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Sepsis induced dysfunction of liver type 1 innate lymphoid cells

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Abstract

Background Sepsis is a life-threatening condition triggered by uncontrolled immune responses to infection, leading to widespread inflammation, tissue damage, organ dysfunction, and potentially death. The liver plays a crucial role in the immune response during sepsis, serving as a major site for immune cell activation and cytokine production. Liver type 1 innate lymphoid cells (ILCs) consist of NK cells and ILC1s. They maintain the local immune microenvironment by directly eliminating target cells and secreting cytokines. However, the specific roles and pathological changes of liver-resident NK cells and ILC1s during sepsis remain poorly understood.

Results This study aims to investigate the pathological changes of NK cells and ILC1s, which might contribute the dysfunction of liver. Sepsis mouse model was established by cecal ligation and puncture (CLP). Mouse immune cells from liver were isolated, and the surface makers, gene expression profiles, cytokine response and secretion, and mitochondrial function of NK (Natural Killer) cells and ILC1s (Innate Lymphoid Cell 1) were analyzed. A significant decrease in the number of mature NK cells was observed in the liver after CLP. Furthermore, the secretion of interferon-gamma (IFN-γ) was found to be reduced in spleen and liver NK cells when stimulated by IL-18. Mitochondrial activities in both liver NK cells and ILC1 were found to be increased during sepsis, suggesting an enhanced metabolic response in these cells to combat the infection. However, despite this heightened activity, liver NK cells exhibited a decreased level of cytotoxicity, which might impact their ability to target infected cells effectively. RNA sequencing supported and provided the potential mechanisms for the proinflammatory effects and exhaustion like phenotypes of liver NK cells.

Conclusions Sepsis induces dysfunction and exhaustion-like phenotypes in liver NK cells and ILC1, which might further impair other immune cells and represent a potential therapeutic target for sepsis.

Keywords Sepsis, Liver resident immune cells, Nature killer cells, Innate lymphoid cell 1

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Introduction

Sepsis refers to the life-threatening organ dysfunction caused by the uncontrolled response to infection [1], which is one of the important causes of death in ICU [2]. Sepsis patients are often accompanied by the dysfunction of organs, like heart, lung, liver, and kidney [3].Compared with the dysfunction of other tissues, the liver dysfunction in sepsis is often more difficult to detect. The liver is an important organ in the human body, which plays critical roles in detoxification, metabolism, bile secretion and immune defense [4]. Once the liver dysfunction happened, it will lead to many physiological function disorders. The increase of bilirubin, an important indicator of liver dysfunction, was also considered to increase the risk of death in severe sepsis patients [5], and liver injury caused the increase of mortality in sepsis patients has been reported [6]. Therefore, how to reduce the liver injury is of great significance for the treatment of sepsis. Liver injury in sepsis is mainly caused by the upregulation of inflammatory factors and various immune cells [7]. Pathological phenotypes of mice with sepsis shows that neutrophils will invade to liver [8]. Additionally, macrophages exhibit increased polarization to the M1 phenotype, characterized by elevated secretion of specific inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), reactive oxygen species (ROS), and nitric oxide synthase (NOS) [9]. In addition to myeloid cells, innate lymphoid cells are also considered to be involved in the progress of sepsis.

There are at least two types of NKp46 positive cells: conventional NK (cNK) cells and ILC1, within the liver tissue. In mice, the surface markers for cNK cells are $CD3^{-}NK1.1^{+}NKp46^{+}CD49a^{-}CD49b^{+}$, while the surface markers for ILC1 are $CD3^{-}NK1.1^{+}NKp46^{+}CD49a^{+}CD49b^{-}$ [10]. Liver cNK cells have similar characteristics to peripheral blood NK cells. They not only directly kill target cells (such as virus-infected cells and malignant tumor cells), but also activate other immune cells through the secretion of cytokines. On the other hand, ILC1s demonstrate strong tissue residency and self-renewal within the tissues [11]. ILC1s have weaker cytotoxicity abilities compared with cNK cells. They primarily exert their function by secreting cytokines. Furthermore, some studies support that liver-resident ILC1s may be associated with immune memory [12].

Although there is substantial research confirming the involvement of NK cells in sepsis progression [13, 14], there is also evidence suggesting that NK cells, or their secretion of IFN-γ, may play a protective role in sepsis [15, 16]. While some studies have reported a decrease in the ability of NK cells to secrete cytokines during sepsis [17], there is still limited research on other aspects of NK cell function in sepsis, such as maturity, cytotoxicity, and gene expression, particularly regarding liver-resident ILC1 cells.

In this study, we investigated the number, maturation, cytokine secretion, and cytotoxicity of NK cells and ILC1 in the liver of septic mice. Our results indicate a decrease in the proportion of terminally mature NK cells following CLP, as evidenced by a diminished $CD11b^{+}CD27^{-}$ subpopulation and reduced Klrg1 expression. Additionally, the secretion of IFN-γ was found to be reduced in NK cells from both the spleen and liver when stimulated by IL-18. Mitochondrial analysis suggests that type 1 ILCs may undergo metabolic adaptation during sepsis. The high expression of Tigit indicates that these cells may acquire exhaustion-like phenotypes. Transcriptome analysis provides potential mechanisms for these changes. These findings contribute to a better understanding of the immune system's complexities during sepsis and may have implications for potential therapeutic interventions in the future.

Materials and methods

Experimental animals

C57BL/6J mice were used in this study and were purchased from Weitonglihua Experimental Animal Technology Co., Ltd. (Beijing, China). β2m-KO mice were purchased from Shanghai Model Organisms Technology Co., Ltd. Mice were kept under specific pathogen free (SPF) conditions. Sepsis model was established by cecal ligation and puncture (CLP) [18]. Male mice aged 8–10 weeks were used for the experiments and they were anesthetized by isoflurane. One of the primary reasons for selecting male mice is to minimize the variability introduced by hormonal fluctuations. By using male mice, we aimed to achieve more consistent and reproducible results. All animals are unconscious before the surgery, which is confirmed by toe pinch. The skin of the mice's abdomen was cut to expose the abdominal cavity of the mice, and then the cecum was ligated at the bottom. The cecum was punctured once near the ligation line with a sterile needle and squeezed until a small amount of feces were discharged. After the operation, the cecum was put back into the abdominal cavity and the incisions were sewn. Isoflurane was used to anesthetize the animal, which loses consciousness during surgery and can wake up within 5 min when the mask is removed. When performing humane endpoints, we use isoflurane to anesthetize the animal before using the cervical dislocation method, ensuring that the animal is unconscious in a very short period of time. For the sham group, CLP were not performed after cecum exposure, and cecum were directly put back and the incisions were sewn. The liver and spleen of the mice were obtained 24 h after the surgery. All animal experiments were approved by the IACUC of Tianjin University and performed

in accordance with institutional animal care and use guidelines.

Isolation of mouse immune cells

To isolate the hepatic immune cells, livers were first perfused to remove the blood cells. Then the liver was cut and digested with collagenase II (Sigma-Aldrich, USA, C6885) at 37 °C for 45 min. Cell suspension was filtered through a 70 μm cell strainer. The immune cells were enriched by Percoll (Solarbio, Beijing, China, NO. P8370) gradient centrifugation. The splenic immune cells were obtained by directly mashing the spleen against the strainer and the cell suspension that passed through were collected. Red blood cells were lysed if necessary.

Flow cytometry analysis

The liver and spleen cell were stained with antibodies including CD45-PC7 (BioLegend, USA, 103114), CD3-PC5.5 (BioLegend, USA, 100327), NK1.1-BV421 (BioLegend, USA, 108741), NKp46-AF647 (BD Biosciences, USA, 560755), Ly6C-BV510 (BioLegend, USA, 108437), CD49a-PE (BD Biosciences, USA, 562115) and CD49b-FITC (BioLegend, USA, 108906). NK cells were defined as $CD45^+CD3^-NK1.1^+Nkp46^+CD49a^-CD49b^+$. ILC1 were defined as $CD45^+CD3^-NK1.1^+Nkp46^+CD49a^+CD49b^-$. Neutrophils were defined as $CD45^+CD11b^+Ly6G^+$. In addition, CD27-BV510 (BioLegend, USA, 124229), CD11b-AF700 (BioLegend, USA, 101222), Klrg1-APC (BD Biosciences, USA, 561620) were used to analyze the maturation of NK cells. IFN-γ-PE (BioLegend, USA, 505807) were used to detect intracellular IFN-γ. For mitochondrial staining, fresh NK cells and ILC1s isolated from the spleen or liver were stained with lineage-specific antibodies and a mitochondrial-relevant dye. Mitochondrial membrane potential was analyzed using TMRM (tetramethylrhodamine methyl ester, Invitrogen, USA, T668). Mito Sox (Invitrogen, USA, M36008) was used to measure mitochondrial superoxide production. Mitochondrial mass was determined using Mito Tracker (Invitrogen, USA, M7514). Flow cytometry was performed on a Beckman CytoFLEX S.

In vivo cytotoxicity test

Spleen cells were isolated from β2m-KO mouse and WT mouse, and fluorescence labeling was performed using ef670 (eBioscience™ Cell Proliferation Dye eFluor™ 670, Invitrogen, 65-0840-85) and CFDA-SE (Invitrogen, USA, C1157), respectively. The labeled cells were mixed in a 1:1 ratio and achieved a final concentration of 0.5×10^7 cells/100 μL (0.25×10^7 cells/100 μL WT cells and 0.25×10^7 cells/100 μL $\beta2m^{-/-}$ cells). 200 μL labeled cells (1×10^7 cells) were injected into CLP or sham mice through spleen and detect the proportion of labeled cells in the liver and spleen after 24 h. The percentage of rejection was calculated by: [100 – (percentage of $\beta2m^{-/-}$ in donor cells / percentage of WT cells in donor cells)] ×100%.

Cytokine stimulation and IFN-γ production analysis

Spleen cells or liver cells were cultured in RPMI1640 medium (KeyGEN BioTECH, Jiangsu, China, KGM31800NH) containing 10% FBS (Gibco, USA), penicillin/streptomycin and β-mercaptoethanol (Gibco, USA, 21985023). The cells were stimulated with IL-12 (Sino-Biological, Beijing, China, CT024-M0208H) only, IL-18 (SinoBiological, Beijing, China, 50073-MNCE) only, or IL-12/IL-18 simultaneously. The cells without any cytokine were used as control. After 18 h of incubation, cells were harvested and analyzed with flow cytometry. Golgi transport inhibitors (BD Biosciences, USA, 554724) were added 4 h before the end of stimulation. The IFN-γ^+ cells in spleen NK cells, liver NK cells and liver ILC1 were detected by flow cytometry.

Transcriptome sequencing and bioinformatics analysis

Spleen NK cells, liver NK cells, and liver ILC1s were sorted by BD FACS Aria III. Total RNA was prepared for reverse transcription. The first strand of cDNA was prepared by SMART, and the second strand was synthesized using KAPA HiFi HotStart DNA Polymerase. DNA samples were loaded into nanoarrays by high-intensity DNA nanochip technique and combinatorial probe-anchor synthesis was used for sequencing. PCA, volcano diagram, heatmap, and GSEA were used for analyzing the gene expression patterns among different samples.

Statistical analysis

The data were presented as mean ± standard deviation, and the comparison between the two groups was performed using the paired t-test. To establish statistical significance, a significance level of $p<0.05$ was applied.

Results

CLP affects the proportion of immune cells in liver

A sepsis mouse model was induced using cecal ligation and puncture (CLP). Observations on day 1 post-procedure revealed a notable decline in the mice's condition, characterized by diminished activity and reduced food intake. One day after the surgical procedure, the mice were humanely euthanized and the spleens and livers were harvested, fixed, and subjected to histopathological or flow cytometry analysis. The cecum in CLP group mice were swelling (Supplementary Fig. 1a). Although no significant inflammation was observed in the liver of CLP mice by HE staining (Supplementary Fig. 1b), there was a noteworthy increase in the percentage of neutrophils (identified as $CD45^+CD11b^+Ly6G^+$) in the liver of CLP group mice ($p<0.01$), indicating a severe acute

inflammatory response (Supplementary Fig. 1c). These findings suggest that the CLP mouse model is an appropriate model for studying the immune response in sepsis and highlight the importance of neutrophil infiltration in the liver during sepsis pathogenesis.

Flow cytometry analysis revealed that nearly all splenic type 1 innate lymphoid cells (ILCs) were CD49b$^+$ cells, which are commonly known as natural killer (NK) cells. The absolute number and percentage of type 1 ILCs (defined as CD45$^+$CD3$^-$NK1.1$^+$NKp46$^+$) in lymphocytes were significantly reduced in the spleen after CLP (Supplementary Fig. 2b, Fig. 1a - c). In contrast, although not statistically significant, the proportion of NK1.1$^+$NKp46$^+$ (type 1 ILCs) cells in lymphocytes increased in the liver after CLP ($p>0.05$, Fig. 1d). These findings suggest that, in addition to neutrophils, type 1 ILCs may also migrate to the liver or undergo local proliferation in response to sepsis. To further investigate the effect of CLP on the proportion of immune cells in the liver, the number of NK cells and ILC1 in CLP-induced sepsis liver was analyzed based on the expression of CD49a and CD49b. The results showed that the proportion of liver NK cells in type 1 ILCs increased significantly ($p<0.01$, Fig. 1g), while the percentage of liver ILC1 decreased in CLP mice ($p<0.01$, Fig. 1g). Although the percentage of NK cells in lymphocytes increased, it did not reach statistical significance ($p>0.05$, Fig. 1e). However, the percentage of ILC1 significantly decreased in CLP-induced sepsis liver ($p<0.05$, Fig. 1f). These findings indicate that CLP significantly alters the quantity and distribution of immune cells within the liver, emphasizing the roles of neutrophils and type 1 ILCs in the immune response during sepsis. Moreover, they suggest that CLP-induced sepsis may selectively influence the distribution of different subsets of type 1 ILCs in the liver.

CLP decreased the proportion of mature NK cells

Maturation is a crucial indicator of the functionality of NK cells. To investigate the effect of sepsis on NK cell maturation, NK cells were firstly gated by CD45$^+$CD3$^-$NKp46$^+$CD49b$^+$CD49a$^-$, and then further analyzed the expression of Klrg1 (Killer cell lectin-like receptor subfamily G member 1), a lectin-like receptor expressed on mature NK cells [19], and CD11b/CD27 by flow cytometry. A four-stage model was proposed to describe the terminal maturation of NK cells based on the expression of CD11b and CD27 [20]. The CD11b$^+$CD27$^-$ subpopulation was considered as the most mature NK cells. One day after CLP, the percentage of CD11b$^+$CD27$^-$ NK cells also decreased in the spleen and liver ($p<0.05$, Fig. 2a - **b**), consistent with Klrg1 based analysis. Compared to sham mice, the proportion of Klrg1$^+$ NK cells in the spleen and liver of CLP-induced sepsis mice significantly decreased ($p<0.01$, Fig. 2c - **d**). One day after CLP, the observed decrease in the proportion of mature NK cells suggests that sepsis may rapidly affect the maturation status of NK cells, potentially impacting their functionality, particularly in the production of IFN-γ. This cytokine plays a crucial role in modulating the inflammatory response during sepsis. Consequently, this prompted further investigation into the functionality of NK cells in modulating the inflammatory pathways through IFN-γ secretion during sepsis.

CLP affects IFN-γ secretion of NK cells and ILC1 in liver

To investigate the capacity of producing IFN-γ, NK cells and ILC1s were stimulated with interleukin-12 (IL-12) and/or IL-18 and the production of IFN-γ was evaluated by flow cytometry. The proportion of IFN-γ$^+$ NK cells in the spleen of mice with CLP-induced sepsis was significantly lower than that in the sham group when stimulated with IL-18 alone ($p<0.05$, Fig. 3a). A statistically significant but slight decrease of IFN-γ secretion was also observed in spleen NK cells from sepsis group when stimulated by IL-18 and IL-12. However, there was no significant difference in IFN-γ$^+$ spleen NK cells stimulated by IL-12 alone between CLP and sham group.

Decreased secretion of IFN-γ was also observed in CLP group derived liver NK cells when stimulated by IL-18 alone. There was no significant difference between the CLP and sham groups when liver NK cells were stimulated with IL-12 alone or with both IL-18 and IL-12 (Fig. 3b). Interestingly, the secretion of IFN-γ increased in liver ILC1s from the CLP group when stimulated with both IL-12 and IL-18. However, there was no significant difference in liver ILC1s between the CLP and sham groups when stimulated with IL-12 or IL-18 alone (Fig. 3c). These findings suggest that sepsis differentially affects IFN-γ production in NK cells and ILC1s. There is a decrease in IFN-γ secretion in NK cells (both spleen and liver) when stimulated with IL-18 alone. This diminished IFN-γ secretion in NK cells highlights a compromised cytokine response under septic conditions, which may impact overall immune efficacy against infections. In contrast, an increase in IFN-γ secretion is observed in liver ILC1s when stimulated with IL-12 and IL-18. The differences in IFN-γ secretion between NK cells and ILC1s under specific stimulations suggest that sepsis affects the cytokine response differently across these cell types.

CLP affects the mitochondrial function of NK cells and ILC1s

Mitochondrial function and metabolism are crucial for immune cell function, and mitochondrial fragmentation has been reported to limit the immunosurveillance capabilities of NK cells [21]. We investigated the changes in mitochondrial-related markers of NK and ILC1 cells

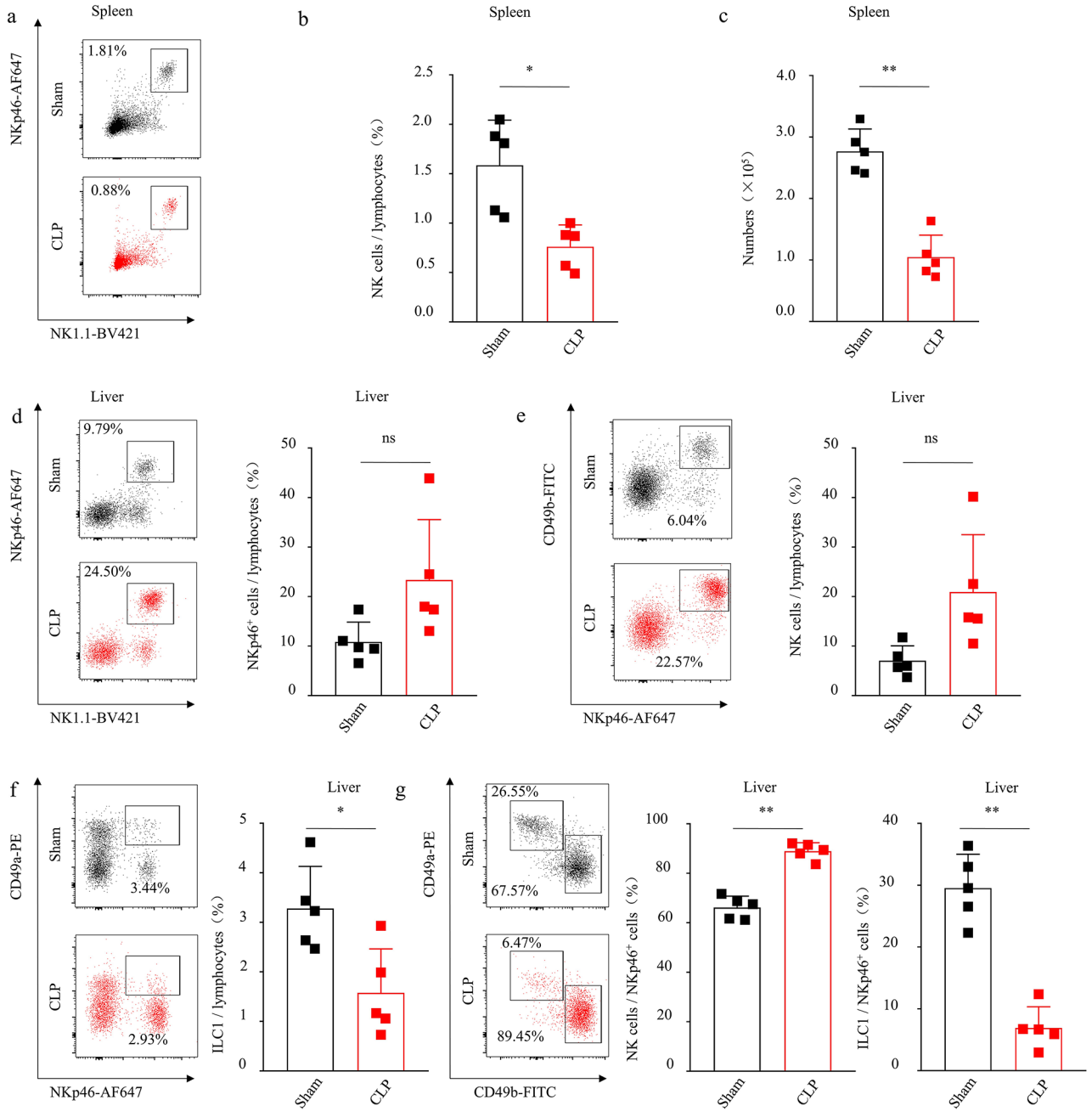

Fig. 1 Effects of sepsis on the distribution of NK cells and ILC1. **a**: Typical flow cytometric plot of Sham and CLP splenic NK cells. The cells were pre-gated as $CD45^+CD3^-$. **b**: The percentage of NK cells in spleen lymphocytes in sham group and CLP group ($n=5$; *: $p<0.05$). **c**: The absolute number of spleen NK cells in the sham and CLP group ($n=5$; **, $p<0.01$). **d**: The percentage of $NK1.1^+NKp46^+$ cells within liver lymphocytes was assessed. A representative flow cytometric plot is presented on the left, while the summary data are displayed on the right ($n=5$; ns: $p>0.05$). **e-f**: Representative and summary data of liver NK cells (**e**) and ILC1 (**f**), and the relative proportion of NK cells and ILC1 within liver $NKp46^+$ cells were generated by flow cytometry. ($n=5$; ns: $p>0.05$; *: $p<0.05$;**: $p<0.01$). **g**: Cells were gated for $CD45^+CD3^-NK1.1^+NKp46^+$, and then the proportion of liver NK cells and ILC1 were measured based on the expression of CD49a and CD49b ($n=5$; **: $p<0.01$)

following CLP. There was no significant change in the mean fluorescence intensity (MFI) of TMRM for spleen NK cells, liver NK cells, or liver ILC1s ($p>0.05$, Fig. 4a, d, g) between sham and CLP group. However, in the Mito Tracker analysis, there was a statistically significant increase among CLP mouse spleen NK cells, liver NK cells, and liver ILC1s ($p<0.01$, Fig. 4b, e, h). Additionally, a noteworthy increase in the percentage of Mito Sox-positive cells was observed specifically in spleen NK cells (Fig. 4c). Conversely, no significant differences were observed in the proportion of Mito Sox^+ cells in liver NK cells or liver ILC1s between the sham and CLP

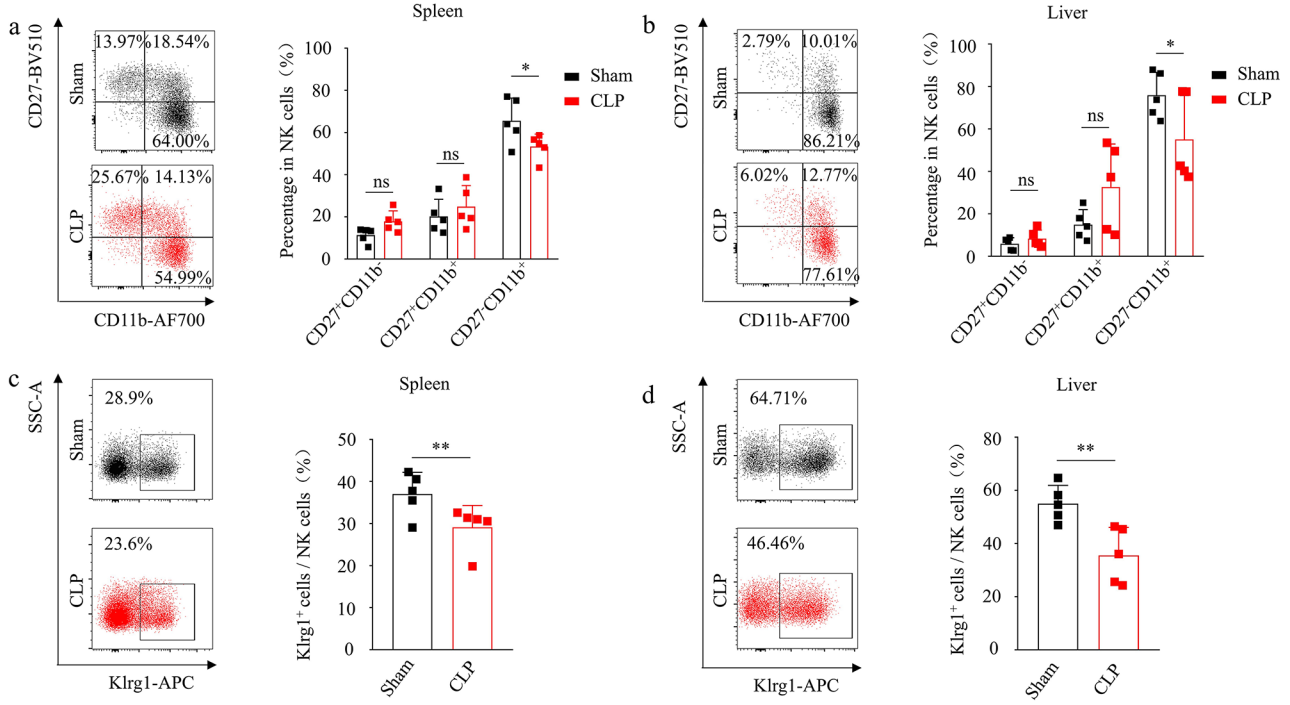

Fig. 2 Effects of sepsis on NK cells maturation. **a** and **b**: The expressions of CD27 and CD11b on spleen (**a**) and liver (**b**) NK cells ($CD45^+CD3^-NK1.1^+NKp46^+$) in septic mice were analyzed by flow cytometry. The proportion of $CD27^+CD11b^-$, $CD27^+CD11b^+$, $CD27^-CD11b^+$ cells were compared. $CD27^-CD11b^+$ NK cells represented the most mature NK cells ($n = 5$; *: $p < 0.05$). **c** and **d**: The expression of Klrg1 on spleen (**c**) and liver (**d**) NK cells in septic and sham mice were measured by flow cytometry. The proportion of Klrg1 positive cells in NK cells were compared ($n = 5$; **: $p < 0.01$)

groups (Fig. 4f and i). The alterations in mitochondrial function observed in NK cells and ILC1s following CLP suggest a metabolic adaptation to sepsis. This shift could potentially enhance or impair the immune cells' ability to manage the energy demands of an intense inflammatory response, directly impacting their effectiveness in sepsis management.

Decreased cytotoxicity and increased exhaustion surface marker on CLP NK cells

One of the key functions of NK cells is to directly kill target cells. To evaluate the effect of CLP on splenic and liver NK cells, an in vivo cytotoxicity assay was conducted using *β2m* deficient cells. MHC-I is the most critical ligand of NK cell inhibitory receptors, and its expression on cell surfaces is essential for establishing self-tolerance in NK cells. *β2m deficiency* results in the inability of MHC-I to express on cell surfaces, thereby rendering the cells without MHC-I as targets of NK cells. One day after the administration, CFDA-SE (WT) or ef670 ($\beta2m^{-/-}$) labeled cells were first gated and the ratio of WT and *β2m* deficient cells were analyzed by flow cytometry. NK cell specific lysis of target cells was calculated by the ratio of donor derived WT and *β2m* deficient cells. A noteworthy reduction in cytotoxicity was observed in liver NK cells (Fig. 5b). Although the cytotoxicity of spleen NK cells in the CLP group showed a decrease compared to the sham group, this difference did not reach statistical significance (Fig. 5a). Considering the observed decrease in cytokine secretion and cytotoxicity of NK cells following CLP, we investigated the expression of markers associated with NK cell exhaustion. TIGIT is one such marker known to be expressed on the surface of exhausted NK cells [20]. Our results revealed that compared with spleen NK cells, liver NK cells exhibited a higher expression level of TIGIT. Moreover, a significant increase in the expression level of TIGIT on NK cells in both the spleen and liver was detected after CLP (Fig. 5c **and d**). These findings strongly indicate that acute sepsis can rapidly induce NK cell expressing exhaustion markers in multiple organs, even within a very short timeframe, potentially compromising the broader immune defenses crucial for managing septic challenges.

Transcriptome analysis of NK cells/ILC1 after CLP

To investigate changes in gene expression in NK cells and ILC1 following CLP, we sorted splenic NK cells, liver NK cells, and liver ILC1, and subsequently performed RNA sequencing to evaluate their gene expression profiles. The mRNA sequencing data were first presented by principal component analysis (PCA). As shown in Fig. 6a, the gene expression patterns of liver NK cells and splenic NK cells are more similar to each other, while they differ significantly from the gene expression pattern of liver ILC1.

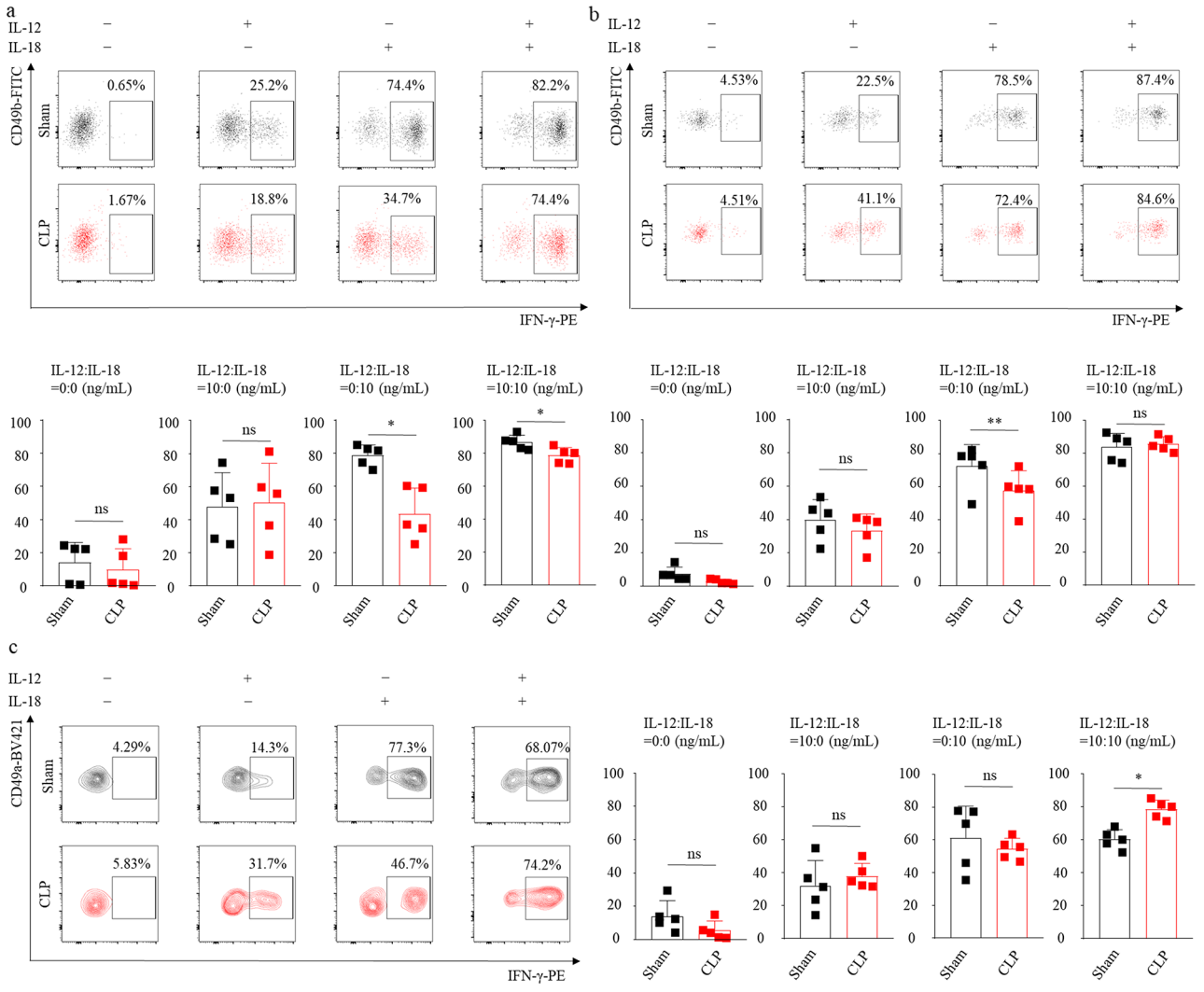

Fig. 3 IFN-γ production in NK cells and ILC1. Spleen cells and liver cells from sham mice and CLP mice were subjected to different treatments: IL-12 (10 ng/mL), IL-18 (10 ng/mL), or a combination of IL-12 and IL-18 (10 ng/mL each). Cells without any cytokine stimulation were used as the control group. The proportion of IFN-γ positive cells in NK cells and ILC1 was detected using flow cytometry. Representative flow cytometry plots and summary data for spleen NK cells (**a**), liver NK cells (**b**), and liver ILC1 (**c**) were included ($n=5$; ns: $p>0.05$; *: $p<0.05$)

This phenomenon is observed in both the Sham and CLP groups. These results validate previous research suggesting that despite their anatomical proximity, liver NK cells exhibit greater similarity with splenic NK cells than with liver ILC1. Comparing splenic NK cells from Sham and CLP groups, their gene expression patterns are relatively similar. However, the difference in gene expression between liver NK cells from sham and CLP groups are relatively larger. Similar phenomenon was also observed in liver ILC1s. This indicated that CLP may have a more significant impact on the liver.

3317 differentially expressed genes were identified in liver NK cells after CLP, with 1976 upregulated and 1341 downregulated genes. In liver ILC1 cells, 3246 differentially expressed genes were found, with 2068 upregulated and 1178 downregulated genes. In splenic NK cells, 2781 differentially expressed genes were detected, with 832 upregulated and 1949 downregulated. These changes in gene expression were also illustrated by volcano plots and heatmaps (Fig. 6b – d, Supplementary Fig. 3). Moreover, genes involved in calcium binding, S100a8, and S100a9, were upregulated, potentially contributing to neutrophil recruitment (Fig. 6c). Gene-related heatmaps were generated for liver NK cells, ILC1 cells, and splenic NK cells after CLP to show the change of their function relevant genes. Liver ILC1 cells exhibited lower Klrg1 expression but higher Klrc1 (NKG2A) and Lag3 expression, compared with liver NK cells or splenic NK cells (Fig. 6e). Flow cytometry was utilized to measure the expression of Ncr1 protein. Contrary to the RNA-seq results, no significant changes in the protein levels of Ncr1 were observed post-CLP (Fig. 6e). This might be attributed to the role

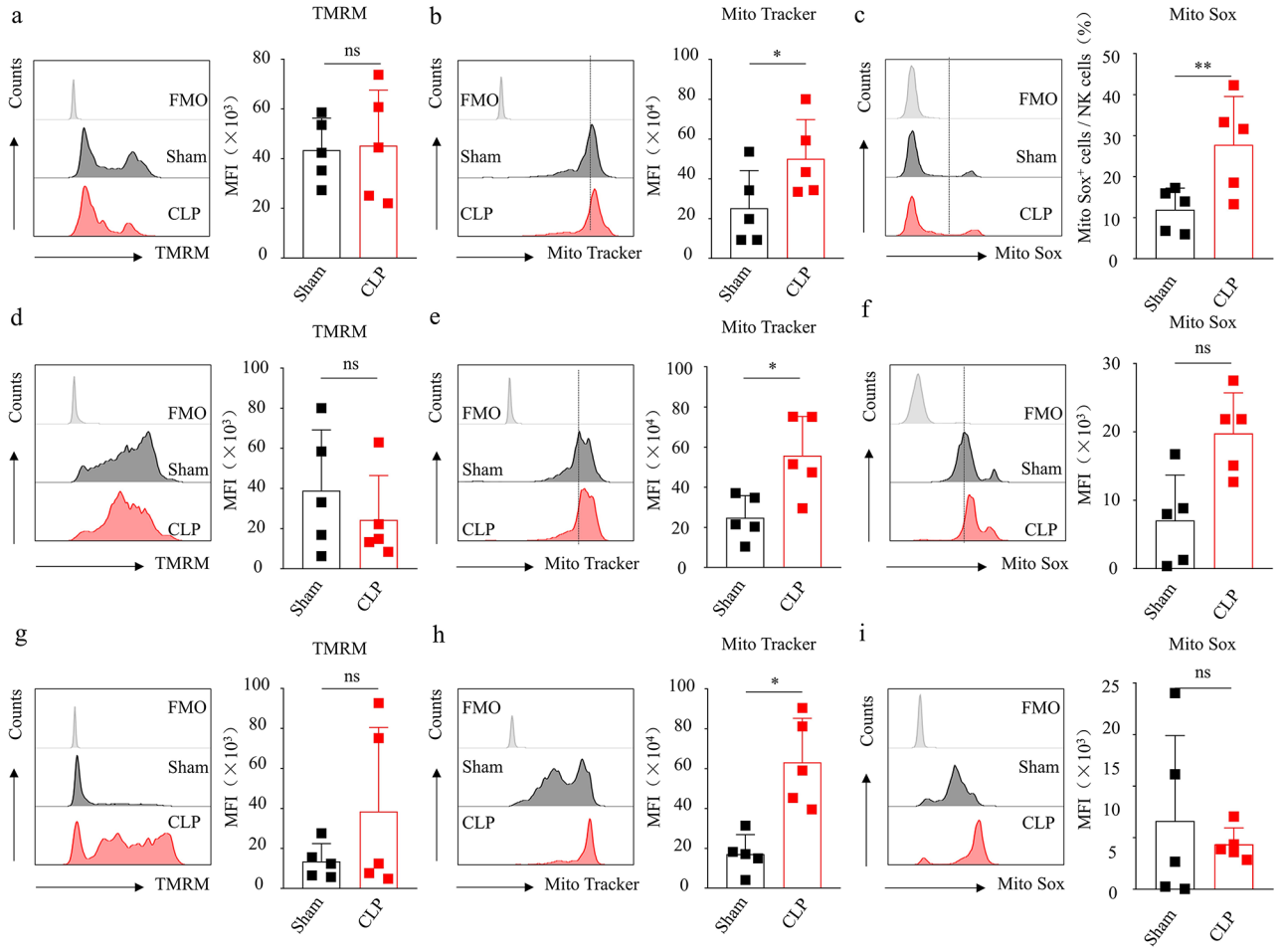

Fig. 4 Effects of CLP on the mitochondrial function of NK cells and ILC1. The spleen NK cells, liver NK cells, and liver ILC1s were stained with a final concentration of 5 μM TMRM, Mito Tracker or Mito Sox in dark at 37 °C, and detected by flow cytometry. **a** - **c**: Representative flow cytometry histograms and summary data of the MFI (mean fluorescence intensity) for TMRM (**a**), Mito Tracker (**b**), and Mito Sox (**c**) staining in spleen NK cells. **d** - **f**: Representative flow cytometry histograms and summary data of the MFI (mean fluorescence intensity) for TMRM (**d**), Mito Tracker (**e**), and Mito Sox (**f**) staining in liver NK cells. **g** - **i**: Representative flow cytometry histograms and summary data of the MFI (mean fluorescence intensity) for TMRM (**g**), Mito Tracker (**h**), and Mito Sox (**i**) staining in liver ILC1 cells. ($n = 5$;ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$)

of Ncr1 as a critical marker gene for group 1 ILCs, where mRNA is expressed robustly in cells. Therefore, short-term variations in mRNA levels may not immediately impact protein expression. Both liver and spleen NK cells showed decreased Klrg1 expression after CLP, consistent with previous flow cytometry results (Fig. 2). Furthermore, immune checkpoint TIGIT expression increased in both liver NK and ILC1 cells after CLP, likely linked to NK cell depletion, which is also consistent with flow cytometry data (Fig. 5c and d). Some active receptors and cytotoxicity relevant genes, such as CD226 and Prf1, were downregulated after CLP, which further support the exhaustion like phenotype of NK cells in sepsis mice. The expression of Tbx21 (encoding transcription factor T-bet) decreased in liver ILC1, liver NK cells, and splenic NK cells after CLP. This reduction in T-bet expression may be one of the reasons for the decreased maturation of NK cells and the lower secretion of IFN-γ by NK cells.

In addition, differential expressed genes related to the immune response were also analyzed by GO analysis (Supplementary Fig. 4a-f) and GSEA analysis (Supplementary Fig. 5). Gene enrichment analysis revealed significant results for differentially expressed genes in liver NK, ILC1, and spleen NK cells between the Sham and CLP groups. Hemoglobin-related metabolism pathway genes were significantly enriched in spleen NK cells after CLP, indicating increased metabolic activity in these cells (Fig. 6f). This might support the Mito Tracker staining results. The inflammation response gene set was significantly enriched in liver NK cells and liver ILC1 cells after CLP, suggesting a more pronounced inflammatory response in the liver during sepsis. Notably, the IL6/JAK/STAT3 signaling pathway was significantly enriched in

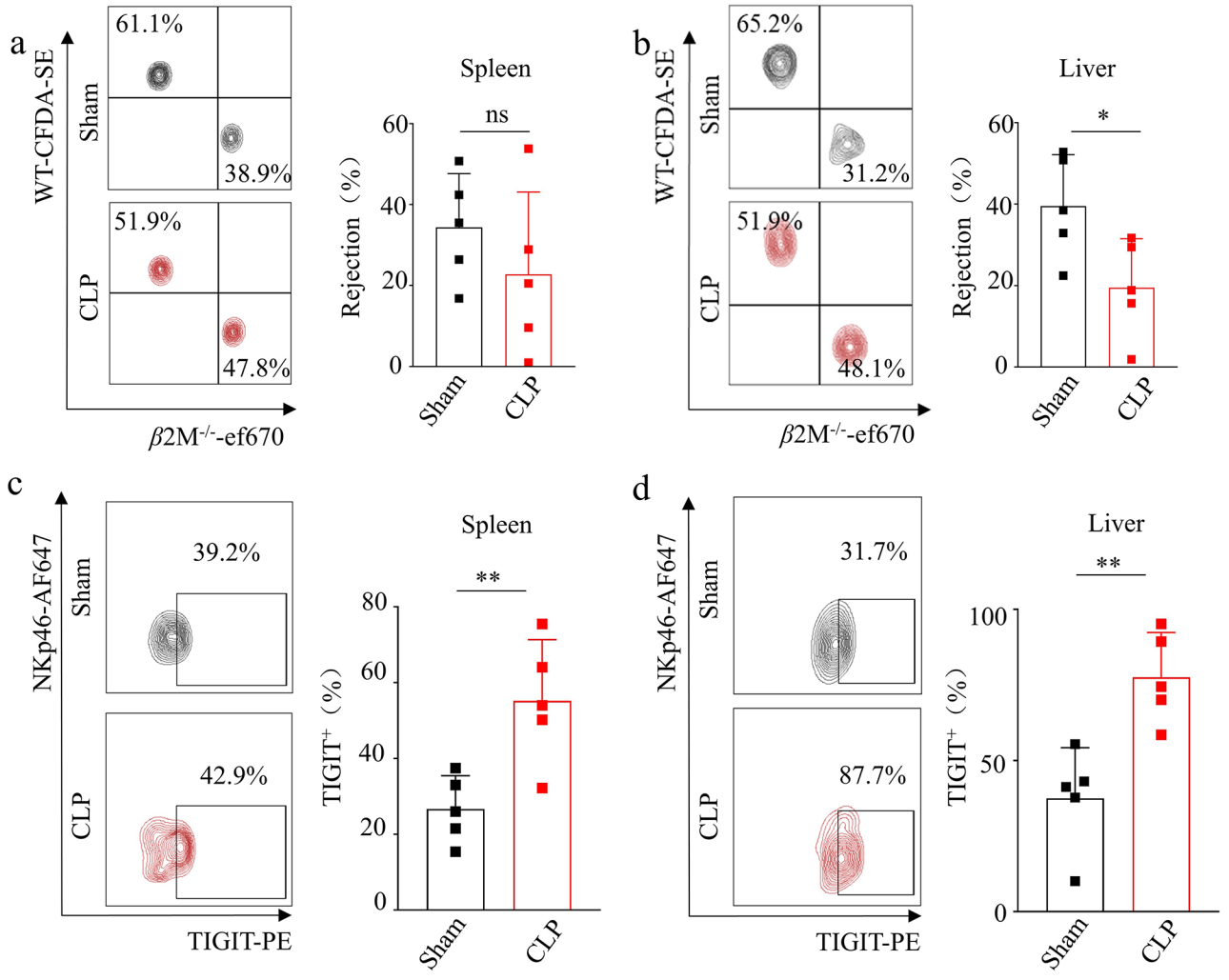

Fig. 5 *iv vivo* cytotoxicity and exhaustion marker assay. **a** and **b**: Representative and summary flow cytometry data showing the rejection of $\beta 2m^{-/-}$ cells by the spleen (**a**) and liver (**b**) of sham and CLP mice. **c** and **d**: Representative histogram and summary flow cytometry data show the expression of exhaustion marker TIGIT of spleen (**c**), liver (**d**) NK cells, and liver ILC1s ($n = 5$; ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$)

liver NK cells and ILC1 cells after CLP, indicating elevated IL-6 expression and JAK/STAT3 pathway activation in sepsis, further exacerbating the inflammatory response (Fig. 6g). The G2M checkpoint was enriched in Sham ILC1 cells, indicating reduced proliferation characteristics in liver ILC1 cells during sepsis (Fig. 6h). Transcriptomic changes observed in NK cells and ILC1s following CLP reveal significant molecular alterations that underline the immune cells' responses to sepsis.

Discussion

We observed significant changes in liver NK cells and ILC1 in sepsis, including a decrease in the number of full mature liver NK cells and a decrease in the number of ILC1 cells, accompanied by an increased capacity of ILC1 to secrete IFN-γ. Additionally, alterations were detected in the mitochondrial function and transcriptome of liver NK cells and ILC1. These changes in NK cells and ILC1 may also have regulatory effects on other immune cells, as evidenced by associated changes observed in macrophages. While the role of myeloid immune cells, such as macrophages [22–24] and neutrophils [25, 26] in sepsis has been extensively studied, our findings suggest that liver resident ILCs, including NK cells and ILC1, also undergo significant changes during sepsis. Further research is needed to elucidate the specific regulatory functions and mechanisms of these cells in the context of sepsis.

Our data showed a decrease in the proportion of terminally mature NK cells following CLP, as evidenced by diminished $CD11b^{+}CD27^{-}$ subpopulation and Klrg1 expression. Given that the NK cells in our study were analyzed only 24 h after CLP, it is improbable that CLP would directly influence the development of NK cells in peripheral organs. A reasonable explanation could be that mature NK cells were subjected to overactivation, leading

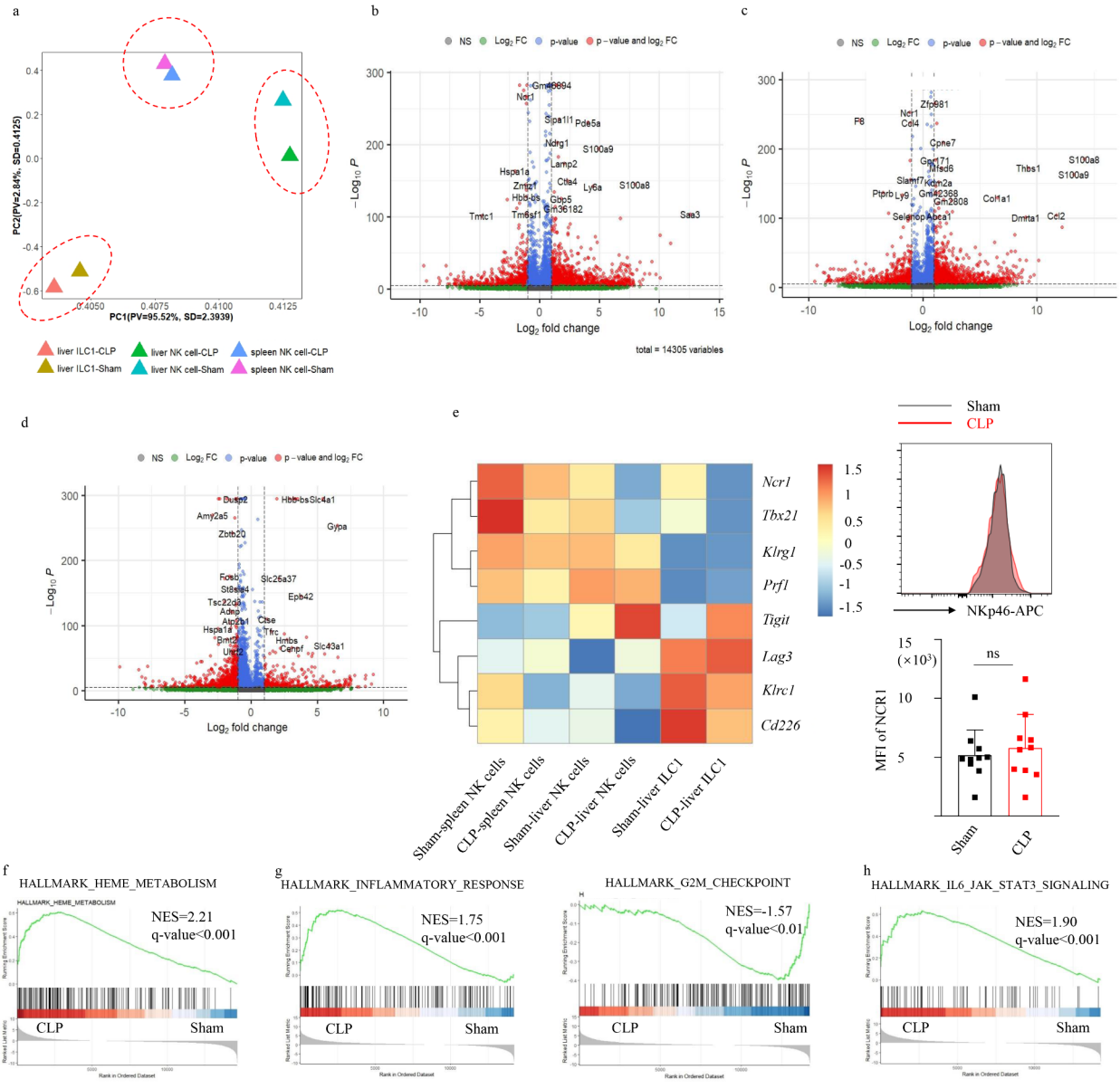

Fig. 6 Transcriptome analysis of NK cells and ILC1 after CLP. **a**: PCA analysis of gene expression data of spleen NK cells, liver NK cells, and liver ILC1s. **b** - **d**: The volcano diagrams illustrated the differentially expressed genes of liver NK cells (**b**), liver ILC1 (**c**), and spleen NK cells (**d**) between sham and CLP conditions. In each volcano plot, up-regulated genes in CLP samples are represented on the right, while down-regulated genes are shown on the left. Significant genes ($|\log2FC| \geq 1$, $p\text{-value} < 0.05$) were denoted by red dots. **e**: Heat maps for NK cells and ILC1 function relevant genes. The expression of Ncr1 was analyzed by flow cytometry. **f** - **h**: GSEA of the genes differentially expressed between sham and CLP mice. Gene sets significant changed in spleen NK cells (**f**), liver ILC1s (**g**), and liver NK cells (**h**)

to cell death of the mature subset [27]. Another possibility is that under the stimulus of sepsis, NK cell progenitors increasingly differentiate into NK cells, displaying signs of immaturity. These newly produced NK cells have not yet had sufficient time to undergo their final differentiation. Additionally, our data indicate that the average number of immature NK cells actually increases after CLP; however, due to substantial variation among individual animals, this increase did not reach statistical significance. This scenario accounts for both the reduction in terminally mature NK cells and the observed rise in the total number of NK cells.

Furthermore, changes in mitochondrial function in NK cells were detected, suggesting a potential association between these alterations and mitochondrial dynamics. The role of Klrg1 in NK cells is multifaceted, serving as a marker of NK cell proliferation, maturation, or exhaustion [19, 28]. In our study, we observed a decrease

in Klrg1 expression in NK cells during sepsis, which may be attributed to the reduced proportion of mature NK cells, as supported by our flow cytometry analysis targeting CD11b/CD27. The decrease in Klrg1 expression is unlikely to reflect NK cell exhaustion in the current study, as exhaustion typically occurs in the context of persistent chronic inflammation [19, 28], whereas sepsis is characterized by acute inflammation.

Emerging research suggests that the distinctions between liver NK cells and liver ILC1 may extend beyond their developmentally distant lineages. Liver NK cells are derived from bone marrow hematopoietic stem cells, while liver ILC1 originate from progenitor cells residing in the liver [29, 30]. While NK cells are pivotal in combating viral infections by destroying infected cells, tissue-resident ILC1 are even more vital, providing early host protection against viral infections through their secretion of IFN-γ [31]. In certain inflammatory diseases, like alcoholic steatohepatitis, NK cells are known to undergo apoptosis, while ILC1 exacerbate liver steatosis through the secretion of IL-17 [32]. In cancer research, it is commonly accepted that cNK cells are more potent in killing tumor cells compared to ILC1. In some tumor microenvironments, cNK cells can even be converted into less cytotoxic ILC1, allowing tumor cells to evade immune surveillance [33]. However, research also indicates that while cNK cells mainly limit tumor growth, ILC1 play a crucial role in controlling metastatic seeding and their effectiveness seems less influenced by the tumor microenvironment [34]. To the best of our knowledge, before this study, the impact of sepsis on liver NK cells and ILC1, as well as their respective responses to sepsis, had not been thoroughly investigated. By analyzing the gene expression patterns from sequencing data of the sham group, we confirmed that although liver NK cells and liver ILC1 are anatomically close, liver NK cells share a more similar gene expression profile with spleen NK cells than with liver ILC1 under resting conditions. Although previous studies have found that NK cells from different tissues respond differently to sepsis [35], these studies did not include the liver and did not examine the effects of sepsis on the two distinct types of type 1 ILCs in the liver.

IFN-γ secretion plays a crucial role in the functionality of both NK cells and ILC1 [36–38]. This cytokine serves as a vital regulatory factor, actively participating in anti-infection immunity by influencing the polarization of T cells [39, 40] and macrophages [41, 42], ultimately promoting the inflammatory response. We observed that the secretion of liver NK cells for IFN-γ remained unchanged between the sepsis group and the sham group following IL-12/IL-18 stimulation. However, the ability of liver ILC1 to secrete IFN-γ increased in the sepsis group. Considering the pro-inflammatory and anti-bacterial effects of IFN-γ [43, 44], the enhanced IFN-γ secretion by liver ILC1 may contribute to bacterial clearance during sepsis. Nevertheless, it is important to note that this elevated immune response of liver ILC1 could potentially lead to excessive inflammation and subsequent organ injury. We also observed that the IFN-γ production of both spleen and liver NK cells decreased significantly in CLP group when stimulated by IL-18 only. This might be supported by the finding that the expression of IL-18 receptor was downregulated in sepsis [16].

ILC1s are primarily tissue-resident cells that contribute to local immune homeostasis and early defense mechanisms against infections. Unlike NK cells, ILC1s do not typically display potent cytotoxic activity but rather exert their influence through cytokine secretion. In the context of sepsis, the increased IFN-γ production by ILC1 despite their reduced numbers suggests a compensatory mechanism aimed at enhancing local immune responses. IFN-γ is crucial for activating macrophages and other immune cells, which is essential in the early phases of an immune response, especially in a liver local environment, which is a frontline organ in bacterial clearance during sepsis. On the other hand, liver conventional NK (cNK) cells are recognized for their cytotoxic capabilities and their ability to circulate between tissues and blood, contributing to systemic immune surveillance. These cells are adept at secreting cytokines like IFN-γ, which are integral in managing infections and modulating immune responses. The observed decrease in IFN-γ production by cNK cells in both the spleen and liver during sepsis may reflect a disrupted or inhibited functional state, possibly due to exhaustion or systemic regulatory feedback mechanisms. However, our analysis focused on a single exhaustion-related marker, TIGIT, for liver NK cells. Further investigation is necessary to fully understand the impact of exhaustion on these cells and to assess whether TIGIT could also serve as an exhaustion marker for ILC1.

Conclusions

In conclusion, our study provides evidence that sepsis induces dysfunction and transcriptome alterations in liver NK cells and ILC1. The upregulation of genes associated with the inflammatory response indicates the involvement of these immune cells in sepsis. However, further investigations are required to elucidate the specific mechanisms by which type 1 ILCs regulate liver injury during sepsis.

Supplementary Information

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Supplementary Material 1

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Author contributions
W.P.Y. and W.Y.W. designed experiments, performed experiments, analyzed the data and wrote the manuscript. W.P.Y., Z.Y.R., Z.Y.M. and S.J.M. performed experiments. W.C., L.Y., L.X.H., and Y.Z.X. analyzed the data, interpreted results and reviewed the manuscript.

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Data availability
Sequence data that support the findings of this study have been deposited in China National Center for Bioinformation with the accession code OMIX006043.

Declarations

Ethical approval
The animal use protocol has been reviewed and approved by the animal ethical and welfare committee of Tianjin University (TJUE-2023-016).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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