Orai1 Mediated Calcium Influx Improves Sepsis-induced T Lymphocyte Immunosuppression and Acute Organ Dysfunction

Longwang Chen

The First Affiliated Hospital of Wenzhou Medical University

Guangju Zhao

The First Affiliated Hospital of Wenzhou Medical University

Heliang Ke

The First Affiliated Hospital of Wenzhou Medical University

Xinyong Liu

The First Affiliated Hospital of Wenzhou Medical University

Jie Lian

The First Affiliated Hospital of Wenzhou Medical University

Miaotong Lin

The First Affiliated Hospital of Wenzhou Medical University

Guangliang Hong

The First Affiliated Hospital of Wenzhou Medical University

Zhongqiu Lu (✉ lzq_640815@163.com)

First Affiliated Hospital of Wenzhou Medical University <https://orcid.org/0000-0002-0569-4182>

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Abstract

Sepsis-triggered immune paralysis, particularly CD4⁺ T-cell dysfunction, increases susceptibility to infections. Ca²⁺ signals arising from store-operated calcium entry (SOCE) in T lymphocytes are critical mediators to infection, inflammation, and autoimmunity. Orai1 is a major component of SOCE. The role of Orai1 and SOCE in sepsis-induced immunosuppression remain to be elucidated. In this study, we first identified the immunosuppression of splenic CD4⁺ T cells and CD4⁺CD25⁺Treg cell/T helper 17 (Th17) cell imbalance in septic mice. Following this, we found that Ca²⁺-calcineurin-calcineurin-nuclear factor of activated T cell (NFAT) signaling pathways as well as SOCE were inhibited in septic mice. Further, Upregulation of Orai1 not only can improve immune function of T cell in sepsis but also reduce the mortality and organ damage in septic mice. Lastly, Overexpression of Orai1 can partially recovery of SOCE in sepsis. These data suggest that Orai1 mediated calcium influx can improve sepsis-induced T lymphocyte immunosuppression and acute organ dysfunction.

Introduction

- Sepsis, defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection, is a leading cause of intensive care unit (ICU) mortality worldwide [1]. Many recent studies have shown that immunosuppression plays a critical role in the late phase of sepsis, contributing to multiple organ dysfunction [2, 3]. Immunosuppression is mainly caused by T cell dysfunction [4]. Despite several strategies targeting immunosuppression, the outcome of sepsis remains poor [5]. Th17 cells characteristically secrete interleukin (IL)-17, which could induce the production of pro-inflammatory cytokines such as TNF- α , IL-6, IL-1 β , thereby exacerbating the inflammatory response [6, 7]. However, Tregs could maintain immune homeostasis by secreting anti-inflammatory mediators e.g. IL-10 and TGF- β to exert an anti-inflammatory role of inhibiting excessive inflammatory responses [8]. The imbalance of Th17 and Treg is related to the occurrence and development of sepsis [9].
- Ca²⁺ signals in T lymphocytes are critical mediators of infection immunity, inflammation, and autoimmunity [10]. Elevations in intracellular Ca²⁺ concentrations result from two sources: the release of Ca²⁺ from intracellular stores and influx of extracellular Ca²⁺ through the plasma membrane Ca²⁺ Channels [11]. The best described Ca²⁺ influx pathway in T lymphocytes is store-operated calcium entry (SOCE) through Ca²⁺ release-activated Ca²⁺ (CRAC) channels, triggered by Ca²⁺ release from ER stores. SOCE is accomplished by the pore-forming Ca²⁺ channel subunits Orai1, Orai2, and Orai3 [12]. As a major component of calcium store-operated channels (SOCs), Orai1 appears to be both necessary and sufficient to reconstitute SOCE [13, 14].
- The role of Orai1 and SOCE in sepsis-induced immunosuppression remain to be elucidated. Therefore, in this study, we tested the immunosuppressive function of splenic CD4⁺ T cells, expression of Orai1 protein, Ca²⁺-calcineurin-NFAT signaling pathway, and SOCE channels in septic mice. We further validated the mechanism by regulating Orai1.

Materials And Methods

Animals

All experimental procedures were performed in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation at WenZhou Medical University, China (Ethics number:WYDW2020-0457). Male BALB/c mice (aged 6–8 weeks, purchased from the Institute of Laboratory Animal Sciences, Shanghai, China) were housed in an animal facility with a 12/12-h light/dark cycle at 22°C and fed a laboratory diet and water *ad libitum*. The mice were anesthetized via an intraperitoneal injection of 4 mg of sodium pentobarbital per 100 g body weight.

Experimental protocol of sepsis induction

Polymicrobial sepsis was induced in mice by a cecal ligation and puncture (CLP) procedure, as previously described [15]. Briefly, the mice were anesthetized, and a midline incision was made to expose the cecum, filled with feces by milking stool backwards from the ascending colon, and 50% of the cecum was ligated with a 5 – 0 silk suture. The cecum was soaked in phosphate-buffered saline (PBS) (pH 7.4) and was then punctured once with a 21-gauge needle on the antimesenteric border. The cecum was then returned into the peritoneal cavity, and the abdominal incision was sutured. Sham mice underwent the same laparotomy and resuscitation procedures, but the cecum was neither ligated nor punctured in these mice. Animals were reanesthetized after CLP or sham procedure to collect samples for further analysis. The mice were classified into three groups: sham operation group (sham), 12 hour post CLP group (CLP 12h), and 24 hour post CLP group (CLP 24h). There were six mice included in each group.

Isolation of splenic CD4 + T cells

Spleens were removed from mice and teased in 5 ml RPMI 1640 (Tianrun Shanda Biotech Co. LTD, Beijing, China). Cells were dispersed through a 30-µm stainless steel mesh and collected after centrifugation at 300 *g* for 10 min, and resuspended in 4 ml RPMI 1640. Mononuclear cells were then obtained by Ficoll-Paque density gradient centrifugation. CD4⁺ T cells were isolated from splenocytes by positive selection using magnetic-activated cell sorting (MACS) CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as per the manufacturer's directions. In brief, CD4⁺ T cells were separated by passing the cell suspension over a magnetic-activated cell sorter MS column held in an MACS magnetic separator (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD4⁺T cells adhering to the column were then harvested for further use.

Enzyme-linked immunosorbent assay

Interleukin (IL)-2, IL-4, and interferon (IFN)-γ levels in the culture supernatant were measured using commercially available enzyme linked immunosorbent assay (ELISA) kits for mice. ELISA was performed strictly following the manufacturer's protocols. ELISA kits of IL-2, IL-4, and IFN-γ were purchased from Biosource (Worcester, MA).

Cell Proliferation Assay

Cell proliferation was assayed using a Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) assay kit. A total of 5×10^4 isolated CD4⁺ T cells were pre-incubated in 96-well plates (cultured in 5% CO₂ at 37°C) for 72 h stimulated with anti-CD3 and anti-CD28 (1 µg/ml). Following this, 10 µl of CCK-8 solution was added to each well, and the wells were incubated for 4 h at 37°C. The optical density of each solution was then measured at 450 nm.

Flow cytometry

To assess the CD4⁺CD25⁺ Treg and the IL-17 expression, the cells were stained with anti-mouse CD4-BV510 antibody, CD25-BB515 antibody and IL-17-BV650 antibody (BD Biosciences, San Jose, CA, USA) for 30min at 4°C in the dark. Following two more washing steps, the cells were analyzed via flow cytometry.

Western blot analysis

Total proteins were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following this, they were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were first blocked with 5% non-fat milk for 2 h at room temperature, followed by incubation with rabbit polyclonal antibody against target proteins at 4°C overnight. β-actin or lamin B was detected as a loading control. The blots were then developed by incubation with biotinylated anti-rabbit antibody (1:5000). Signals were detected using an ECL kit and X-ray films.

Cytosolic calcium concentration measurement

A total of 1×10^6 Cells were incubated for 30 min at room temperature with 2.5 µM Fura-2AM in darkness. Supernatant fluid was changed twice to remove extracellular dye. About 1×10^6 cells were placed in a quartz cuvette, and their fluorescence at 510 nm was measured on a fluorescent spectrometer by alternating between wavelengths of 340 nm and 380 nm. Calcium concentration was expressed as a ratio of the fluorescence at 340 nm to the fluorescence at 380 nm (F₃₄₀/F₃₈₀).

Intracellular calcium and SOCE measurement

T cells were labeled with 5 mM Fluo-3/AM (Molecular Probes, Eugene, OR) in PBS containing 0.02% Pluronic F-127 (Molecular Probes, Eugene, OR) at 37°C for 45 min. Subsequently, cells were washed twice and allowed to “rest” for 20 min in the dark. Labeled cells were washed, resuspended with prewarmed Hanks’ balanced salt solution (HBSS), and analyzed by flow cytometry using a FACScan (BD Biosciences, Mountain View, CA). Ca²⁺ store depletion was triggered by incubating cells with 1 nM thapsigargin (Tg) (Sigma) in Ca²⁺-free HBSS. SOCE was measured by monitoring the increase in [Ca²⁺]_i after adding 2 mM CaCl₂ [Hanks Balanced Salt Solution (with Ca²⁺ & Mg) (Beyotim)] to the Tg-containing medium.

Assay of NFAT activity

Nuclear was extracted by Nuclear extract kit (Active Motif). The TransAM NFAT Transcription Factor ELISA Kits (Active Motif) was used to quantify the amount of active NFAT in nuclei. Briefly, the active

NFAT were purified from a nuclear lysate upon binding an immobilized oligonucleotide containing a 50-AGGAAA-30 motif, and detected by ELISA.

Confocal Fluorescence Microscopy

A total of 5×10^4 spleen leukocytes were rested in DPBS for 30 min before the cells were stimulated with 50 ng/ml PMA plus 500 ng/ml ionomycin. The stimulation was stopped by adding 500 ml precooled PBS, and the cells were then centrifuged onto slides using Cytospin 4. The cells were fixed with prechilled acetone for 15 min, and then with 0.5% Triton X-100 for another 15 min. After blocking with 3% BSA in PBS at room temperature for 1 h, the cells were stained with mouse-anti NFAT1 (sc-7296; Santa Cruz Biotechnology) as primary Abs at room temperature for 1 h. After three washes with PBS, the cells were incubated with goat-anti-mouse cy3 (Proteintech SA00009-1) as secondary Abs for 1 h. After washing and mounting with mounting solution containing Hoechst. The experimental results were observed under a laser scanning confocal microscope.

Overexpression of Orai1

According to the results of the previous experiment, the time point of 24 hours after operation was selected for the next experiment. The 12 mice were classified into two groups: 24 hour post CLP group (CLP 24h) and Overexpression of Orai1 group (LV-Orai1). The LV-Orai1 group of Orai1 uses lentivirus to carry the full-length Orai1 gene fragment (Shanghai Genechem Co., Ltd.). The lentivirus targeting Orai1 was diluted to a total volume of 300 μ l containing 4×10^7 TU was injected into the tail vein of BALB/c mice twice a week for 4 weeks. After 4 weeks of treatment, the mice were performed by CLP.

Survival analysis

In the survival experiment, BalB/c mice ($n = 40$) were randomly divided into the following two groups ($n = 20$ /group): CLP 24h and LV-Orai1 group. All mice were provided with food and water *ad libitum* and were monitored every day. The mice were monitored for 7 days to analyze the long-term effects of Orai1.

Histopathological Examination

The kidneys and lungs were collected at 24 h after operation. They were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Lung injury was scored from 0–4 (normal to severe) based on neutrophil infiltration, hemorrhage, alveolar, and edema. Kidney injury was scored from 0–3 (normal to severe) based on tubular dilatation, glomerular epithelial hyperplasia, protein cast formation, and inflammatory cell infiltration. The score was performed by the pathologist. The total score for each sample was calculated.

Statistical analysis

Data are represented as mean $\pm$ standard deviation (SD), and analyzed with one-way ANOVA. Fisher's least significant difference (LSD) was used to evaluate significant differences between groups. The

survival rate was determined using the Kaplan-Meier estimator. $P < 0.05$ were considered statistically significant.

Results

CD4⁺ T cell immune dysfunction in sepsis

Generation of IL-2, IL-4, and IFN- γ is critical in T-cell-dependent immunity. IL-2, IL-4, and IFN- γ secretion from splenic CD4⁺ T cells is induced by challenge with anti-CD3/CD28 antibody stimulation. We showed that septic splenic CD4⁺ T cells fail to fully produce IL-2, IL-4, and IFN- γ in response to TCR/CD28 costimulation compared with sham (Fig. 1). It is well known that CD4⁺ helper T cells can differentiate antigen stimulations and divide their responses into two distinct subsets of effector cells, Th1 and Th2, based on their distinct cytokine expression profiles and their subsequent immune regulatory functions. Th1 cells mainly secrete IL-2 and IFN- γ , whereas Th2 cells secrete IL-4, our study has shown that on 12 hour and 24 hour following sepsis, there is a shift from Th1 to Th2 response, which contributes to immunosuppression. Moreover, we detected the proliferation activity of septic splenic CD4⁺ T cells. As shown in Fig. 2, the proliferation activities of CLP 12h and CLP 24h groups were lower than those of the sham group.

A regulatory T (Treg) cell/T helper 17 (Th17) cell imbalance is involved in sepsis

The imbalance of Treg/Th17 have a crucial role in the development of many disease. Therefore, we further detect the frequency of splenic CD4⁺CD25⁺Treg cells and Th17 cells in septic mice. As shown in Fig. 3, the proportion of Treg cells and Th17 cells increased in sepsis, which was obvious at 24 hours. Moreover, the ratio of Treg and Th17 was also increased in sepsis mice.

Inhibition of Ca²⁺-calcineurin-NFAT signaling pathways in sepsis

- Previous study has shown that Ca²⁺-calcineurin-NFAT signaling pathways are closely associated with apoptosis of CD4⁺ T lymphocytes [16]. In the current experiment, we detected the Ca²⁺-calcineurin-NFAT signaling pathways of splenic CD4⁺ T cells in septic mice. As shown in Fig. 4, the levels of [Ca²⁺]_i in CLP 12h and CLP 24h groups were reduced compared with those in the sham group (A, B and C). Consistent with the [Ca²⁺]_i levels, the activity of NFAT in the nuclear fraction (D) and the protein levels of calcineurin (E) were also markedly reduced in splenic CD4⁺ T cells. Moreover, the NFAT translocation was abated in splenic CD4⁺ T cells (Fig. 5). These results indicated that Ca²⁺-calcineurin-NFAT signaling pathways were inhibited in sepsis.
- Inhibition of Tg-dependent Ca²⁺ influx in splenic CD4⁺ T cells in septic mice

Addition of Tg in a Ca^{2+} -free solution produced a transient rise in Ca^{2+} most likely reflecting Ca^{2+} release from the ER stores; when the medium was subsequently supplemented with 2 mM Ca^{2+} , the CD4^{+} T cells displayed a large increase in Ca^{2+} levels. This increase reflects Ca^{2+} influx through the plasma membrane SOCs, triggered by Ca^{2+} release from ER stores. Therefore, we detected the Tg-dependent Ca^{2+} influx in splenic CD4^{+} T cells in septic mice. As shown in Fig. 6, the Tg-dependent Ca^{2+} influx was inhibited in splenic CD4^{+} T cells in septic mice. These results reflected that the SOCE was decreased in septic mice.

Inhibition of Orai1 expression in splenic CD4^{+} T cells of septic mice

For signaling experiments, splenic CD4^{+} T cells were stimulated with crosslinked anti-CD3 + anti-CD28 mAbs for 30 min. After anti-CD3/CD28 mAbs stimulation, we detected that the level of Orai1 protein was significantly lower in septic mice on 12 hour and 24 hour compared with that in sham animals (Fig. 7). As major components of calcium SOCs, Orai1 appears to be both necessary and sufficient to reconstitute SOCE. Thus, these results suggest that downregulation of Orai1 expression is associated with CD4^{+} T cell immune suppression.

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

To further clarify the role of Orai1 in sepsis, another 40 mice were randomly divided into 2 groups (CLP group and LV-Orai1 group). We followed the animals for 7days. As shown in Fig. 8, the survival rate was significantly improved up to 50% in LV-Orai1 group in comparison with 20% in CLP mice. The damage of lung and kidney were evaluated by histological observation and scores. As shown in Fig. 9A, the CLP 24h group exhibited hemorrhage, alveolar edema and neutrophil infiltration. As shown in Fig. 9B, the CLP 24h group exhibited tubular epithelial necrosis, tubular dilatation, protein cast formation and inflammatory cell infiltration. However, the extent of tissue injury was alleviated in the LV-Orai1 group.

Overexpression of Orai1 can improve immune function of T cell in sepsis

- Moreover, we detected the proliferation activity of splenic CD4^{+} T cells and the ratio of Th17/Treg in septic mice after overexpression of Orai1. As shown in Fig. 10, the proliferation levels in the LV-Orai1 group decreased compared with those in the CLP 24h group. The Th17/Treg imbalance was also attenuated after overexpression of Orai1 (Fig. 11). These results suggest that Orai1 can improve immune function of T cell in sepsis.

Overexpression of Orai1 can partially recovery of SOCE in sepsis

Orai1 appears to be both necessary and sufficient to reconstitute SOCE, therefore we detected the levels of SOCE after upregulation of Orai1. The increase of fluorescence intensity represents the level of SOCE. As shown in Fig. 12, overexpression of Orai1 can partially recovery of SOCE in sepsis.

Discussion

CLP is currently the most widely used animal model of sepsis and generally recognized as a reliable and clinically relevant animal model of the human septic condition. CLP surgery is straightforward: ligation distal to the ileocecal valve and needle puncture of ligated cecum cause leakage of fecal contents into the peritoneum, with subsequent polymicrobial bacteremia and sepsis. Multiple species of bacteria are found in the bloodstream, and progressive systemic inflammatory response syndrome followed by septic shock and multiorgan injury ensues. CLP-induced sepsis models show a cytokine profile similar to that in human sepsis [15]. Sepsis initiates a complex immune response that varies over time, with the concomitant occurrence of both pro-inflammatory and anti-inflammatory mechanisms. As a result, most patients with sepsis rapidly display signs of profound immunosuppression, which is associated with deleterious consequences [17, 18]. Previous studies have suggested that following the onset of sepsis, the production of both Th1 and Th2 cell-associated cytokines is decreased. Moreover, a shift from a Th1 to a Th2 response occurs, contributing to immunosuppression during sepsis [19, 20]. Our findings are consistent with those reported in previous studies, and indicate that anti-CD3/CD28 antibody-induced IL-2, IFN- γ , and IL-4 secretion from septic CD4⁺ T cells was markedly decreased on 12 h and 24 h. The proliferation activities of splenic CD4⁺ T cells in CLP 12h and CLP 24h groups were also lower than those in the sham group.

Th17 cells, characterized by the production of proinflammatory cytokines IL-17A, are a major contributor in multiple diseases [21, 22]. In contrast, Treg cells have been identified as dedicated suppressors of diverse immune responses and inflammations through the secretion of the anti-inflammatory cytokine IL-10 [23, 24]. These two cells play opposite roles in the inflammatory responses to jointly maintain immune homeostasis. The potential role of the Th17/Treg balance in sepsis has not been fully elucidated. In this study, we observed that septic mice exhibited a significant increase in the frequency of Th17 cells and CD4⁺CD25⁺Treg, compared to the sham group. Furthermore, we confirmed that the Treg/Th17 ratio in septic mice were higher as compared to the sham group. These results are consistent with previous studies. A study also showed an imbalance in cell mediated immune response and disturbance in Th1/Th2/Th17 and Treg population of T helper cells in patients with post traumatic sepsis [25]. Therefore, the skewing of the Treg/Th17 ratio along with Th2/Th1 may contribute to the pathogenesis of sepsis.

Calcium (Ca²⁺) is a ubiquitous intracellular signaling entity responsible for controlling numerous cellular processes. Calcium SOC_s activated by the depletion of Ca²⁺ from the ER are a primary Ca²⁺ entry pathway in nonexcitable cells and are essential for T cell activation and adaptive immunity [26–28]. SOCE is required not only for Treg development but also for their suppressive function [29]. Our study

confirmed that Ca^{2+} -calcineurin-NFAT signaling pathways as well as SOCE channels were inhibited in septic mice. Research also shows that CaN-NFAT pathway of T cells were inhibited in *Cryptococcus neoformans* infected rats [30]. However, it has been reported that inhibition of Ca^{2+} channels, rather than activation of SOCE process, is beneficial for the survival of septic patients [31]. In sepsis, T-cell immune response changes with time, and the level of SOCE may change with time. Therefore, more time points are needed to clarify its changing law in the CLP model. In this study, we found that the level of SOCE of CD4^+ T cells in sepsis was slightly decreased, while the proliferation of CD4^+ T cells was significantly decreased. The reason is that the proliferation ability is not only affected by SOCE, but also by other factors, such as apoptosis and immunosuppression.

Calcium SOCs compose of poreforming Ca^{2+} channel subunits Orai1, Orai2, and Orai3 as well as ER Ca^{2+} sensors STIM1 and STIM2 [32, 33]. As major components of SOCs, Orai1 appear to be both necessary and sufficient to reconstitute SOCE [13, 14]. In septic mice, the protein expression of Orai1 was decreased compared with that in the sham group. Interestingly, Upregulation of Orai1 not only can improve immune function of T cell in sepsis and reduce the mortality and organ damage in septic mice but also can partially recovery of SOCE in sepsis. These results proved that SOCE channels were suppressed during sepsis, and this may be involved in sepsis-induced immunosuppression of T lymphocytes and acute organ dysfunction. However, a study has shown that Orai1-deficient mice had normal Treg development; this is presumably because of residual SOCE in naive CD4^+ T cells, which is likely to be mediated by Orai2 and/or Orai3 [34]. We will further validate this mechanism by knocking out Orai1 in septic mice in future studies.

Taken together, the skewing of the Treg/Th17 along with Th2/Th1 may contribute to the pathogenesis of sepsis. SOCE channels were suppressed during sepsis, Orai1 can regulate SOCE. Lower SOCE levels may be associated with the sepsis-induced immunosuppression of T lymphocytes and acute organ dysfunction. Upregulation of Orai1 can partially block this effect. This will be a potential target for treatment of sepsis.

Declarations

AUTHORS' CONTRIBUTIONS

LC and GZ performed the experiments, analyzed the data, wrote the manuscript. ZL designed and supervised the project, contributed to the discussion and reviewed and edited the manuscript. HK, JL, XL and ML performed the *in vivo* experiment and reviewed and edited the manuscript. GH analyzed the data, contributed to the discussion, and reviewed/edited the manuscript.

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Conflicts of Interest. The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

DATA AVAILABILITY

The data that support this study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate.

All animal experiments were approved by the Regional Ethical Committee for Animal Experimentation at WenZhou Medical University, China. Significant efforts were made to minimize both the number of animals and their suffering. All procedures were strictly conducted in accordance with the code of ethics.

Consent for Publication: Not applicable

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Figures

Figure 1

Changes in cytokines secreted by splenic CD4+ T cells in septic mice. Mice underwent CLP or identical laparotomy and resuscitation procedures without the ligation or puncture of the cecum (sham group). CD4+ T cells were harvested 12 h and 24 h after CLP induction. Levels of IL-2, IFN-γ, and IL-4 in CD4+ T cells were determined 24 h after incubation with anti-CD3/CD28 antibodies by ELISA as described in the Materials and Methods. Data are presented as means $\pm$ SD. (*P < 0.05, n = 6)

Figure 2

Changes in the proliferation activity of splenic CD4⁺ T cells in septic mice. BalB/c mice were randomly divided into three groups, they were sacrificed at the set time. The proliferation activity of splenic CD4⁺ T cells was measured by the CCK-8 assay. Further, 5×10^4 isolated CD4⁺ T cells were co-incubated for 72 h with 1 mg/ml of soluble anti-CD3 antibody (eBioscience) and anti-CD28 antibody (eBioscience) in a humidified environment with 5% CO₂ at 37°C. Following this, 10 μ L CCK-8 solution was added to each well. The cells were incubated for 4 h at 37°C with 5% CO₂, and the OD at 450 nm was measured for all samples using a microplate reader. The proliferation levels in the CLP group decreased compared with those in the sham group (*P < 0.05, n = 6).

Figure 3

The frequency of Treg cells and Th17 cells in septic mice. BALB/c mice were sacrificed at 12 h and 24 h after operation. The spleen was collected. The proportion of Treg cells and Th17 cells were analyzed by flow cytometry. n = 6 mice per group. Data are shown as mean values $\pm$ SD are presented, p values were calculated using Unpaired T-test, *p < 0.05 compared with sham group.

Figure 4

Changes in Ca²⁺-calcineurin-NFAT signaling pathways of splenic CD4⁺ T cells in septic mice. BalB/c mice were randomly divided into three groups: they were sacrificed at the set time. Splenic CD4⁺ T cells were isolated. The Ca²⁺-calcineurin-NFAT signaling pathways were detected. (A and B) [Ca²⁺]_i was indicated by Fluo-3. (C) [Ca²⁺]_i was indicated by a Fura-2 ratio of 340 nm/380 nm. (D) NFAT activity in the nuclear fraction of CD4⁺ T cells as measured by ELISA. (E) Protein levels of calcineurin in each group. Data are presented as means ± SD. (*P < 0.05, n = 6).

Figure 5

Changes in NFAT1 translocation of splenic CD4⁺ T cells in septic mice. BalB/c mice were randomly divided into three groups: they were sacrificed at the set time. Splenic CD4⁺ T cells were isolated. (A) The NFAT1 translocation were analysed by laser scanning confocal microscope. The yellow arrow represents NFAT1 intraconuclear transfer. Mean Optical Density was recorded for statistic. Data are presented as means $\pm$ SD. (* $P < 0.05$, $n = 6$). (B) Splenic CD4⁺ T cells stimulated with anti-CD3 and anti-

CD28 for 2 h for total cell lysate extraction or nuclear extraction. β -actin or lamin B was analyzed as a control for protein loading and nuclear extraction. Data are presented as means $\pm$ SD. (#P >0.05, n = 3; *P < 0.05, n = 3).

Figure 6

Tg-dependent Ca²⁺ influx in splenic CD4⁺ T cells in septic mice. CD4⁺ T cells were harvested from each group. They were treated with Fluo-3/AM for measuring the calcium concentration. Depletion of Ca²⁺ stores was triggered by incubating cells with thapsigargin (Tg). SOCE was measured by monitoring the increase in [Ca²⁺]_i after the addition of 2 mM Ca²⁺. Representative flow cytometry results from six fields of cells were observed for each group. Data are presented as means $\pm$ SD. (*P < 0.05 versus sham group, n = 6).

Figure 7

Protein level expression of Orai1 in response to anti-CD3/CD28 mAbs in septic mice. CD4⁺ T cells from each group were incubated with anti-CD3/CD28 mAbs for 30 min. The figure shows a typical Western blot and average expression levels of Orai1 protein in response to anti-CD3/CD28 mAbs in septic mice on 12 h and 24 h. Data are presented as means $\pm$ SD. (* $P < 0.05$ versus sham group, $n = 3$).

Figure 8

Overexpression of Orai1 can reduce the mortality in septic mice. 40 BalB/c mice were randomly divided into 2 groups (CLP group, LV-Orai1 group). (A) The expression of Orai1 protein from splenic CD4⁺ T cells was detected by Western blot. (B) The mice were monitored daily for 7 days. The survival rate was analyzed by Kaplan-Meier survival analysis and compared by the log-rank test. *P < 0.05 vs. CLP.

Figure 9

Overexpression of Orai1 can effectively alleviate lung injury in septic mice. BalB/c mice were randomly divided into 3 groups (sham group, CLP 24h group, LV-Orai1 group). Pathological damage of lung and kidney in mice were evaluated by histological observation and scores. The above pathological images represent the results of one representative experiment. * $P < 0.05$ vs. sham; # $P < 0.05$ vs. CLP 24h.

Figure 10

Overexpression of Orai1 can improve proliferation activity of splenic CD4⁺ T cells in sepsis. BalB/c mice were randomly divided into 2 groups (CLP 24h group, LV-Orai1 group). CD4⁺ T cells were collected 24 hours after operation. The proliferation activity of splenic CD4⁺ T cells was measured by the CCK-8 assay. The proliferation levels in the LV-Orai1 group decreased compared with those in the CLP 24h group (*P < 0.05, n = 6).

Figure 11

Overexpression of Orai1 can attenuate the Th17/Treg imbalance in septic mice. BalB/c mice were randomly divided into 2 groups (CLP 24h group, LV-Orai1 group). The spleen was collected. The proportion of Treg cells and Th17 cells were analyzed by flow cytometry. n=6 mice per group. Data are shown as mean values $\pm$ SD are presented, p values were calculated using Unpaired T-test, *p < 0.05 compared with CLP 24h group.

Figure 12

Overexpression of Orai1 can partially recovery of SOCE in sepsis. BalB/c mice were randomly divided into 2 groups (CLP 24h group, LV-Orai1 group). The splenic CD4⁺T cells was collected. Then they were treated with Fluo-3/AM for measuring the calcium concentration. Depletion of Ca²⁺ stores was triggered by incubating cells with thapsigargin (Tg). SOCE was measured by monitoring the increase in [Ca²⁺]_i after the addition of 2 mM Ca²⁺. Representative flow cytometry results from six fields of cells were observed for each group. Data are presented as means ± SD. (*P < 0.05 versus CLP 24h group, n = 6).