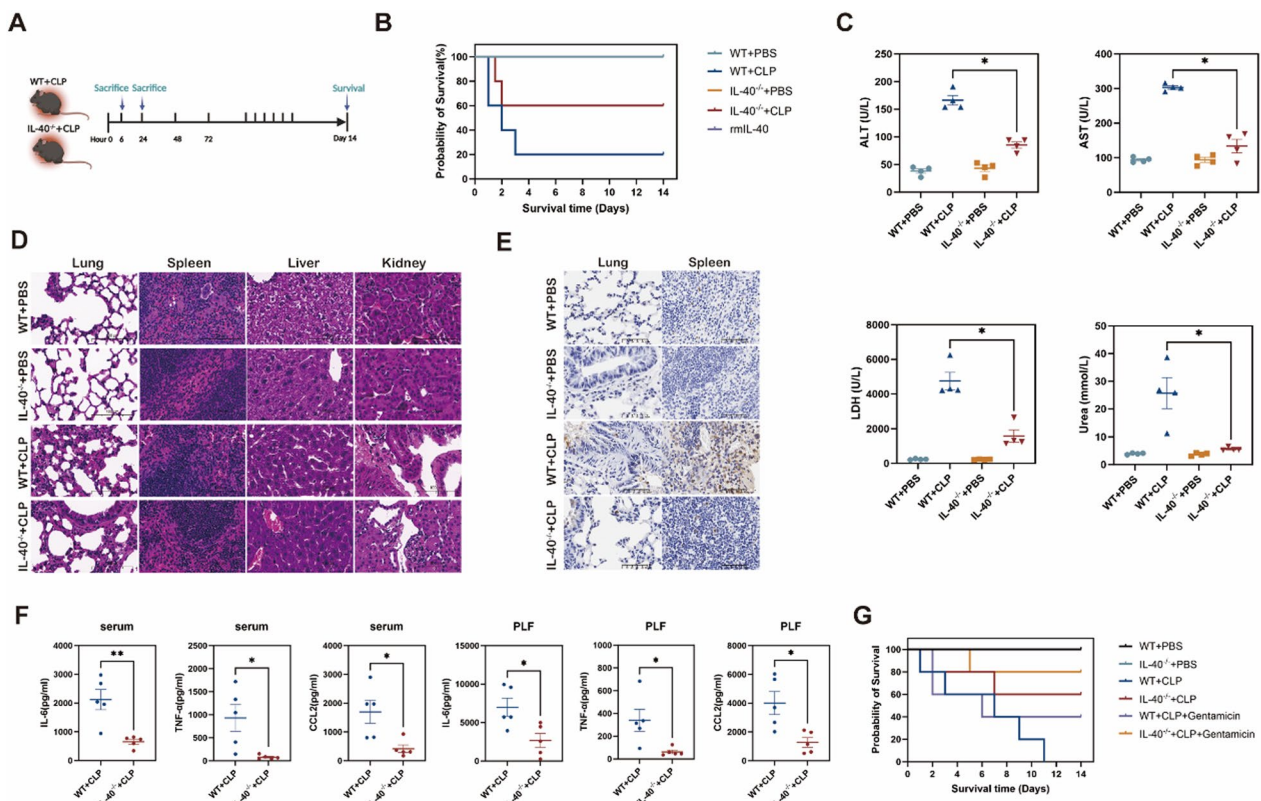


Fig. 3 Deficiency in IL-40 prevents the progression and outcome of polymicrobial sepsis. **A** Experimental design for establishing a polymicrobial sepsis model via CLP in WT and IL-40^{-/-} mice. The mice were sacrificed 24 h after CLP for analysis or monitored for survival for 14 days. **B** 14-day survival of the WT and IL-40^{-/-} septic mice (n=5). **C** Serological markers of organ damage, including ALT, AST, LDH, and urea, were measured in WT and IL-40^{-/-} septic mice 24 h after CLP (n=4). **D** Representative examples of H&E-stained lung, liver, spleen, and kidney sections from WT and IL-40^{-/-} septic mice 24 h after CLP (n=5). Scale bars, 100 μ m. **E** Representative examples of TUNEL-stained lungs and spleens from WT and IL-40^{-/-} septic mice 24 h after CLP showing apoptotic cells (n=5). Scale bars, 50 μ m. **F** Multiplex detection of IL-6, TNF- α , and CCL2 in the blood and PLF of WT and IL-40^{-/-} septic mice 24 h after CLP (n=5). **G** Gentamicin (3 mg/kg) was injected *i.p.* two hours before CLP and at 2, 24, 48, and 72 h after CLP, and survival was monitored for 14 consecutive days (n=5). The data are expressed as the means $\pm$ SEMs and represent three independent tests. Statistical analysis was performed with the Mann-Whitney U test (**C**, **F**) and log-rank test (**B**, **G**). * P < 0.05 and ** P < 0.01

monocytes, macrophages, dendritic cells, NK cells, mast cells, T cells, and B cells, were obtained from these 23 clusters (Fig. 4D). Notably, neutrophils annotated from clusters 5, 6, 8, and 16 accounted for a large proportion of immune cells and were considerably less abundant in the IL-40^{-/-} septic mice than in the WT septic mice (Fig. 4E and F). The identification of the top three DEGs revealed that these neutrophils highly expressed the proinflammatory genes S100A8 and S100A9 (S100A8/9^{hi} neutrophils) (Figs. 4G and S4B). In this context, we further identified the DEGs whose expression was most significantly altered between the IL-40^{-/-} and WT S100A8/9^{hi} neutrophils and performed Gene Ontology Biological Process (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses to elucidate the key roles of these S100A8/9^{hi} neutrophils in the IL-40's regulation during sepsis pathogenesis. We detected 260 DEGs

that were downregulated in the IL-40^{-/-} S100A8/9^{hi} neutrophils compared with the WT S100A8/9^{hi} neutrophils; these DEGs included those related to proinflammatory and chemoattractant IL-1RN, IL-1A, CCL4, CXCL1 and CXCL3 (Fig. S4C). Among these downregulated DEGs, the represented GO-BP terms less enriched in IL-40^{-/-} S100A8/9^{hi} neutrophils were “cytokine-mediated signaling pathway” and “response to lipopolysaccharide”, et al. (Fig. 4H), and the KEGG terms less enriched in IL-40^{-/-} S100A8/9^{hi} neutrophils than in WT neutrophils were “NOD-like receptor signaling pathway” and “apoptosis”, et al. (Fig. 4I). Consistent with the scRNA-seq results, flow cytometry analysis confirmed that the frequency of infiltrating CD11b⁺Ly6G⁺ neutrophils in the peritoneum was significantly lower in the IL-40^{-/-} mice than in the WT mice 24 h after CLP (Fig. 4J and K). As S100A8/9 are thought to induce prolonged inflammation and

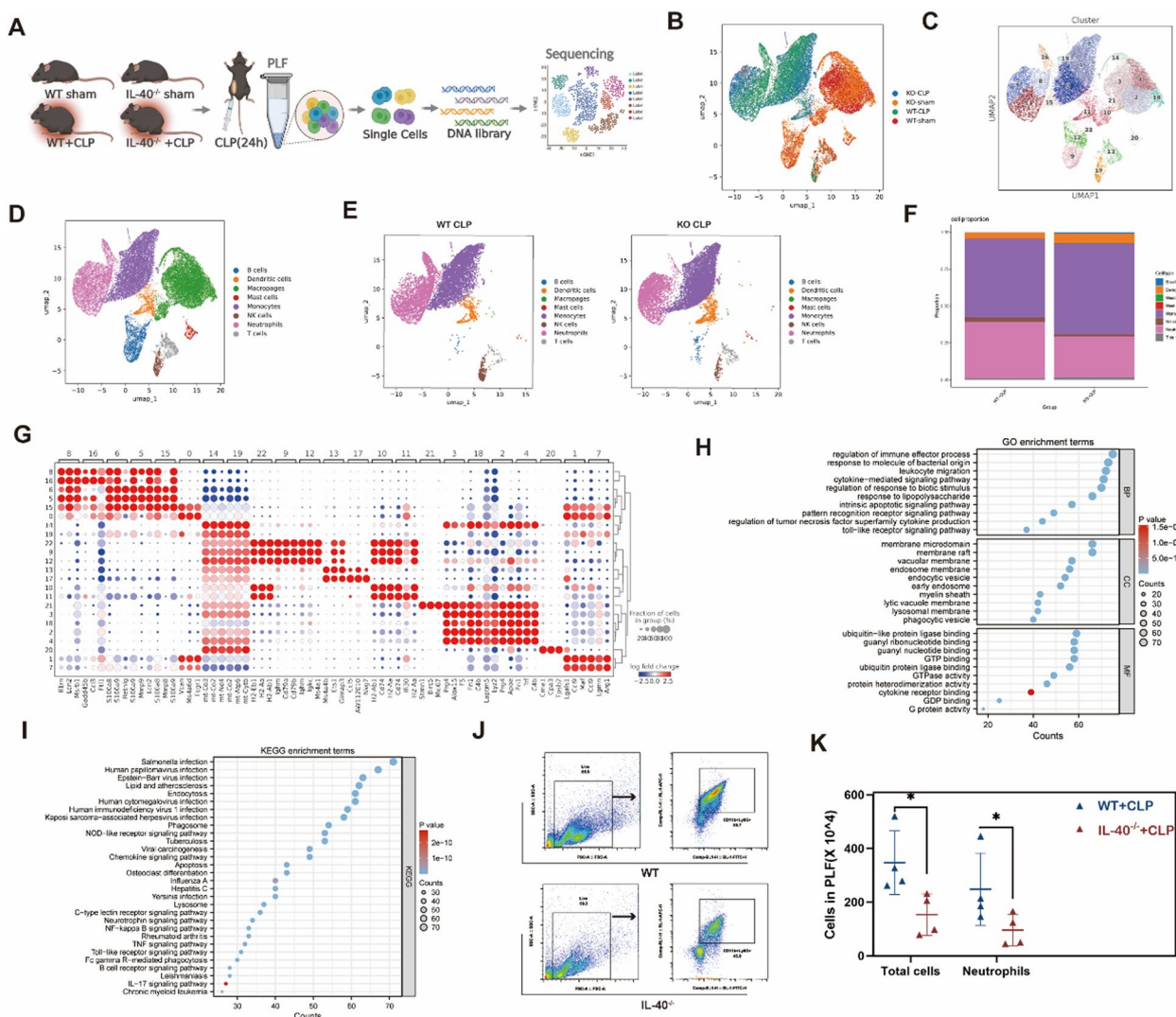


Fig. 4 IL-40 promotes sepsis by promoting S100A8/9^{hi} neutrophil recruitment and activation. **A** Schematic workflow of scRNA-seq of PLFs from IL-40^{-/-} and WT mice 24 h after CLP. **B** Uniform manifold approximation and projection (UMAP) representation of 42,896 single peritoneal cells from WT sham, WT + CLP, IL-40^{-/-} sham, and IL-40^{-/-} + CLP mice colored according to inferred cluster identity and sample origin. **C** The clusters were annotated into cell populations based on the marker genes and were distributed in UMAP colored by cell type. **D** UMAP plot showing the annotated cell types from the clusters in **C** based on the marker genes and colored by cell type. **E** UMAP plot showing the distributions of annotated cell types in WT and IL-40^{-/-} septic mice. **F** Proportions of annotated cell populations in WT and IL-40^{-/-} septic mice. **G** Dot plot showing the top three marker genes differentially expressed per cluster. The dot size represents the percentage of cells in each cluster with more than one read of the corresponding gene. **H** and **I** The top 10 enriched GO terms are categorized by biological process, molecular function, and cellular component, and the top 30 enriched KEGG terms are between those of WT and IL-40^{-/-} neutrophils. **J** Flow cytometry gating strategies for counting peritoneal neutrophils via flow cytometry according to CD11b and Ly6G expression. Neutrophils were identified as CD11b⁺Ly6G⁺ populations. **K** Bar chart showing the total number of inflammatory cells and CD11b⁺Ly6G⁺ neutrophils in the PLF (n=4). The data are expressed as the means ± SEMs and represent three independent tests. Statistical analysis was performed with the Mann–Whitney U test. *P<0.05.

endothelial injury in the septic microenvironment and serve as independent risk factors for poor prognosis in septic patients [23, 24], these data suggest a potential role for IL-40 in S100A8/9^{hi} neutrophil recruitment

and activation and that proinflammatory S100A8/9^{hi} neutrophils might mediate the detrimental effects of IL-40 in sepsis.

Deficiency in IL-40 protects against sepsis by mitigating NETosis both in vivo and in vitro

Recently, it was reported that S100A8/9^{hi} neutrophils may accelerate organ dysfunction during sepsis by disrupting mitochondrial homeostasis to contribute to the formation of NETs, and according to scRNA-seq data, the majority of NETs-related gene⁺ neutrophils are S100A8/A9^{hi} neutrophils [25, 26]. We then sought to explore in depth the mechanisms by which IL-40 regulates S100A8/9^{hi} neutrophil populations to contribute to sepsis progression. By UMAP analysis, a total of 11 cell clusters were identified (Fig. 5A), and were classified into 5 subtypes as G1-G5a (Fig. 5B). The heatmap of the top ten DEGs revealed that S100A8 was highly expressed in G1-G5a (Fig. S5A). Specifically, G1 neutrophils are characterized by RPL12^{hi}; G2 neutrophils are Chil3^{hi}; and G3 neutrophil has abundant expression of Retnlg (Retnlg^{hi} G3), whereas G4 neutrophils constitute more mature populations that highly express CCL6 and CXCL2 (CCL6^{hi} CXCL2^{hi} G4), which are key mediators of host defenses and inflammation. G5a is the most mature neutrophil characterized by high expression of CCL6 and Cstcd4 (CCL6^{hi} Cstcd4^{hi} G5) in the bone marrow, accounting for the majority of neutrophils in peripheral tissues (Fig. 5C), which is consistent with the reported findings on neutrophil classification [27, 28]. Notably, IL-40^{-/-} led to a significant reduction in the proportions of G4 and G5a neutrophils but an increase of G1, G2 and G3 neutrophils (Figs. 5D and S5B). In further investigations, DEGs were defined thereafter between WT and IL-40^{-/-} G1-G5a neutrophils (Fig. S5C). GO analysis of these DEGs revealed enrichment of key pathways involved in NETs formation and neutrophil recruitment, including nitric oxide-mediated signal transduction, neutrophil migration, neutrophil chemotaxis, positive regulation of cytokine production, as well as positive

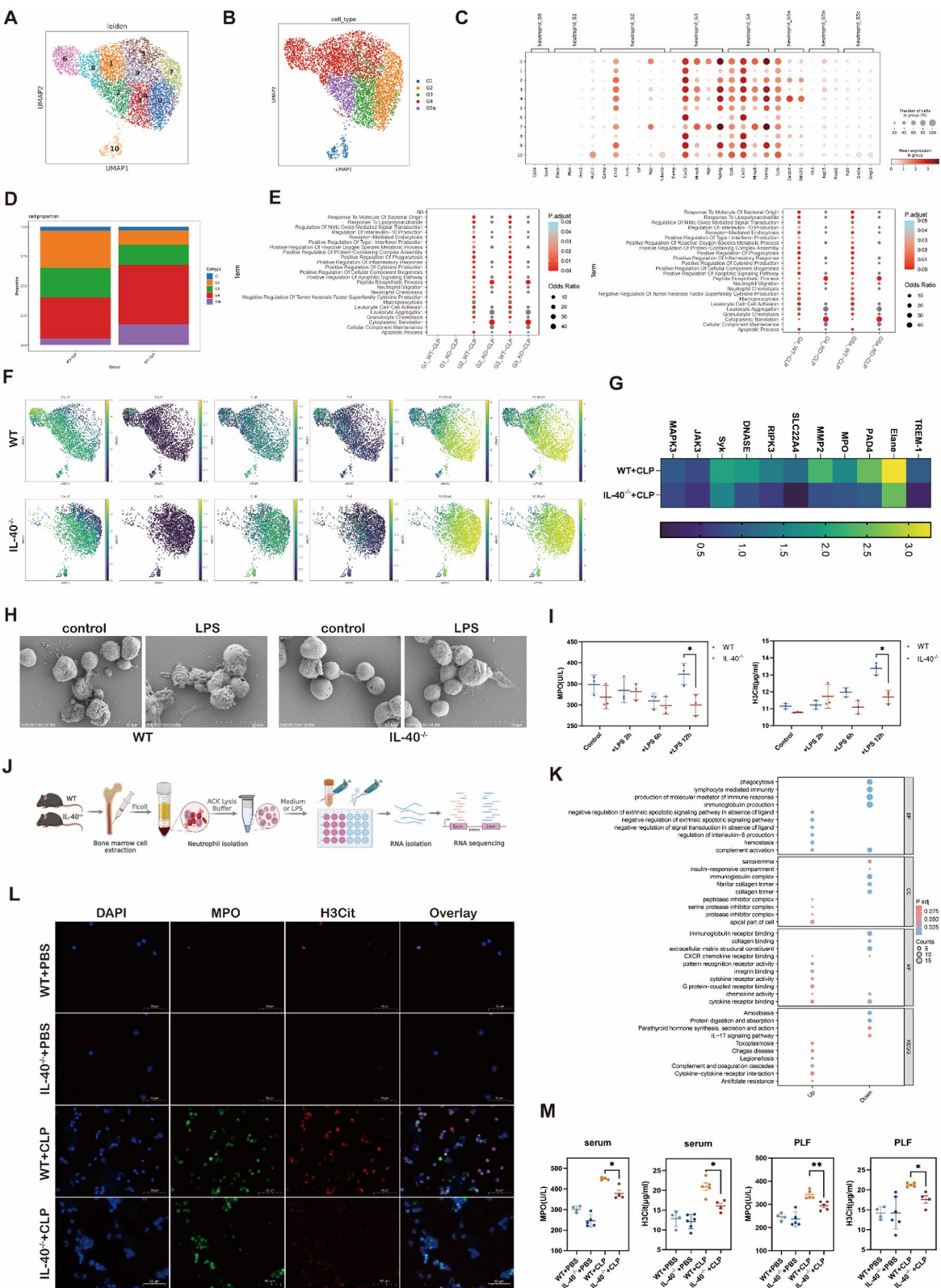
regulation of reactive oxygen species metabolic processes, et al., in WT neutrophils, which was attenuated in the IL-40^{-/-} neutrophils of septic mice (Fig. 5E). In combination with the above findings, genetic knockout of IL-40 also downregulated the expression of proinflammatory genes, including IL-6, CXCL1, CXCL2, S100A8, CCL6, and TNF- α , in neutrophils (Fig. 5F). Notably, IL-40^{-/-} led to reductions in NETs-related markers, including TREM-1, Elane, PAD4, MPO, and the NETosis-related pathways Syk, JAK3, and MAPK3, respectively (Fig. 5G), indicating that deficiency of IL-40 attenuated neutrophil activation and NETosis in sepsis.

We then sought to determine whether IL-40 is involved in NETs release and the physiopathology of sepsis in vitro. In this context, cytosolic LPS triggered NETs production in bone marrow-derived neutrophils isolated from WT mice, which was abrogated after IL-40 deficiency (Fig. 5H). Additionally, the NETosis-related MPO and H3Cit were lower in IL-40^{-/-} neutrophils induced with LPS than in WT neutrophils (Fig. 5I), with decreased PAD4 and ROS expressions as well (Fig. S5D). We then performed RNA sequencing (RNA-seq) of bone marrow-derived neutrophils stimulated with LPS (Fig. 5J). There were 311 DEGs identified between WT and IL40^{-/-} neutrophils following LPS induction. IL-40 deficiency led to 196 downregulated DEGs and 115 upregulated DEGs (Fig. S5E). Similarly, these downregulated genes were enriched in NETosis and proinflammatory conditions (Fig. 5K).

Finally, in vivo immunofluorescence costaining of MPO and H3Cit [29] in neutrophils further demonstrated that sepsis triggered NETosis, which was attenuated directly in IL-40^{-/-} mice (Fig. 5L), and deficiency of IL-40 led to a significant reduction of MPO, H3Cit expressions in serum and PLF as detected via ELISA (Fig. 5M), accompanied with decreased ROS and PAD4 expressions

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Fig. 5 IL-40 deficiency prevents NETosis during sepsis both in vivo and in vitro. **A** UMAP plot showing the 11 clusters from WT and IL-40^{-/-} neutrophils classified based on marker genes. **B** The clusters in (A) were annotated into G1-G5a cell populations and were distributed in UMAP and colored by cell type. **C** Dot plot showing the scaled expression of three selected signature genes for each cluster, colored according to the average gene expression in each cluster scaled across all clusters. The dot size represents the percentage of cells in each cluster with more than one read of the corresponding gene. **D** The proportions of G1-G5a neutrophil populations in WT and IL-40^{-/-} septic mice. **E** The top 24 enriched GO terms were categorized by biological process, molecular function, and cellular component between WT and IL-40^{-/-} G1-G5a neutrophils. **F** Representative UMAP plot of proinflammatory CXCL2, CXCL1, CCL6, TNF- α , S100A8 and S100A9 in WT and IL-40^{-/-} G1-G5a neutrophils. **G** Relative expression of NETs associated markers and pathways in WT and IL-40^{-/-} G1-G5a neutrophils. **H** and **I** Bone marrow-derived neutrophils were isolated from WT and IL-40^{-/-} mice and stimulated with LPS to induce NETs; the cells were collected for electron microscopy visualization of NETs, while the supernatants were collected for MPO and H3Cit detection via ELISA (n = 3). **J** Experimental scheme for bulk RNA-seq of bone marrow-derived neutrophils from septic WT and IL-40^{-/-} mice stimulated with LPS. **K** GO enriched terms categorized by biological process, molecular functions, and cellular components and KEGG enriched terms between WT and IL-40^{-/-} neutrophils based on the DEG. **L** Representative images of IF staining for the NET-related MPO and H3Cit in peritoneal cells from WT and IL-40^{-/-} septic mice in vivo. The nuclei were stained with DAPI (blue). MPO: green; H3Cit: red. **M** WT and IL-40^{-/-} mice were sacrificed 24 h after CLP, and serum and PLF were collected for MPO and H3Cit detection via ELISA (n = 4–6). The data are representative of three individual experiments. The values are expressed as the means $\pm$ SEMs. Statistical analysis was performed with the Mann–Whitney U test (**I**, **M**). * $P < 0.05$, and ** $P < 0.01$



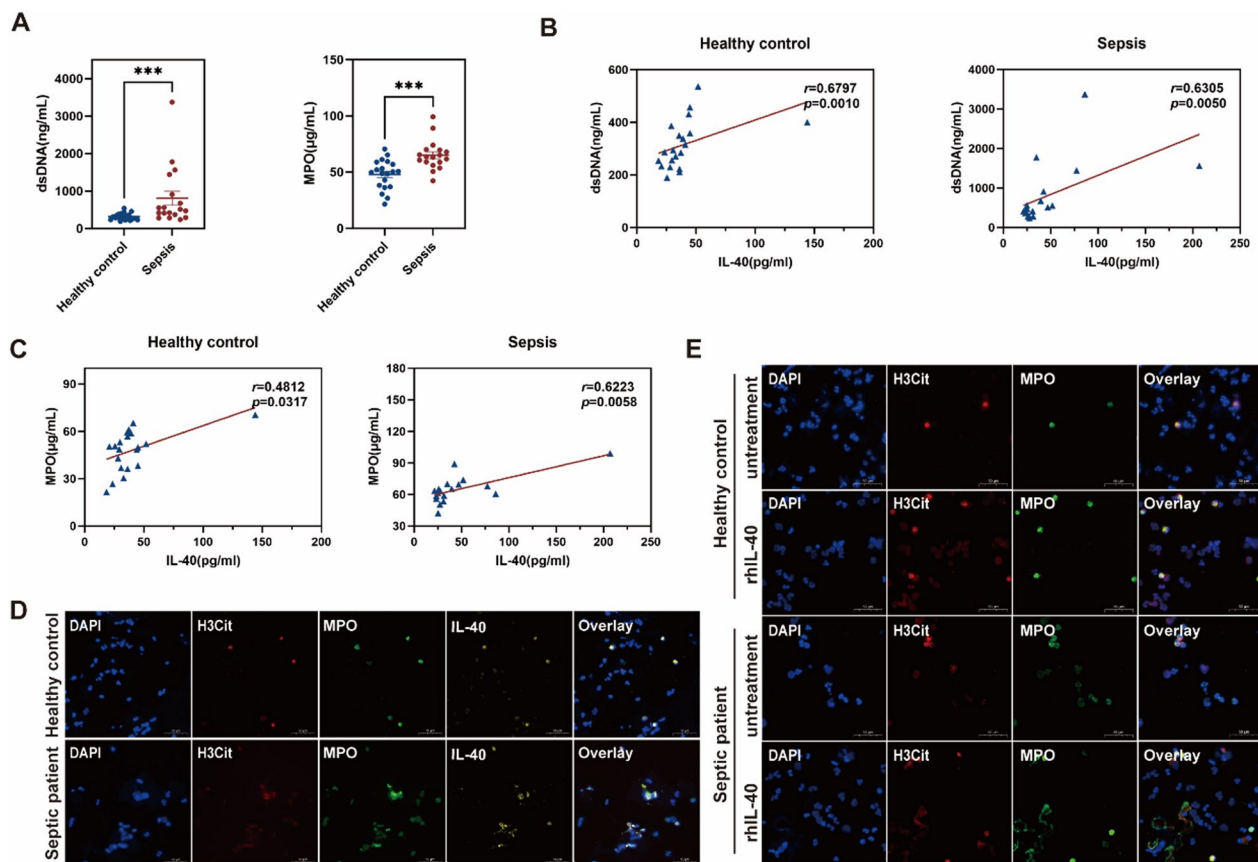


Fig. 6 IL-40 was associated with NETs release in septic patients. **A** Adult septic patients ($n = 18$) and healthy controls ($n = 20$) were enrolled, and serum was collected for NETs-related dsDNA and MPO detection. **B** and **C** Correlations between circulating IL-40 levels and dsDNA and MPO in septic patients ($n = 18$) and healthy controls ($n = 20$). **D** Representative images showing the colocalization of IL-40 (orange), MPO (green), and H3Cit (red) in neutrophils from septic patients and healthy controls; the nuclei were stained with DAPI (blue). **E** Neutrophils from septic patients and healthy controls were isolated and induced with LPS for 4 h, with or without rhIL-40 treatment (500 ng/mL). The cells were collected for MPO (green) and H3Cit (red) staining; nuclei were stained with DAPI (blue). The data are representative of at least two individual experiments. The values are expressed as the means $\pm$ SEMs and were analyzed with the Mann–Whitney U test (**A**) and Spearman's correlation coefficient test (**B** and **C**). *** $P < 0.001$

(Fig. S5F), which is consistent with the sequencing data. Together, these data indicate that deficiency of IL-40 fails to shape into the pro-inflammatory phenotype and less production of NETs.

IL-40 is associated with NETs release in patients with sepsis

To demonstrate the clinical significance of our studies, we enrolled 18 patients with sepsis and 20 healthy volunteers as controls. The demographic and clinical characteristics of the septic patients are shown in Table S5. We confirmed that septic patients had higher levels of circulating NETs than healthy volunteers (Fig. 6A) and that IL-40 expression was significantly positively correlated with MPO and dsDNA (Fig. 6B and C). By immunofluorescence co-staining for MPO, H3Cit, and IL-40 in neutrophils isolated from septic patients, we found that these primary neutrophils produced NETs and expressed

IL-40, demonstrating that IL-40 expression was positively synchronous with NETs release (Fig. 6D). Additionally, ex vivo rhIL-40 treatment further strengthened the NETs productions by circulating neutrophils (Fig. 6E). Collectively, these results provide further evidence that the IL-40 pathway is involved in the release of NETs during sepsis.

Discussion

In recent years, the roles of novel immunoregulatory factors such as IL-17D, IL-27, and IL-34 in regulating the uncontrolled inflammatory immune response during sepsis have garnered significant attention [30–32]. Owing to their short half-life, stability, and easy detection, these immunoregulatory proteins hold groundbreaking value in the early diagnosis and treatment of sepsis, representing a new direction for screening candidate biomarkers

for the diagnosis and prognosis of sepsis. In our study, we suggest that the novel cytokine IL-40 may be a novel therapeutic target for sepsis. In both our adult and pediatric cohort analyses, septic patients with elevated circulating IL-40 levels at admission had a greater risk of 28-day mortality. High plasma IL-40 levels were used to stratify the early death of critically ill patients with sepsis, along with elevated PCT, CRP, and LDH levels; higher SOFA scores; and increased absolute WBC/neutrophil counts. These findings indicate that sepsis patients who might benefit from an anti-IL40 injection could be identified and treated at the right stage of sepsis if plasma levels of IL-40 are assessed at the time admission. Our clinical study has several advantages. First, we used two cohorts of septic patients, including both adults and children, which may strengthen the prognostic application of IL-40 in sepsis. Second, as a novel cytokine, IL-40 is stable, and its elevated expression in the circulation can persist for up to 3 days after admission, thus contributing to monitoring the disease status and prognosis of sepsis patients. Additionally, as a small secretory protein, IL-40 has the advantages, such as being easily measured and beneficial for clinical application.

The correlation of IL-40 levels and their roles in a polymicrobial sepsis mouse model reproduced human data, where mice with IL-40 treatment had worsened organ dysfunction and a greater chance of death from sepsis, while deficiency in IL-40 presented mitigated cytokine storm, organ failure, and mortality, indicating that secreted IL-40 activates genes associated with the hyperinflammatory sepsis phenotype and that anti-IL-40 therapy may constitute a novel therapeutic strategy for life-threatening sepsis. To the best of our knowledge, this is the first study illustrating the therapeutic targeting of IL-40 in sepsis.

In our further investigations, by using sc-RNA-seq and validated by Bulk RNA-seq, our pilot study demonstrated that the role of IL-40 in promoting sepsis progression was highly associated with its regulation on S100A8/9^{hi} neutrophils, which is consistent with findings showing that neutrophils are induced to differentiate into the S100A8/9^{hi} phenotype to induce septic mortality [23]. Importantly, S100A8/9 heterodimers, are known biomarkers of NETs formation; sepsis-derived S100A8/9 leads to the release of oxidized mitochondrial DNA that contributes to NETosis, which then, in turn, releases S100A8/9, forming a positive feedback loop and thereby amplifying the proinflammatory response [25, 33]. Specifically, by further annotating the S100A8/9^{hi} neutrophils into subtypes, we found that deficiency in IL-40 decreased the proportions of the more mature G4-G5a subtype neutrophils, which is closely associated with neutrophil migration, NETosis,

apoptosis, etc. Therefore, our findings regarding the regulatory effect of IL-40 on S100A8/9^{hi} neutrophils during sepsis are consistent with the reported evidence and reveal a novel mechanism driving sepsis progression by controlling NETs extrusion. The role of NETs in controlling infection was first described by Brinkmann et al., whereas NETs have been shown to act as a double-edged sword during sepsis in the past decade [34–36]. In 2008, NETs-related markers (cf-DNA-NETs) were first found in large quantities in the peripheral blood of septic patients and were significantly positively correlated with poor prognosis in sepsis patients [37], revealing a novel role of NETs in sepsis beyond their primary bactericidal function. Subsequent mechanistic studies confirmed that NETs and their components are cytotoxic and can exacerbate endothelial damage, and mediate macrophage pyroptosis, thereby exacerbating the uncontrolled inflammatory response in sepsis [38–42]. Our study herein provides an upstream key molecule that regulates the formation of NETs in sepsis and that NETosis during sepsis is an IL-40-dependent event.

The clinical relevance of our findings is supported by the observation that neutrophils from septic patients produce higher levels of NETs and IL-40 has a positive correlation with NETs, and we confirmed for the first time that neutrophils undergoing NETosis expressed IL-40 on cell membranes, suggesting that IL-40 is synchronous with NETs release [15]. Moreover, primary neutrophils from septic patients treated with rhIL-40 produce more NETs, similar to what has been observed in mice, indicating that IL-40 drives NETosis [18]. Considering that neutrophil activation-related pathways were highly associated with non-surviving septic patients compared with healthy volunteers [43], our study herein proved that IL-40 is an upstream factor of sepsis by targeting NETs.

There are several limitations to our study. The sample size within our study cohort was relatively small, which limits the strength of our findings regarding the AUC of IL-40 in predicting sepsis prognosis to only moderate capacity. Despite this, IL-40 exhibited a higher AUC compared to established sepsis prognostic markers such as PCT, CRP, and SOFA scores, suggesting that IL-40 may be a more reliable predictor of sepsis survival than those markers. Future clinical studies involving enlarged sample sizes are therefore needed to illustrate this point. Additionally, the functions of IL-40 rely on its receptors. Nevertheless, there are no investigations regarding the receptor of IL-40 yet which also warrants further in-depth studies.

In summary, within the current medical situation where sepsis mortality remains high in the absence of reliable biomarkers and targeted therapy, our study

herein provides a novel prognostic marker and a therapeutic target for sepsis. Our pilot study revealed that the novel IL-40 is active in neutrophils from both septic mice and patients and plays a crucial role in NETosis and organ dysfunction during sepsis. Therefore, therapy against IL-40 could be clinically applicable for improving sepsis outcomes. Additionally, considering that IL-40 is the effector of NETosis, promising inhibitors of IL-40 could have a pharmacologic impact on treating a broader spectrum of diseases characterized by NET-mediated immunopathology, in addition to sepsis.

Methods and materials

Human study

This study involved the analysis of prospective cohorts of adult patients with sepsis ($n=53$) or septic shock ($n=63$) and healthy controls ($n=50$) from Zhongda Hospital, as well as pediatric septic patients ($n=88$) or septic shock patients ($n=18$) and healthy controls ($n=90$) from the Children's Hospital of Nanjing Medical University. In addition, 50 non-septic adult patients as well as 50 non-septic pediatric patients in critical conditions under mechanical ventilation (for example, intracranial hemorrhage, cerebrovascular accidents and hypertensive emergencies, et al., following successful resuscitation with no infection) were recruited from ICU as patient control subjects as we previously described [20]. To determine NETs, another cohort of 18 septic patients with 20 age and sex-matched healthy controls from Zhongda Hospital were enrolled. All the patients were eligible and diagnosed according to the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) [1]. 3 mL of whole blood samples were collected from all patients within 24 h of ICU admission or on days 3, 7, and 10 when applicable. The serum was separated and stored at -80°C until analysis. Baseline demographics and clinical details, including SOFA scores, were collected in the first 24 h after admission to the ICU. Laboratory data, including absolute WBC/neutrophil counts and C-reactive protein (CRP), procalcitonin (PCT), and lactate (LDH) levels, were recorded during blood punctures. The relevant outcomes on the basis of 28-day survival were also monitored.

Murine model of sepsis

Inbred pathogen-free IL-40^{-/-} mice were obtained from Cyagen Biosciences (Guangzhou, China); Wild type C57BL/6 mice (WT) were obtained and housed under specific pathogen-free conditions at the Laboratory Animal Services Center, Southeast University. Cecal ligation puncture (CLP) was used as a model of polymicrobial sepsis, as described previously [21]. Briefly, the mice were anesthetized with a mixture of xylazine (4.5 mg/kg) and

ketamine (90 mg/kg) intraperitoneally. Under sterile conditions, a 1-cm incision was made parallel to the midline, and the cecum was exteriorized and ligated. The cecum was then punctured twice with a 22-gauge needle and placed back in the peritoneal cavity. Mice that underwent CLP either received saline (5 mL/100 g body weight) subcutaneously for resuscitation or were treated with IL-40. For the treatment of mice with rmIL-40, the mice were given rmIL-40 (800 ng/ 100 μL) (MyBioSource Inc., USA) via *i.p.* injection immediately after CLP. To treat septic mice with antibiotics when applicable, gentamicin (3 mg/kg) was administered *i.p.* 2 h before CLP and at 2, 24, 48, and 72 h after CLP. The mice were monitored for survival for 14 consecutive days or sacrificed at the indicated times for analysis. After sacrifice, blood was collected via cardiac puncture, and peritoneal lavage fluid (PLF) was collected by instilling and aspirating 5 mL of precooled PBS into the murine abdominal cavity two times. Tissues, including the lung, spleen, kidney, and liver, were obtained for the next step analysis.

Enzyme-linked immunosorbent assay (ELISA) of IL-40

IL-40 levels in the plasma of patients with sepsis were determined via human IL-40 ELISA kits according to the manufacturer's instructions (Lun Chang Shuo Biotech, China). Mouse IL-40 was detected with murine IL-40 ELISA kits (MyBiosource, Inc.). Briefly, immunoplates were coated with anti-IL-40 at 4°C overnight and blocked with 1% BSA at 37°C for 1 h; samples were incubated at 37°C for 2 h with an IL-40 detection antibody, followed by incubation with HRP-conjugated anti-rabbit IgG at 37°C for 1 h; 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added, and the samples were incubated at 37°C for 20 min; then, sulfuric acid solution was added to terminate the reaction. The results were obtained via a Spark[®] multimode microplate reader (Tecan Life Sciences, Switzerland) at 450 nm and 570 nm.

Calculation of bacterial loads

Inbred mice underwent CLP as described above. The PLF, blood, and spleen were harvested under sterile conditions 24 h after CLP. The blood was diluted 10 and 10^2 fold; the spleen homogenates were diluted 10^2 and 10^3 fold; and the PLFs were diluted 10^4 , 10^5 , and 10^6 fold with serial PBS. 100 μL diluted blood, PLF, and spleen were plated on blood agar plates (Kemajia Microbiology Inc., Shanghai, China) and incubated at 37°C for 18 h for counting the colonies.

Histopathology examination

The mice were euthanized 24 h after sepsis. The lung, spleen, kidney, and liver were harvested and fixed in 4% buffered formalin and infused for 24–48 h. After dehydration and immersion in xylene, the tissues were embedded in paraffin, cut into Sects. (5 μ m thick), stained with H&E, and digitized with a Digital Pathology Slide Scanner (KFBIO Technology, China). To detect apoptotic cells, lung and spleen sections were dewaxed, protease K was added, the sections were stained with a TUNEL detection solution and visualized with a Digital Pathology Slide Scanner.

Measurement of biochemistry biomarkers of organ injury

The mice were euthanized 24 h after CLP; plasma was collected to assess LDH, alanine transaminase (ALT), aspartate transaminase (AST) and urea levels via the routine chemical pathology service at the clinical laboratory affiliated with Zhongda Hospital, Southeast University, Nanjing, China.

Longitudinal cytokine profile

The concentrations of murine TNF- α , IL-6, CCL2, IFN- γ and IL-10 were quantified with a Mouse Cytokine Milliplex MAP assay kit (Millipore Corporation, Billerica, MA, USA) via a Cytometric Bead Array (CBA) Flex Sets (BD Biosciences, San Jose, CA, USA) on a BD FACSVia flow cytometer (BD Biosciences). The data were analyzed via BD Biosciences FCAP array software (V3.0).

Determination of neutrophils in the PLF

Peritoneal cell suspensions were blocked with anti-CD16/32 (BioLegend, 1:100), followed by staining with monoclonal antibodies (mAbs) against CD11b (BioLegend, 1:100) conjugated with FITC, lymphocyte antigen six complex locus G (Ly6G, BioLegend, 1:100) conjugated with APC. After washing and resuspending, the cell populations were analyzed with an Attune Nxt flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA). At least 10,000 cells were collected.

Single-cell RNA sequencing

Freshly isolated PLFs were lysed with RBC lysis buffer (Solarbio Life Sciences, Beijing, China) and washed with 1 $\times$ PBS containing 2% FBS. Cell viability was assessed via a Count-star, in which cells with greater than 80% viability were qualified for library construction. The prepared single cells were then paired with beads in a gel bead-in emulsion (GEM). After exposure to cell lysis buffer, polyadenylated RNA molecules were hybridized into the beads, and reverse transcription

was performed to generate cDNA. During cDNA synthesis, each cDNA molecule was tagged on the 5' end with a UMI and a cell label indicating its cell of origin. The 10 $\times$ beads were then subjected to second-strand cDNA synthesis, adaptor ligation, and universal amplification. The sequencing libraries were prepared via the use of randomly interrupted whole-transcriptome amplification products to enrich the 3' ends of the transcripts linked with the cell barcode and UMI. The sequencing libraries were quantified via a high-sensitivity DNA chip (Agilent) on a Bioanalyzer 2100 and a Qubit high-sensitivity DNA assay (Thermo Fisher Scientific). The libraries were sequenced on a NovaSeq 6000 (Illumina, Inc., San Diego, CA, USA), generating 150 bp paired-end reads. The sequencing and bioinformatics analysis were performed by Hangzhou Cosmos Wisdom Co., Ltd. (Hangzhou, China).

Single-cell RNA-seq data processing

The reads were processed via the Cell Ranger 4.0 pipeline with the default and recommended parameters. FASTQs generated from Illumina sequencing output were aligned to the mouse genome, version GRCm38, via the STAR algorithm. Next, gene-barcode matrices were generated for each sample by counting UMIs and filtering non-cell-associated barcodes. Finally, a gene-barcode matrix containing the barcoded cells and gene expression counts was generated. This output was then imported into the Seurat (v3.2.0) R toolkit for quality control and downstream analysis. Low-quality cells were excluded on the basis of the number of detected transcripts or genes and the percentage of reads mapped to mitochondrial genes. The expression of mitochondrial genes was calculated via the percentage feature set function of the Seurat package. Normalized data were used for principal component analysis (PCA). Uniform manifold approximation and projection for dimension reduction (UMAP) were used to visualize the clusters on a two-dimensional map to identify the cell types. The cells were subsequently clustered or subclustered via graph-based clustering of the PCA-reduced data via the Louvain method after a shared nearest-neighbor graph was computed. We used the Wilcoxon rank-sum test for each cluster to find significantly differentially expressed genes (DEGs) when the remaining clusters were compared. SCINA and known marker genes were used to identify cell types. DEGs between different samples or clusters were determined via FindMarkers in Seurat for differential expression analysis and functional enrichment. Essentially, DEGs with $|\log_2FC| > 0.25$ and a Q value ≤ 0.05 were considered significantly differentially expressed genes. In addition, functional enrichment analysis of GO (<https://github.com/tanghaibao/Goatools>), as well as Kyoto

Encyclopedia of Genes and Genomes (KEGG, <https://www.kegg.jp/>) pathway analysis, was carried out with a Bonferroni-corrected P value ≤ 0.05 compared with the whole-transcriptome background.

Bulk RNA sequencing and functional annotations

Total RNA was extracted from bone marrow-derived neutrophils via QIAzol reagent (Qiagen Inc., Valencia, CA, USA). Briefly, mRNA was purified from total RNA (100 ng) via poly-T oligo-attached magnetic beads and fragmented randomly via the addition of fragmentation buffer for library construction. The RNA quality was determined via a 5300 Fragment Bioanalyzer (Agilent Technologies, Beijing, China) and quantified via an ND-2000 (NanoDrop Technologies, Inc., Wilmington, DE, USA). Only high-quality RNA samples ($OD_{260}/OD_{280} = 1.8 \sim 2.2$, $OD_{260}/OD_{230} \geq 2.0$, $RIN \geq 6.5$, $28S:18S \geq 1.0$, $>1 \mu g$) were used to construct a sequencing library. The sequencing libraries were created via the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (Illumina Inc., San Diego, CA, USA). Libraries were sequenced on the NovaSeq 6000 sequencer platform to generate 150 bp paired-end reads according to the manufacturer's instructions. The sequenced reads/raw reads containing low-quality reads or reads with adaptors were filtered to obtain clean reads, and the clean reads were subsequently mapped to the reference genome via HISAT (version 2.0.4). To analyze gene expression levels, the expression level of each transcript was calculated according to the transcripts per million reads (TPM) method, and RSEM was used to quantify gene abundances. Differential gene expression analysis was performed via DESeq2 (version 1.10.1, Bioconductor); genes with $|\log_2 FC| \geq 1$ and $FDR \leq 0.05$ were considered DEGs. Essentially, GO functional enrichment and KEGG pathway analyses were carried out with Goatools and KOBAS, respectively.

Isolation of primary neutrophils

To isolate primary neutrophils from mice, bone marrow was collected as previously described [44]. The cells were then suspended in Hank's balanced saline solution (HBSS) with 15 mM ethylenediaminetetraacetic acid (EDTA). After centrifugation ($400 \times g$, 10 min, $4^\circ C$), the cells were resuspended in 2 mL of HBSS with 2 mM EDTA. Next, according to the manufacturer's instructions, neutrophils were isolated with a mouse bone marrow neutrophil isolation kit (Solarbio Life Sciences). The purity of the isolated neutrophils was greater than 90%, as characterized by the use of anti-CD11b plus anti-Ly6G mAb, as described above. For the isolation of human

circulating neutrophils from peripheral blood, 10 mL of whole blood was collected from either septic patients or healthy controls; phosphate-buffered saline-diluted fresh human blood was centrifuged with 1.082 g/mL isotonic Percoll solution (GE Healthcare Life Sciences) for 20 min at 1800 rpm. After RBC lysis to eliminate erythrocytes, flow cytometry revealed a neutrophil population with a viability $>90\%$. The isolated neutrophils were resuspended in RPMI 1640 medium supplemented with 5% FBS and 1% penicillin/streptomycin (P/S) for further experiments.

In vitro NETs induction

Primary neutrophils from the bone marrow of WT and IL-40^{-/-} mice or human peripheral blood were separated as described above. Neutrophils were seeded in 24-well cell culture plates at a concentration of 5×10^5 /mL and induced with sterile LPS (30 μg /mL) for 4 h, with or without rm/rhIL-40 treatment (500 ng/mL). The cells were then prepared for electron microscopy or immunofluorescence staining, and the supernatants were collected for NETs-related MPO/DNA, H3Cit, ROS and PAD4 detection when appropriate.

Immunofluorescence microscopy

Primary human and mouse neutrophils were isolated, and NETs were induced as described above. Neutrophils were then fixed in 4% paraformaldehyde for 30 min at room temperature (RT), followed by blocking with 3% BSA for 30 min. The target markers were subsequently stained via multiplex immunohistochemistry (mIHC) with a multiplex IHC detection kit. Briefly, the cells were stained with the corresponding primary antibodies against MPO (1:2000, Abcam Limited, Cambridge, UK), H3Cit (1:1000, Abcam Limited) and IL-40 (1:500, MyBiosource Inc.). when appropriate at $4^\circ C$ overnight. After being washed with PBS, the cells were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 50 min at room temperature. Next, the cells were incubated with the appropriate fluorophore-conjugated tyramide signal amplification (TSA) mixture for 10 min at RT. The cells were then subjected to microwave treatment to strip the tissue-bound primary/secondary antibody complexes, followed by repeated staining with primary antibodies until all the markers were fully labeled. Finally, the slides were stained with DAPI in the dark for 10 min at room temperature and mounted with anti-fade mounting medium. The slides were subsequently visualized via an automatic digital slide scanner (3D HISTECH Ltd., Budapest, Hungary).

NETs quantification

Primary neutrophils from mice or humans were separated as described above. MPO, H3Cit, PAD4, and ROS were detected with the corresponding ELISA Kit (MEIKE Biotech, China) according to the manufacturer's instructions, and the results were read via the Spark® multimode microplate reader (Tecan life sciences) at 450 nm. For dsDNA detection, the amount of DNA was quantified via a Quant-iT™ PicoGreen™ dsDNA Reagent and Kit (Thermo Fisher Scientific). The fluorescence intensity was quantified via the Spark® multimode microplate reader (Tecan Life Sciences) with excitation at 488 nm and emission at 525 nm.

Scan electron microscopy (SEM)

NETs were induced as described above. Next, neutrophils were transferred to slides and fixed with electron microscope fixative for 2 h at RT in the dark, followed by fixation again with 1% osmic acid for 1–2 h at RT in the dark. The fixed sample was rinsed with 0.1 M PBS (pH 7.4) 3 times. Next, the samples were dehydrated with an ascending acetone series. After desiccation, the samples were attached to metallic stubs via carbon stickers and sputter-coated with gold for 30 s. Finally, the NETs in the samples were visualized, and images were taken with an ultrahigh-resolution scanning electron microscope (Hitachi SU8000 Series, Hitachi High-Tech Corp, Tokyo, Japan).

Statistics

All the statistical analyses were performed via GraphPad Prism software (version 10.0; La Jolla, CA, USA) and IBM SPSS (version 26.0; Armonk, New York, USA). Each experiment was replicated at least 2–3 times as independent biological replicates. Unless otherwise noted, all values are expressed as the mean ± SEM. Significant data comparisons between 2 groups were analyzed via a 2-tailed Student's *t* test or nonparametric Mann–Whitney *U* test. Variances of multiple comparisons were examined via one-way ANOVA followed by Tukey post hoc analysis. A nonparametric Spearman's rank correlation test was used to evaluate the correlation of the IL-40 concentration with the concentrations of multiple cytokines/chemokines, laboratory parameters, and NETs-related markers. Receiver operating characteristic (ROC) curves were constructed, and the area under the curve (AUC) was determined to evaluate the predictive and prognostic value of IL-40, PCR, CRP and SOFA scores in sepsis patients. Significance was set at **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13054-025-05257-2>.

Supplementary file 1.

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Author contributions

X.G. designed the experiments. X.G. and G.Q.W. obtained the funding. Y.Q.J. helped with the clinical experiments. S.J.C., X.L., C.Z., Z.H., Y.H.L., S.M., and Y.M.Y. performed the experiments and analyzed the data. X.G. and S.J.C. wrote the manuscript. C.K.W. and G.Q.W. revised and edited the manuscript.

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Availability of data and materials

Datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The human study was approved by the affiliated Zhongda Hospital of Southeast University Clinical Research Ethics Committee (Registration No. 2023ZDSYLL463-P01), and written informed consent was obtained from patients or their legally authorized representatives before enrollment, according to the Declaration of Helsinki. All animal experiments complied with the guidelines of the Animal Experimentation Ethics Committee (AEEC) Guide for the Care and Use of Laboratory Animals and were approved by the AEEC, Southeast University.

Competing interests

The authors declare no competing interests.

Author details

¹Center of Clinical Laboratory Medicine, Zhongda Hospital, Southeast University, Nanjing, Jiangsu, China. ²Department of Laboratory Medicine, Medical School of Southeast University, Nanjing, Jiangsu, China. ³Department of Laboratory Medicine, Children's Hospital of Nanjing Medical University, Nanjing, Jiangsu, China. ⁴Institute of Chinese Medicine and State Key Laboratory of Research on Bioactivities and Clinical Applications of Medicinal Plants, The Chinese University of Hong Kong, Shatin, Hong Kong, China. ⁵Department of Chemical Pathology, The Chinese University of Hong Kong, Shatin, Hong Kong, China. ⁶Li Dak Sum Yip Yio Chin R & D Centre for Chinese Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China.

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