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Research article

Orai1 overexpression improves sepsis-induced T-lymphocyte immunosuppression and acute organ dysfunction in mice

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HIGHLIGHTS

- Orai1 overexpression improves sepsis-induced T lymphocyte immunosuppression.
- Orai1 overexpression reverses increases in Treg and Th17 cell numbers in sepsis.
- Orai1 overexpression reduces organ dysfunction in septic mice.

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ABSTRACT

Immune paralysis induced by sepsis, especially dysfunction of $CD4^+$ T cells, leads to an increased risk of infection. In sepsis, abnormal differentiation of T lymphocytes is associated with multiorgan dysfunction syndrome. In T lymphocytes, the Orai1/nuclear factor of activated T Cells (NFAT) pathway is a critical mediator of infection, inflammation, and autoimmunity. In this study, we confirmed immunosuppression of splenic $CD4^+$ T cells and abnormal differentiation of T lymphocytes in septic mice. Furthermore, we found that the Orai1/NFAT signaling pathway was inhibited in septic mice; however, the overexpression of Orai1 not only improved immune function of T cells in sepsis but also reduced the mortality and organ damage in septic mice. Moreover, the overexpression of Orai1 could reverse the increases in the numbers of T regulatory and T helper 17 cells in septic mice. These data suggest that the Orai1-mediated NFAT signaling pathway can improve sepsis-induced T-lymphocyte immunosuppression and acute organ dysfunction.

1. Introduction

Sepsis is an organ dysfunction resulting from a disordered infection response of the host, which can pose a threat to life and causes death at intensive care units globally [1]. As shown in many recent studies, immunosuppression has an important effect on late-stage sepsis and induces multiorgan dysfunction syndrome [2, 3]. Immunosuppression mostly results from T-cell dysfunction [4]. Sepsis still has a dismal prognosis, although multiple measures have been proposed to target immunosuppression [5]. T helper (Th) 17 cells can produce interleukin (IL)-17, which activates the synthesis of proinflammatory factors, including IL-1β, IL-6, and TNF-α, thus amplifying the inflammatory response [6, 7]. However, T regulatory cells (Tregs) can maintain immune homeostasis through the secretion of anti-inflammatory factors, such as TGF-β and IL-10, thus suppressing an excess inflammatory response [8]. The imbalance between Th17 cells and Tregs is related to the occurrence and development of sepsis [9].

The Ca^{2+}/calcineurin/NFAT signaling pathway in T lymphocytes is a critical mediator of anti-infection immunity, inflammation, and autoimmunity [10]. Intracellular Ca^{2+} concentrations are elevated through the release of Ca^{2+} from intracellular reserves and extracellular Ca^{2+} influx via Ca^{2+} channels in the plasma membrane [11]. The best-described

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Ca^{2+} influx pathway in T lymphocytes is store-operated calcium entry (SOCE) through Ca^{2+} release-activated Ca^{2+} (CRAC) channels, triggered by Ca^{2+} release from endoplasmic reticulum stores [12]. As a major part of calcium store-operated channels (SOCs), ORAI1 appears to be both necessary and sufficient to reconstitute SOCE [13, 14]. NFAT activation is achieved through voltage-gated Ca^{2+} channels in the plasma membrane, as well as through Ca^{2+} nanodomains close to open store-operated ORAI1 [15]. Therefore, the ORAI1/NFAT signaling pathway may be relevant to T-cell immune function.

The present study tested immunosuppressive function of splenic $CD4^+$ T cells and the expression of ORAI1/NFAT signaling pathway and further validated the involved mechanism by overexpressing ORAI1.

2. Materials and methods

2.1. Animals

Experiments were carried out following animal protection laws, and the study was approved by the Regional Ethical Committee for Animal Experimentation at Wenzhou Medical University, China. We raised male BALB/c mice within an animal facility at 22 °C under 12/12-h light/dark cycle conditions, with free access to water and food.

2.2. Overexpression of Orai1

A total of 18 mice were divided into the following three groups: a sham group, 24-h post-cecal ligation and puncture (24-h CLP) group, and ORAI1-overexpressing (LV-ORAI1) group. In the LV-ORAI1 group, a lentivirus carrying a full-length *Orai1* gene fragment (Shanghai Genechem Co., Ltd.) was used. The *Orai1*-harboring lentivirus was diluted to a final volume of 300 μl, containing 4×10^7 toxic units, and the suspension was administered to BALB/c mice via the tail vein twice weekly for 4 weeks. CLP was performed in mice after treatment completion.

2.3. Experimental protocol of sepsis induction

The CLP procedure was conducted to induce polymicrobial sepsis, according to a previous description [16]. In brief, after anesthesia, the cecum was exposed by making a midline incision, and the stool was then milked backward from the ascending colon to fill the cecum with feces, followed by ligation of 1/2 of the cecum using a 5-0 silk suture. After being immersed in phosphate-buffered saline (PBS), a 21-G needle was used to puncture the cecum through the antimesenteric border. Afterward, the cecum was placed back into the peritoneal cavity, followed by the suture of the abdominal cavity. The sham mice underwent the same laparotomy and resuscitation procedures but without cecal ligation or puncture.

2.4. Isolation of splenic $CD4^+$ T cells

After removing the spleen from each mouse, splenic samples were cultured in 5 ml of RPMI-1640 medium (Tianrun Shanda Biotech Co., Ltd., Beijing, China). Thereafter, the cells were dispersed through a stainless-steel mesh (30 μm) and centrifuged at 300× *g* for 10 min, followed by resuspension in 4 ml of RPMI-1640 medium. Subsequently, Ficoll–Paque density gradient centrifugation was conducted to obtain mononuclear cells. CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for magnetic-activated cell sorting (MACS) were used to isolate $CD4^+$ T cells from splenocytes through positive selection, following the manufacturer instructions. Briefly, the suspension was passed through an MS column in a MACS magnetic separator (Miltenyi Biotec) to separate $CD4^+$ T cells, while those that adhered to the MS column were collected for subsequent analyses.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Commercial ELISA kits for mice (Biosource, Worcester, MA, USA) were used to measure interferon (IFN)-γ and IL-4 concentrations in culture supernatants, following the manufacturer instructions.

2.6. Cell proliferation assay

Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used to measure cell proliferation. Briefly, 5×10^4 $CD4^+$ T cells were incubated in 96-well plates at 37 °C under 5% CO_2 conditions for 72 h, followed by the induction with 1 μg/ml anti-CD3 and anti-CD28 antibodies. Thereafter, the CCK-8 reagent (10 μl) was added to all wells, followed by an additional incubation for 4 h at 37 °C. Finally, absorbance was measured at 450 nm.

2.7. Flow cytometry (FCM)

To assess the expression of CD3, CD4, CD8, CD25, FOXP3, and IL-17, cells were incubated with anti-mouse CD3-APC-R700, CD4-BV510, CD8-PerCP-Cy5.5, CD25-BB515, and IL-17-BV650 antibodies (BD Biosciences, San Jose, CA, USA) for 30 min in the dark at 4 °C. Then, the membrane was broken and stained with the FOXP3 antibody for 20 min in the dark at 4 °C. After rinsing twice, FCM was performed for cell analysis.

2.8. Western blot (WB) analysis

Total proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. The membranes were incubated with 5% skim milk at ambient temperature for 2 h, followed by incubation with an anti-ORAI1 rabbit polyclonal antibody () overnight at 4 °C. GAPDH served as an endogenous control. Blots were developed by incubating membranes with a biotinylated anti-rabbit antibody (1:5000). Signals were detected using an enhanced chemiluminescence kit and X-ray films.

2.9. Confocal fluorescence microscopy

Prior to stimulation with 500 ng/ml ionomycin combined with 50 ng/ml phosphomolybdic acid, 5×10^4 spleen lymphocytes were incubated for 30 min with PBS. The stimulation process was terminated by adding prechilled PBS (500 ml), followed by cell centrifugation on slides using a Cytospin 4 cytocentrifuge. After 15-min fixation with cold acetone, the cells were treated for 15 min with 0.5% Triton X-100. Then, the cells were blocked with 3% bovine serum albumin in PBS for 1 h at ambient temperature, followed by cell staining with a mouse anti-NFAT1 primary antibody (sc-7296; Santa Cruz Biotechnology) for 1 h at ambient temperature. Cells were then rinsed three times with PBS and probed with goat anti-mouse cyanine 3-labeled secondary antibody (SA00009-1; Proteintech) for 1 h. Afterward, the cells were washed and mounted with a Hoechst-containing mounting solution and were observed under a laser scanning confocal microscope.

2.10. Measurement of cytosolic calcium concentrations

A total of 1×10^6 cells were incubated with 2.5 μM Fura-2/AM for 30 min at room temperature in the dark. The supernatant fluid was changed twice to remove extracellular dye. Approximately 1×10^6 cells were placed in a quartz cuvette, and their fluorescence was measured at 510 nm using a fluorescent spectrometer by alternating between the excitation wavelengths of 340 and 380 nm. Calcium concentrations were expressed as a ratio of the fluorescence at 340 and 380 nm (F340/F380). The calcium signal was also detected with Fluo-3/AM. T cells were labeled with 5 mM Fluo-3/AM (Molecular Probes, Eugene, OR, USA) in PBS containing 0.02% Pluronic™ F-127 (Molecular Probes) at 37 °C for 45 min. Subsequently, the cells were washed twice and allowed to rest for

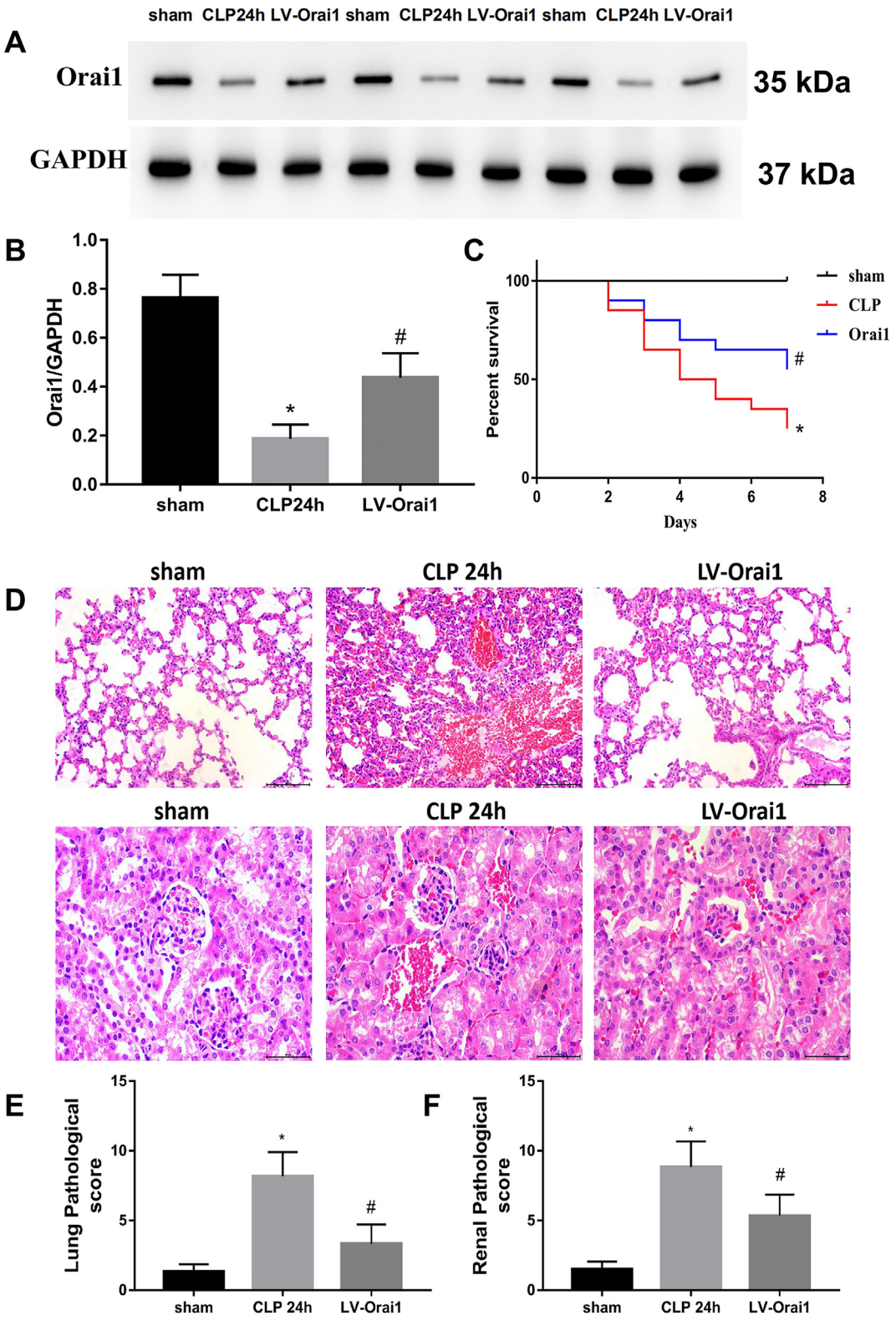

Figure 1. Effects of ORAI1 overexpression on organ damage and mortality in septic mice. (A and B) $CD4^+$ T cells were incubated for 30 min with anti-CD3 and anti-CD28 monoclonal antibodies. Expression levels of ORAI1 were measured by western blotting. Results are displayed as the mean ± SD ($n = 3$). $^{\#}P < 0.05$ vs. the 24-h CLP group, $^{*}P < 0.05$ vs. the sham group. (C) BALB/c mice ($n = 60$) were randomized into three groups and monitored daily for 7 consecutive days. A Kaplan–Meier curve was plotted to analyze the survival rates, and a log-rank test was used for comparison. $^{\#}P < 0.05$ vs. the 24-h CLP group, $^{*}P < 0.05$ vs. the sham group. (D–F) Renal and hepatic pathological damage was assessed using histology and scores. Representative pathological images are shown for results obtained based on a typical assay. $^{\#}P < 0.05$ vs. the 24-h CLP group, $^{*}P < 0.05$ vs. the sham group.

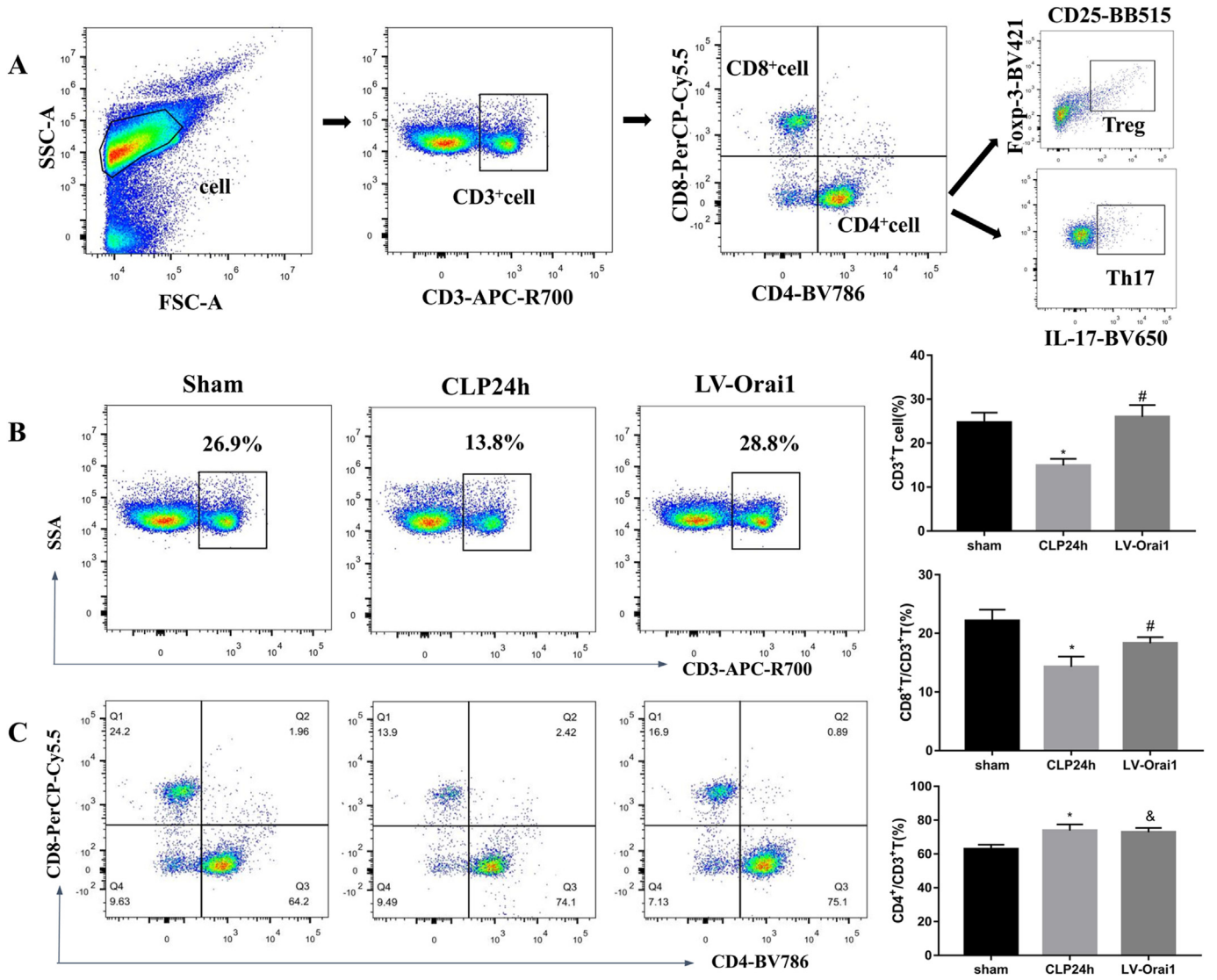

Figure 2. Effects of ORAI1 overexpression on the proportions of $CD3^+$ and $CD8^+$ T cells in the spleen of septic mice. BALB/c mice were randomized into three groups. The expression of CD3, CD4, and CD8 was detected by flow cytometry. (A) First, $CD3^+$ T cells were circled from total spleen cells, then $CD4^+$ and $CD8^+$ T cells were circled from $CD3^+$ T cells, and finally, Th17 cells and Tregs were circled from $CD4^+$ T cells. (B) Proportion of $CD3^+$ T cells in total cells in each group. Results are displayed as the mean ± SD ($n = 6$). $^{\#}P < 0.05$ vs. the 24-h CLP group, $^{*}P < 0.05$ vs. the sham group. (C) Proportions of $CD4^+$ and $CD8^+$ cells in $CD3^+$ T cells in each group. Results are displayed as the mean ± SD ($n = 6$). $^{\#}P < 0.05$ vs. the 24-h CLP group, $^{\&}P > 0.05$ vs. the 24-h CLP group, $^{*}P < 0.05$ vs. the sham group.

20 min in the dark. The labeled cells were washed, resuspended with prewarmed Hanks' balanced salt solution, and analyzed by FCM using a FACScan (BD Biosciences, Mountain View, CA, USA).

2.11. Survival analysis

To analyze survival, we randomized 60 BALB/c mice into the same three groups (sham, LV-ORAI1, and 24-h CLP), with 20 mice per group. Each mouse had free access to water and food and was monitored daily for 7 consecutive days to analyze the long-term effect of ORAI1 overexpression.

2.12. Histopathological examination

Lungs and kidneys were harvested at 24-h postoperatively, and samples were fixed with 10% neutral buffered formalin, followed by embedding in paraffin, sectioning, and hematoxylin and eosin staining. Lung injury was rated according to alveolar edema, hemorrhage, and neutrophil infiltration from 0 to 4 (normal to severe). Likewise, we rated kidney injury according to glomerular epithelial hyperplasia, tubular dilatation, inflammatory cell infiltration, and protein cast occurrence from 0 to 3 (normal to severe). The scoring was performed by a pathologist. Finally, overall scores were determined for individual samples.

2.13. Statistical analysis

Results are presented as the mean ± standard deviation (SD) and were analyzed using one-way analysis of variance. Significance of differences between groups was assessed using Fisher's least significant difference test. The survival rate was determined using the Kaplan–Meier method. A value of $P < 0.05$ was considered to indicate statistically significant difference.

3. Results

3.1. Overexpression of Orai1 can reduce the mortality and organ damage in septic mice

To better clarify the effect of ORAI1 on sepsis, we randomized 60 mice into three groups (sham, LV-ORAI1, and 24-h CLP) (Figure 1A, B).

Each mouse was observed for a 7-day period. As shown in Figure 1C, compared with that of the CLP mice, the survival rate significantly improved in the LV-ORAI1 group. The damage to the lung and kidney was evaluated by histological observation and pathological scores. As shown in Figure 1D–F, the lung tissue in the 24-h CLP group showed neutrophil infiltration, alveolar edema, and hemorrhage, while the kidney tissue showed tubular dilatation, tubular epithelial necrosis, inflammatory cell infiltration, and protein cast occurrence. However, the LV-ORAI1 group showed mitigated tissue injury.

3.2. Overexpression of Orai1 can affect lymphocyte differentiation in sepsis

As shown in Figure 2A–C, the proportion of $CD3^+$ T cells significantly decreased in the spleen of septic mice and significantly increased after overexpressing ORAI1. FCM showed that the proportion of $CD8^+$ T cells significantly decreased in the spleen of septic mice but increased upon overexpression of ORAI1. The proportion of $CD4^+$ T cells in $CD3^+$ T cells significantly increased but did not significantly change after overexpression of ORAI1. We further estimated the proportions of splenic Th17 cells and Tregs in septic mice. As shown in Figure 3A, B, the proportions of Th17 cells and Tregs were elevated in septic mice. After overexpression of ORAI1, the proportion of Tregs in $CD4^+$ T cells significantly decreased, and that of Th17 cells slightly decreased.

3.3. Overexpression of ORAI1 increases NFAT1 translocation in splenic $CD4^+$ T cells

A previous study has shown that NFAT signaling pathways are closely associated with apoptosis of $CD4^+$ T lymphocytes [17]. In this study, we detected the components of the ORAI1/NFAT signaling pathway in splenic $CD4^+$ T cells from septic mice. As shown in Figure 4, the NFAT translocation was abated in splenic $CD4^+$ T cells, while ORAI1 overexpression increased NFAT1 translocation in these cells.

3.4. Overexpression of Orai1 improves immune function of $CD4^+$ T cells in sepsis

The secretion of IFN-γ and IL-4 by splenic $CD4^+$ T cells was stimulated by anti-CD3 and anti-CD28 antibodies. The results showed that upon T-cell receptor (TCR)/CD28 costimulation, splenic $CD4^+$ T cells from patients with sepsis did not produce IFN-γ, IL-2, and IL-4 at the same levels as those in the sham group (Figure 5A–C). $CD4^+$ Th cells have differential responses to antigen stimulations and are divided into two different effector cell subpopulations, Th1 and Th2, according to different expression levels of cytokines as well as different immunoregulatory effects. Th1 cells are mostly responsible for secreting IFN-γ and IL-2, while Th2 cells are mostly in charge of secreting IL-4. Our study showed a Th1-to-Th2 response shift 24 h following sepsis induction, which contributed to immunosuppression. Moreover, we measured the proliferation of splenic $CD4^+$ T cells in patients with sepsis (Figure 5D). The proliferation activity was lower in the 24-h CLP group than in the sham group, but this change was reversed after ORAI1 overexpression. These results suggest that ORAI1 overexpression can improve immune function of T cells in sepsis.

3.5. Overexpression of ORAI1 enhances calcium signaling in $CD4^+$ T cells in sepsis

The Ca^{2+}/calcineurin/NFAT signaling pathway is closely associated with apoptosis of $CD4^+$ T lymphocytes [17]. In this study, we observed calcium signaling in septic mice. As shown in Figure 6A–C, the levels of the intracellular calcium ion ($[Ca^{2+}]i$) in the 24-h CLP group were reduced compared with those in the sham group. The overexpression of ORAI1 enhanced calcium signaling in splenic $CD4^+$ T cells.

4. Discussion

At present, CLP has been extensively adopted as a sepsis animal model that shows a well-recognized reliability and clinical relevance to human

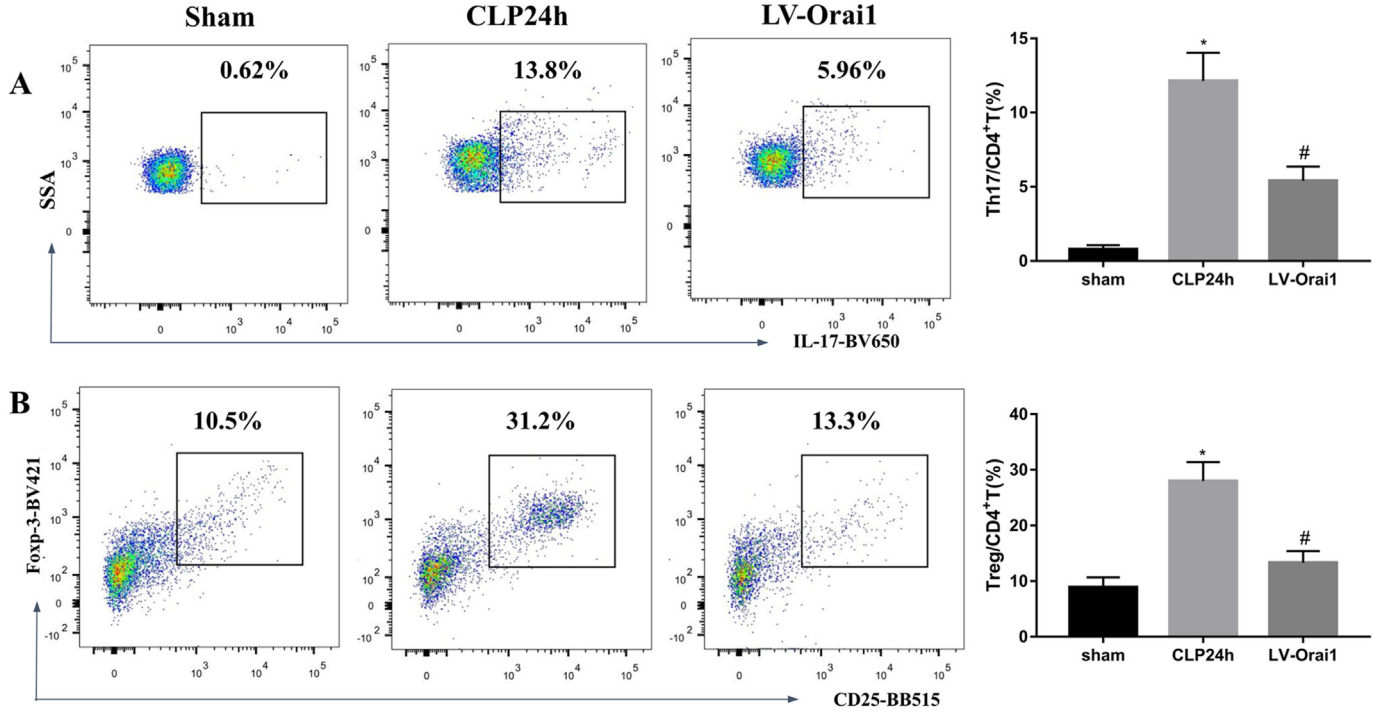

Figure 3. Effects of ORAI1 overexpression on the proportions of Th17 cells and Tregs in septic mice. BALB/c mice were randomized into three groups. The proportions of Th17 cells and Tregs in $CD4^+$ T cells from the spleen were examined by flow cytometry. Results are displayed as the mean ± SD ($n = 6$). $^{\#}P < 0.05$ vs. the 24-h CLP group, $^{*}P < 0.05$ vs. the sham group.

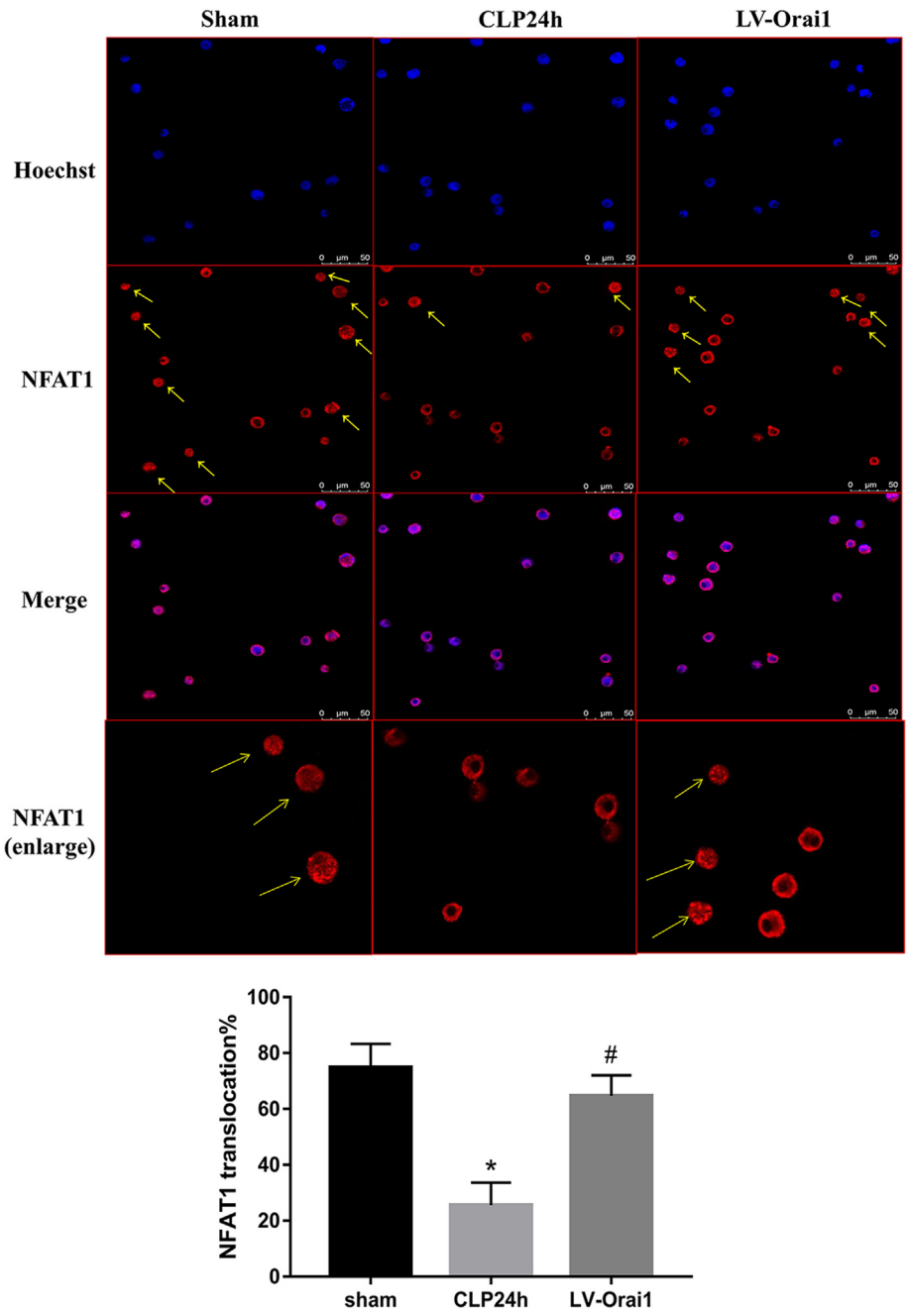

Figure 4. Effects of ORAI1 overexpression on NFAT1 translocation in splenic $CD4^+$ T cells. BALB/c mice were randomized into three 3 groups. NFAT1 translocation was analyzed by laser scanning confocal microscopy. The yellow arrow represents NFAT1 intranuclear transfer. The mean optical density was recorded for statistics. Results are displayed as the mean ± SD. $^{\#}P < 0.05$ vs. the 24-h CLP group, $^{*}P < 0.05$ vs. the sham group.

sepsis. The CLP procedure is simple and involves ligation of the distal ileocecal valve; after cecal ligation, needle puncture leads to the leakage of the fecal contents to the peritoneum, thereby causing polymicrobial bacteremia as well as sepsis. There are diverse bacterial species within the bloodstream, which can subsequently result in continuous systemic inflammatory response syndrome, septic shock, and multiorgan impairment.

In the CLP-induced sepsis model, cytokine profiles are similar to those observed in patients with sepsis [16]. Sepsis starts a complicated immune response, which varies over time, and the pro- and anti-inflammatory mechanisms coexist simultaneously. Consequently, many septic cases quickly show substantial immunosuppression signs, which is related to unfavorable outcomes [18, 19]. As previously reported, after sepsis has occurred, Th1- and Th2-type cytokines are released at lower amounts. In addition, a Th1-to-Th2 response shift is observed, thus facilitating immunosuppression during sepsis [20, 21]. Our results were consistent with the data of previous reports, indicating that anti-CD3 and anti-CD28 antibody-induced IFN-γ and IL-4 secretion by septic $CD4^+$ T cells markedly decreased at 24 h. The 24-h CLP group had a reduced proliferation activity of splenic $CD4^+$ T cells compared with that in the sham group.

Abnormalities in T-cell differentiation play an important role in the pathogenesis of sepsis. In this study, we observed a significant decrease in the proportion of $CD3^+$ T lymphocytes. Th17 cells synthesize a proinflammatory factor (IL-17A) and are the main contributors to several disorders [22, 23]. By contrast, Tregs have been suggested to suppress inflammation and immune responses by secreting the anti-inflammatory

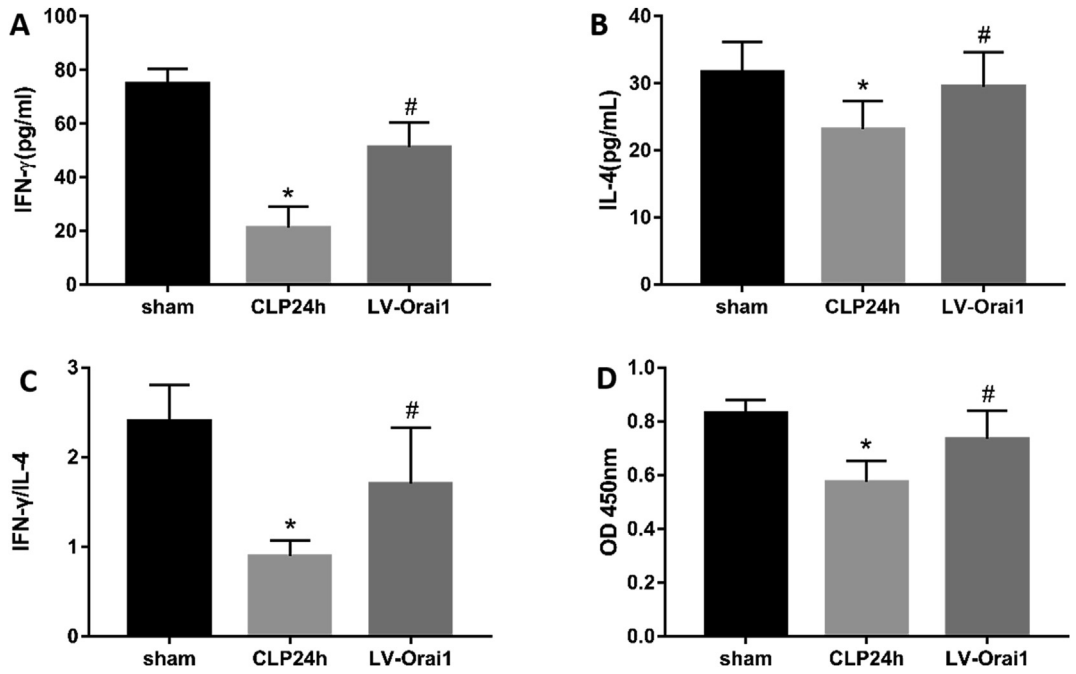

Figure 5. Effects of ORAI1 overexpression on immune dysfunction of septic splenic $CD4^+$ T-cells. BALB/c mice were randomized into three groups. $CD4^+$ T cells were collected 24 h after the operation. IL-4 and IFN-γ expression in $CD4^+$ T cells was measured by ELISA after 24-h incubation with anti-CD3 and anti-CD28 antibodies. Results are displayed as the mean ± SD ($n = 6$). $^*P < 0.05$. The CCK-8 assay was performed to measure splenic $CD4^+$ T-cell proliferation. $^{\#}P < 0.05$ vs. the 24-h CLP group, $^*P < 0.05$ vs. the sham group.

factor IL-10 [24,25]. Th17 cells and Tregs have distinct functions in inflammatory responses, thus maintaining immune homeostasis together. In this study, we observed that septic mice showed markedly increased proportions of Th17 cells and $CD4^+CD25^+FOXP3^+$ Tregs compared with those in the sham group. Furthermore, the proportion of Tregs in $CD4^+$ T cells significantly decreased, and that of Th17 cells slightly decreased, which supported the findings of prior reports. A study has also shown that cell-regulated immune responses were imbalanced and the

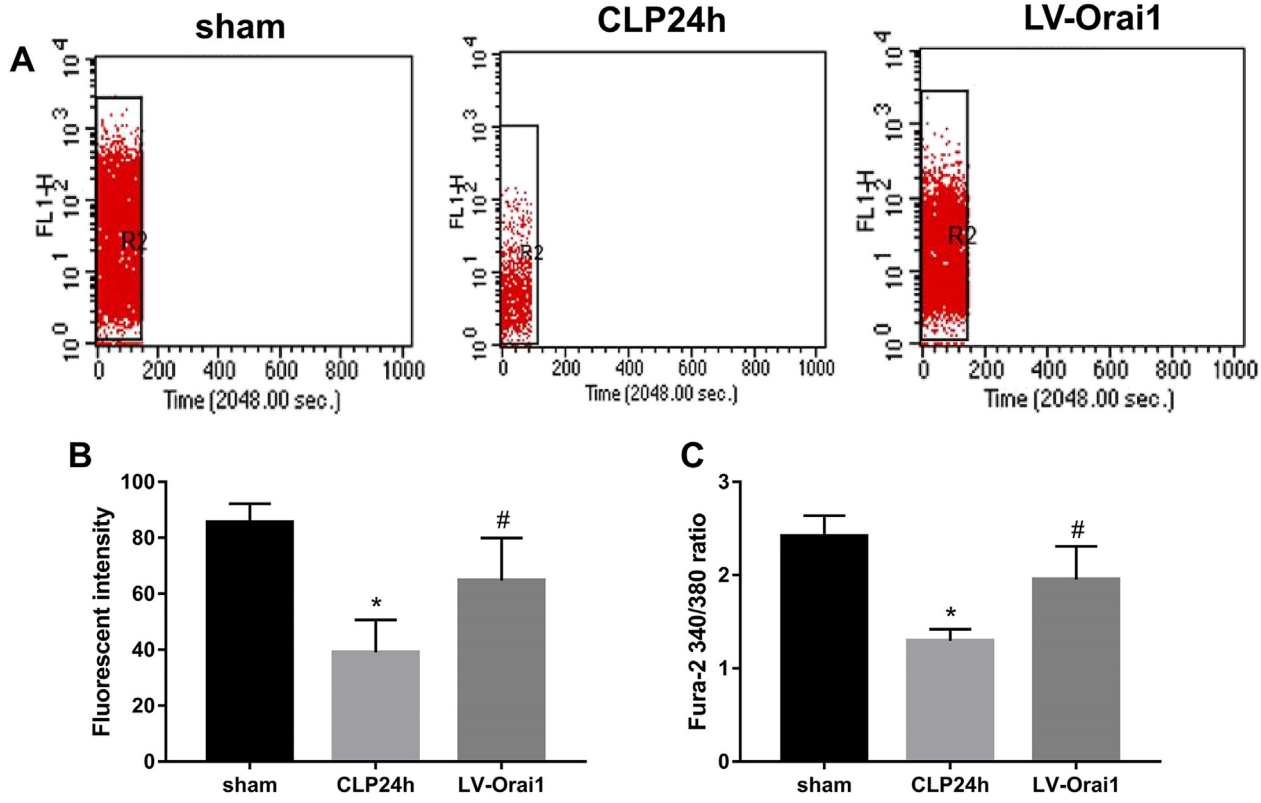

Figure 6. Effects of ORAI1 overexpression on calcium signaling in $CD4^+$ T cells in sepsis. BALB/c mice were randomly divided into three groups. (A and B) $[Ca^{2+}]i$ was measured using Fluo-3. (C) $[Ca^{2+}]i$ was measured using a Fura-2 ratio at 340 and 380 nm. Results are displayed as the mean ± SD ($n = 6$). $^{\#}P < 0.05$ vs. the 24-h CLP group, $^*P < 0.05$ vs. the sham group.

proportions of Tregs and Th1/Th2/Th17 populations of Th cells were disordered among cases with post-traumatic sepsis [26]. Therefore, the imbalanced Th2/Th1 and Treg/Th17 proportions are possibly associated with the pathogenesis of sepsis.

CRAC channels represent the SOC prototype within the well-characterized immunocytes. As a pore subunit, ORAI1 sheds substantial light on CRAC channels while providing the molecular approach to investigating physiological outcomes of Ca^{2+} signaling in the process of immunity [27]. T The downstream NFAT transcription factors are involved in the selective transcriptional process within T cells, which restricts immunity [28]. Our study confirmed that the ORAI1/NFAT signaling pathway was inhibited in septic mice. Interestingly, the overexpression of ORAI1 not only improved immune function of T cells in sepsis and reduced the mortality and organ damage in septic mice but also partially recovered the NFAT activity in sepsis. These data suggest that the ORAI1-mediated NFAT signaling pathway can improve sepsis-induced T-lymphocyte immunosuppression and acute organ dysfunction. However, a previous study has shown a normal proliferation of Tregs in ORAI1-deficient mice, mainly due to remaining SOCE in naïve $CD4^+$ T cells, which is possibly regulated via ORAI2 and/or ORAI3 [29]. In future studies, this mechanism needs to be further verified by knocking out Orai1 in septic mice.

Ca^{2+} is a ubiquitous intracellular signaling entity responsible for controlling numerous cellular processes. Our study confirmed that Ca^{2+} signaling was inhibited in septic mice, while the overexpression of ORAI1 enhanced calcium signaling in $CD4^+$ T cells in sepsis. Therefore, we speculated that the NFAT1 translocation or the change in the ratio of Th17 cells and Tregs in ORAI1-overexpressing mice might be due to altered calcium signaling. Early reports also showed decreased calcium signaling and decreased T-cell proliferation in sepsis [30, 31, 32]. However, a recent study has reported that immediate downstream signaling and TCR stimulation were not altered in circulating CD4 lymphocytes from patients with septic shock. The cells exhibited no deregulation of intracytoplasmic calcium influx after TCR ligation compared with that in cells from healthy controls [33]. In sepsis, T-cell immune responses change over time, and calcium signaling may also change. Therefore, more time points are needed to clarify its changing status in the CLP model.

Taken together, abnormal differentiation and immunosuppression of T lymphocytes may contribute to the pathogenesis of sepsis. The ORAI1/NFAT signaling pathway was found to be suppressed during sepsis, which may be related to sepsis-mediated acute organ dysfunction and T-lymphocyte immunosuppression. The overexpression of ORAI1 could partially reverse this effect by regulating calcium signaling, and ORAI1 may be a candidate antisepsis therapeutic target.

Declarations

Author contribution statement

Longwang Chen, Heliang Ke and Yaolu Zhang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pinpin Jin, Xinyong Liu and Guangliang Hong: Analyzed and interpreted the data; Performed the experiments; Contributed reagents, materials, analysis tools or data.

Guangju Zhao, Zhongqiu Lu, Bin Wu: Conceived and designed the experiments; Analyzed and interpreted the data.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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