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Trichostatin A modulates the macrophage phenotype by enhancing autophagy to reduce inflammation during polymicrobial sepsis

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ABSTRACT

Sepsis is a syndrome of life-threatening organ dysfunction caused by dysregulated host responses to infection. Macrophage polarization is a key process involved in the pathogenesis of sepsis. Recent evidence has demonstrated that autophagy participates in the regulation of macrophage polarization in different phases of inflammation. Here, we investigated whether trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, promotes the macrophage M2 phenotype by enhancing autophagy to counteract excessive inflammation in a cecal ligation and puncture (CLP) mouse model. TSA stimulation increased the proportions of M2 marker (CD206, CD124 and CD23)-labeled RAW264.7 macrophages. Furthermore, with increasing TSA doses, autophagy was enhanced gradually. Interestingly, the autophagy activator rapamycin (Rap), also known as an mTOR inhibitor, unexpectedly decreased the proportions of M2 marker-labeled macrophages. However, TSA treatment reversed the Rap-induced decreases in CD206-labeled macrophages. Next, we stimulated different groups of RAW264.7 cells with the autophagy inhibitors MHY1485 or 3-methyladenine (3-MA). Inhibition of autophagy at any stage in the process suppressed TSA-induced macrophage M2 polarization, but the effect was not associated with mTOR activity. In vivo, TSA administration promoted peritoneal macrophage M2 polarization, increased LC3 II expression, attenuated sepsis-induced organ (lung, liver and kidney) injury, and altered systemic inflammatory cytokine secretion. However, 3-MA abolished the protective effects of TSA in CLP mice and decreased the number of M2 peritoneal macrophages. Therefore, TSA promotes the macrophage M2 phenotype by enhancing autophagy to reduce systemic inflammation and ultimately improves the survival of mice with polymicrobial sepsis.

1. Introduction

Sepsis is a significant public healthcare problem worldwide and is a major cause of death in patients in intensive care units [1]. Imbalance between anti- and proinflammatory immune responses, which are mediated by different types of immune cells, is involved in the pathophysiology of sepsis [2]. Macrophages, a crucial cell type in innate immunity, play important roles in host defense against microbial infections and in maintaining tissue homeostasis in inflammatory diseases [3]. Macrophages are classified into a classically activated M1 type and an alternatively activated M2 type, depending on the stimuli present in the tissue microenvironment [4]. M1 phenotype macrophages, which are regulated by signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factor 5 (IRF5) secrete proinflammatory mediators, such as interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α and reactive nitrogen and oxygen species, which promote inflammation and have strong microbicidal activity. However, M2 phenotype macrophages, which are regulated by signal transducer and activator of transcription 3 (STAT3), peroxisome proliferator-activated receptor gamma (PPARγ) and interferon regulatory factor 4 (IRF4), participate in tissue remodeling by producing anti-inflammatory mediators, such as IL-10 and IL-4 [5,6]. It is widely known that M2 phenotype macrophages play positive roles in resolving inflammation, which is necessary for the treatment of sepsis [5,7]. Therefore,

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regulation of macrophage polarization balance may be a promising strategy for the treatment of sepsis.

Autophagy is a homeostatic degradative process that sequesters damaged organelles or misfolded proteins in autophagosomes and degrades them via fusion with lysosomal compartments [8]. In addition, autophagy also participates in innate defense by promoting intracellular microbial pathogen degradation [9]. Recent studies have shown the role of autophagy in regulating macrophage polarization in inflammatory diseases. In thioacetamide (TAA)-induced acute liver injury, spermine (SPM)-induced autophagy inhibits M1 polarization and promotes M2 polarization of liver-resident macrophages by upregulating Atg5 expression to ultimately attenuate liver injury [10]. Furthermore, in obese mice, loss of autophagy promotes inflammation by decreasing anti-inflammatory M2 polarization [11]. The above findings indicate that autophagy may regulate inflammation by influencing macrophage activity. However, the correlation between autophagy and macrophage polarization in sepsis remains unclear.

Trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, has the potential to be used as a therapeutic agent for many diseases due to its notable biological activity [12,13]. In addition to its usefulness for cancer treatment, TSA is effective for alleviating inflammation. In sepsis-induced liver injury, TSA treatment improves survival rate and attenuates liver injury by reversing the acetylation of IκB kinase (IKK) [14]. In addition, in sepsis-associated encephalopathy (SAE), TSA treatment rescues neuronal apoptosis by regulating Bcl-2 family protein expression and improves learning and memory abnormalities in SAE mice [15]. In addition, combinatorial therapy with TSA and a DNA methyl transferase inhibitor (5-aza 2-deoxycytidine) has been found to reduce mortality and attenuates lung vascular hyperpermeability by inhibiting the eNOS-Cav1-MLC2 signaling pathway in an acute lung injury mouse model [16]. Thus, TSA may be effective for the treatment of inflammatory diseases. Recent studies have shown that TSA can induce autophagy in a variety of human cancer cells, and suppresses tumor metastasis by promoting cancer cell death [17,18]. However, its role in regulating macrophage autophagy remains elusive.

Given the findings mentioned above, we explored the role of TSA in modulating autophagy and macrophage differentiation. We hypothesized that TSA treatment may protect septic mice by promoting macrophage M2 polarization via enhancement of autophagy and consequent attenuation of systemic inflammation.

2. Materials and methods

2.1. RAW264.7 cell culture

RAW264.7 cells (CCTCC, Wuhan, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. The cells were passaged once every two days, and cells in the logarithmic growth phase were used for experiments.

2.2. Western blot analysis

Cells were lysed for 30 min in ice-cold buffer containing a phosphatase inhibitor and proteinase inhibitor cocktail. The concentrations of protein were determined with a BCA Protein Assay Kit (KeyGen BioTech, Jiangsu, China). Equal amounts of proteins were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes. After the membranes were blocked with 5% nonfat milk for 1 h, they were probed with anti-LC3 (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-p62 (1:1000; Sigma-Aldrich, St. Louis, MO, USA) or GAPDH antibodies (1:1000; Antgene Biotechnology, Wuhan, China) overnight at 4 °C. The membranes were then incubated with secondary antibodies (1:3000; Antgene Biotechnology) for 1 h. After being washed three times, the membranes were incubated with SuperSignal ECL (Thermo Fisher Scientific, Waltham, MA, USA) and detected with a UVP imaging system (Upland, CA, USA). The images were analyzed with ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.3. Immunofluorescence staining and confocal microscopy

RAW264.7 cells were seeded on coverslips in 24-well plates. At the end of the designated treatment periods, the cells were fixed with 4% paraformaldehyde and 0.2% Triton-X-100 was used to permeabilize the membranes. After incubation in 5% BSA for 1 h, the coverslips were probed with the following primary antibodies: anti-LC3 (1:100; Cell Signaling Technology) and anti-p62 (1:100; Sigma-Aldrich) overnight at 4 °C. After being washed three times, the coverslips were incubated with IFKine Green AffiniPure Goat Anti-Rabbit IgG and Red Goat Anti-Mouse IgG (1:300; Abbkine, CA, USA) for 1 h at room temperature. Cell nuclei were stained with blue-fluorescent DAPI. The images were obtained using confocal microscopy (Zeiss Axiovert, LSM710).

2.4. RT-PCR

Total RNA was isolated from RAW264.7 cells after administration of different doses of TSA. cDNA was produced according to the RT kit instructions (Takara, Shiga, Japan). PCR primers (Tsingke Biological Technology, Beijing, China) were as follows: CD206 forward, 5′-TTCA GCTATTGGACGCGAGG-3′ and reverse, 5′-GAATCTGACACCCAGCG GAA-3′; CD124 forward, 5′-TCTGCATCCCGTTGTTTTGC-3′ and reverse, 5′-GCACCTGTGCATCCTGAATG-3′; CD23 forward, 5′-TCCTAGAAAGC GTTGCTGCT-3′ and reverse, 5′-TCCCAGTGCCACAGAAGAAG-3′; iNOS forward, 5′-GTTCTCAGCCCAACAATACAAGA-3′ and reverse, 5′-GTGG ACGGGTCGATGTCAC-3′. The cycling conditions included 40 cycles with a melting temperature (Tm) of 60 °C.

2.5. Flow cytometry

RAW264.7 cells were seeded in 6-well plates before stimulation with TSA (Sigma-Aldrich) for 24 h. Then, the cell density was adjusted to 2.5×10^6 cells/ml, and 200 μl of the suspension was used to detect M2 markers. AF647 CD206, PE CD124 or PEcy7 CD23 antibodies were used to label different M2 markers. F4/80 antibody was used to label the peritoneal macrophages (PMs). AF647 CD206 and PE CD124 were used to detect the expression of M2 markers in PMs.

2.6. Mice

Six- to eight-week-old male C57BL/6J mice (Hua Fu Kang Co, Beijing, China) weighing 22–25 g were housed in a specific pathogen-free environment with food and water at a fixed temperature under a 12 h light-dark cycle. All animal experiments were approved by the Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology.

2.7. Cecal ligation and puncture (CLP) model

Sepsis was induced in mice by CLP as previously described [19,20]. Briefly, mice were anesthetized by intraperitoneal injection of 1% pentobarbital (50 mg/kg), and the abdomen of each mouse was shaved and disinfected with 75% alcohol. A longitudinal midline skin incision was performed. Then, the cecum was exposed, ligated with sterile silk at the designated position for mid-grade sepsis and double punctured with a 20-gauge needle. A small amount of fecal matter was extruded from the holes, and then the cecum was returned to the peritoneal cavity. For the sham-operated mice, the cecum was exteriorized but not ligated or punctured and then returned to the abdomen. The mice were subcutaneously injected with 1 ml of prewarmed normal saline after the operation. Two hours before the CLP operation, mice were intraperitoneally injected with 3-methyladenine (3-MA; MCE, Deer Park,

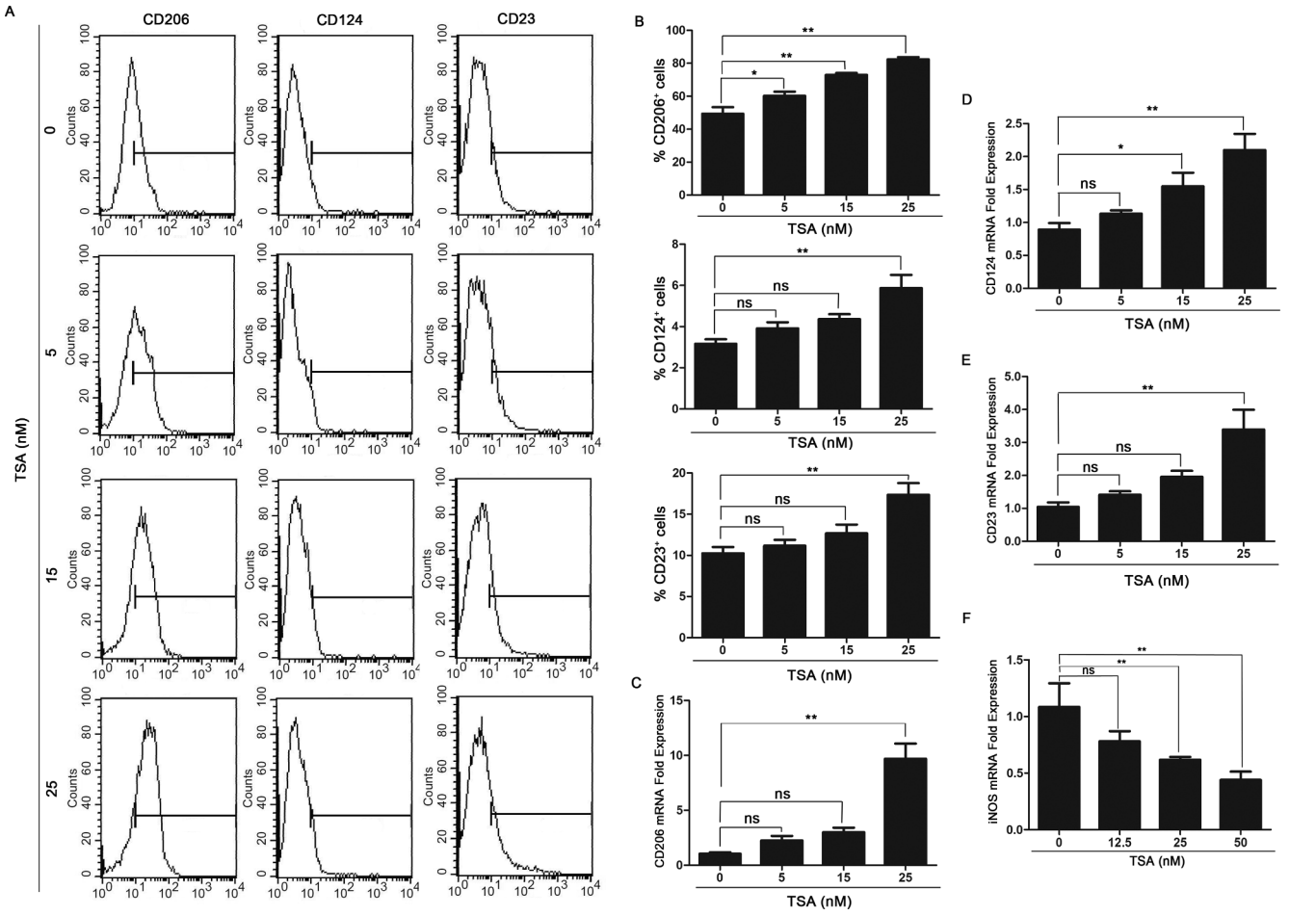

Fig. 1. Effect of TSA on M2 polarization in RAW264.7 cells. RAW264.7 cells were incubated with TSA (0, 5, 15, 25 nM) for 24 h. A. Cells were stained for M2 markers (CD206, CD124 or CD23) to determine M2 polarization using flow cytometry. The shown pictures are representative of four independent experiments. B. Quantification of flow cytometry data from A. The data represent the mean ± s.e.m of four replicated experiments. mRNA levels of CD206 (C), CD124 (D), CD23 (E) and iNOS (F) were analyzed by RT-PCR array. The data represent the mean ± s.e.m of five replicated experiments. (one-way ANOVA; Newman-Keuls post hoc test, $^{*}P < 0.05$, $^{**}P < 0.01$, and ns indicates no significance).

NJ, USA; 30 mg/kg) or phosphate-buffered saline (PBS; 0.15 ml/mouse). One hour after the CLP procedure, the mice were treated with TSA (2 mg/kg) or PBS (0.2 ml/mouse).

2.8. Peritoneal macrophage isolation

PMs were obtained according to previously published methods [21,22]. In brief, mice were euthanized by cervical dislocation. The abdominal skin was removed to expose the peritoneum. A total of 20 ml of PBS was used to lavage the abdominal cavity, and the peritoneal lavage fluid (PLF) was centrifuged for 8 min at 1500 rpm. The supernatant was discarded, and the cell pellet was resuspended in RPMI 1640 medium with 10% FBS and 1% penicillin and streptomycin and incubated for 1.5 h in a humidified 5% CO_2 atmosphere at 37 °C. Then, the nonadherent cells were removed, and the PMs were obtained.

2.9. Histopathological analysis and differential cell counting

Twenty-four hours after the administration of TSA or PBS, mice were anesthetized by the intraperitoneal injection of 1% pentobarbital (50 mg/kg) and sacrificed. Then, the lung, liver and renal tissues were collected, fixed in 4% paraformaldehyde and embedded in paraffin for hematoxylin and eosin staining. Lung injury scores were determined according to published criteria [23]. The specific scoring criteria are shown in Supplementary Table 1. After harvesting, the PLF was centrifuged for 8 min at 1500 rpm to obtain cells. Then, the total cells were counted with a hemocytometer, and the differential cell counts were determined after Giemsa staining.

2.10. Assessment of blood biochemistry and cytokine levels

Whole blood was collected at the indicated time and centrifuged for 10 min at 2000 rpm. Then the serum was isolated. An alanine aminotransferase (ALT) assay kit and a creatinine (Cr) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used according to the manufacturer's instructions. Enzyme-linked immunosorbent assays (ELISAs) were performed on serum using kits for TNF-α, IL-6, and IL-10 (RayBiotech, Inc. Norcross, GA, USA).

2.11. Survival analysis

Mice were intraperitoneally injected with PBS (0.15 ml/mouse) or 3-MA (30 mg/kg) 2 h before CLP and were treated with TSA (2 mg/kg) or PBS (0.2 ml/mouse) one hour after the CLP procedure. The dose of TSA or 3-MA was based on previously published studies [14,24]. Mortality was recorded every 24 h for 8 days for the survival test.

2.12. Statistical analysis

Data are expressed as the mean ± standard error of the mean

(s.e.m.). One-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test was ultilized to compare the difference among multiple groups and *t* test was used to analyze between two groups. Kaplan-Meier survival curves and log-rank (Mantel-Cox) tests were used to assess the survival rate. GraphPad Prism 5 for Windows (San Diego, CA, USA) was used for statistical analysis. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of TSA on M2 polarization in RAW264.7 cells

To explore the effect of TSA on M2 polarization, we performed RT-PCR and flow cytometry to detect the expression of three M2 markers. RAW264.7 cells were stimulated with different doses of TSA (0, 5, 15, and 25 nM) for 24 h. Cells were then harvested and stained for CD206, CD124 and CD23 markers of the M2 phenotype. The flow cytometry data showed that the numbers of macrophages labeled with these M2 markers increased in a dose-dependent manner (Fig. 1A and 1B). Likewise, as the TSA dose increased, the transcript levels of the above M2 markers increased gradually, peaking at 25 nM TSA (Fig. 1C, D and E). In addition, we also detected the transcript levels of nitric oxide synthase (iNOS), an M1 marker. Our data showed reduced expression of iNOS in TSA-stimulated cells (Fig. 1F).

3.2. TSA induces autophagy in RAW264.7 cells

Next, we tested the effect of TSA on autophagy in macrophages. After stimulation with different concentrations of TSA for 24 h, RAW264.7 cells displayed an accumulation of LC3 II. In addition, activation of autophagy was also confirmed by decreases in the levels of the autophagy substrate p62 (Fig. 2A and D). Confocal microscopy of these cells revealed more LC3 puncture numbers after TSA stimulation (Fig. 2E). Consistent with the observed LC3 II accumulation, TSA stimulation also elevated the expression of Atg7, an established autophagy-related protein (Fig. 2B). Mammalian target of rapamycin (mTOR) is a key autophagy regulator in cells, and mTOR activation (by phosphorylation) is known to inhibit autophagy [25]. Thus, we next explored the effect of TSA on mTOR expression. Our data showed that 25 nM TSA reduced the level of phospho-mTOR (pmTOR). However, 5 nM and 15 nM TSA did not affect pmTOR levels (Fig. 2C). All of the above data indicate that TSA enhances autophagy in RAW264.7 cells.

3.3. Autophagy is essential for TSA-induced M2 polarization

To maximize the effects of TSA, we used 25 nM TSA to stimulate RAW264.7 cells for 24 h in subsequent experiments. Rap, MHY or 3-MA was used to regulate autophagy in RAW264.7 cells. Interestingly, stimulation with Rap, an inhibitor of mTOR, slightly enhanced autophagy (Fig. 3A and B), but unexpectedly decreased the proportions of CD206-, CD124- and CD23-labeled macrophages (Fig. 3C and D). However, TSA attenuated the Rap-induced decreases in the numbers of CD206-labeled macrophages (Fig. 3C and D). In addition to activating autophagy, mTOR signaling also adversely affects cellular growth and metabolism [26]. Furthermore, studies have shown that mTOR is essential for M2 polarization [27,28]. We found that 50 nM TSA strongly decreased mTOR expression but reduced cell viability (data not shown). Therefore, we speculated that Rap decreased the proportions of M2 marker-labeled cells mainly by inhibiting cellular metabolism rather than by activating autophagy. Next, we used MHY or 3-MA to inhibit autophagy. MHY, an activator of mTOR, promoted mTOR expression (Supplementary Fig. 1) and inhibited autophagy via the inhibition of autophagosome fusion with lysosomes. In contrast to MHY, 3-MA inhibits autophagy by inhibiting class III PI3K reduced mTOR expression. After cells were stimulated with these two autophagy inhibitors, the TSA-induced macrophage M2 phenotype was inhibited in an mTOR activation-independent manner (Fig. 3C and D). These data demonstrate that autophagy is essential for TSA-induced M2 polarization in RAW264.7 macrophages.

3.4. 3-MA inhibits TSA-induced M2 polarization in septic mice

Having shown the role of TSA in promoting M2 polarization in vitro, we next explored the relationship between autophagy and TSA-induced macrophage polarization in vivo. We used a mouse model of cecal ligation and puncture (CLP); such model has been used extensively to investigate the clinical settings of sepsis and septic shock [19]. PMs were harvested, and autophagy was assessed by determination of the expression of LC3 II and p62. The data showed that compared with those in the CLP group, the PMs in the TSA-treated group displayed enhanced autophagy (Fig. 4A). In addition, F4/80 was used to label macrophages; CD206 and CD124 were assessed as M2 markers by flow cytometry. The separate proportions of CD206- and CD124-labeled macrophages among all the macrophages were calculated. The results showed that the percentage of M2 macrophages was significantly increased in the TSA-treated group compared with the CLP group (Fig. 4B and C). However, in mice intraperitoneally injected with 3-MA to inhibit autophagy, TSA treatment did not increase the proportion of M2 macrophages (Fig. 4B and C). All these data indicate that autophagy plays an important role in TSA-induced macrophage polarization in septic mice.

3.5. 3-MA dampens the protective function of TSA in septic mice

To further confirm the relationship between autophagy and TSA treatment in septic mice, we assessed the degree of major organ injury, the inflammatory response and the survival rate of mice in different experimental groups. The autophagy inhibitor 3-MA, was administered in subsequent studies. The mice in the CLP group exhibited pathological changes such as alveolar congestion, hemorrhage and alveolar wall thickening in lung tissues (Fig. 5A). The liver showed inflammatory cell infiltration and necrosis (Fig. 5C). And the injured kidney exhibited disordered epithelium and interstitial edema (Fig. 5E). Likewise, CLP significantly increased the serum levels of Alanine transaminase (ALT; a biomarker of liver health) and Creatinine (Cr; an important indicator of renal health) (Fig. 5D and F). However, administration of TSA attenuated the organ injury, decreased the lung injury score (Fig. 5B) and reduced the levels of serum ALT and Cr. Furthermore, administration of 3-MA two hours before CLP abolished the TSA-mediated prevention of organ injury in septic mice. Next, we examined the levels of cytokines in the serum. As shown in Fig. 6A-C, the levels of proinflammatory cytokines (TNF-α and IL-6) were lower in TSA-treated mice than in CLP mice. Furthermore, TSA treatment promoted the production of the anti-inflammatory cytokine IL-10. However, compared with TSA-treated mice, 3-MA + TSA-treated mice showed reduced IL-10 expression and increased TNF-α and IL-6 levels. Then, Giemsa staining was used to evaluate the quantities of different cell types in the peritoneum. We observed an increase in the number of total cells and neutrophils in 3-MA + TSA-treated mice compared with TSA-treated mice (Fig. 6D). Similarly, the number of macrophages was reduced in 3-MA + TSA-treated mice (Fig. 6D). Finally, we observed the survival rate of mice in different groups. As shown in Fig. 7, TSA treatment significantly improved the survival rate of CLP mice. In contrast, administration of 3-MA decreased the survival rate of CLP, mice even when treated with TSA. Taken together, the above data demonstrate that autophagy is essential for reducing inflammation in CLP and that the suppression of autophagy dampens the protective function of TSA in septic mice.

4. Discussion

In this study we report a correlation between autophagy and the macrophage phenotype. Our results demonstrate that TSA induces the

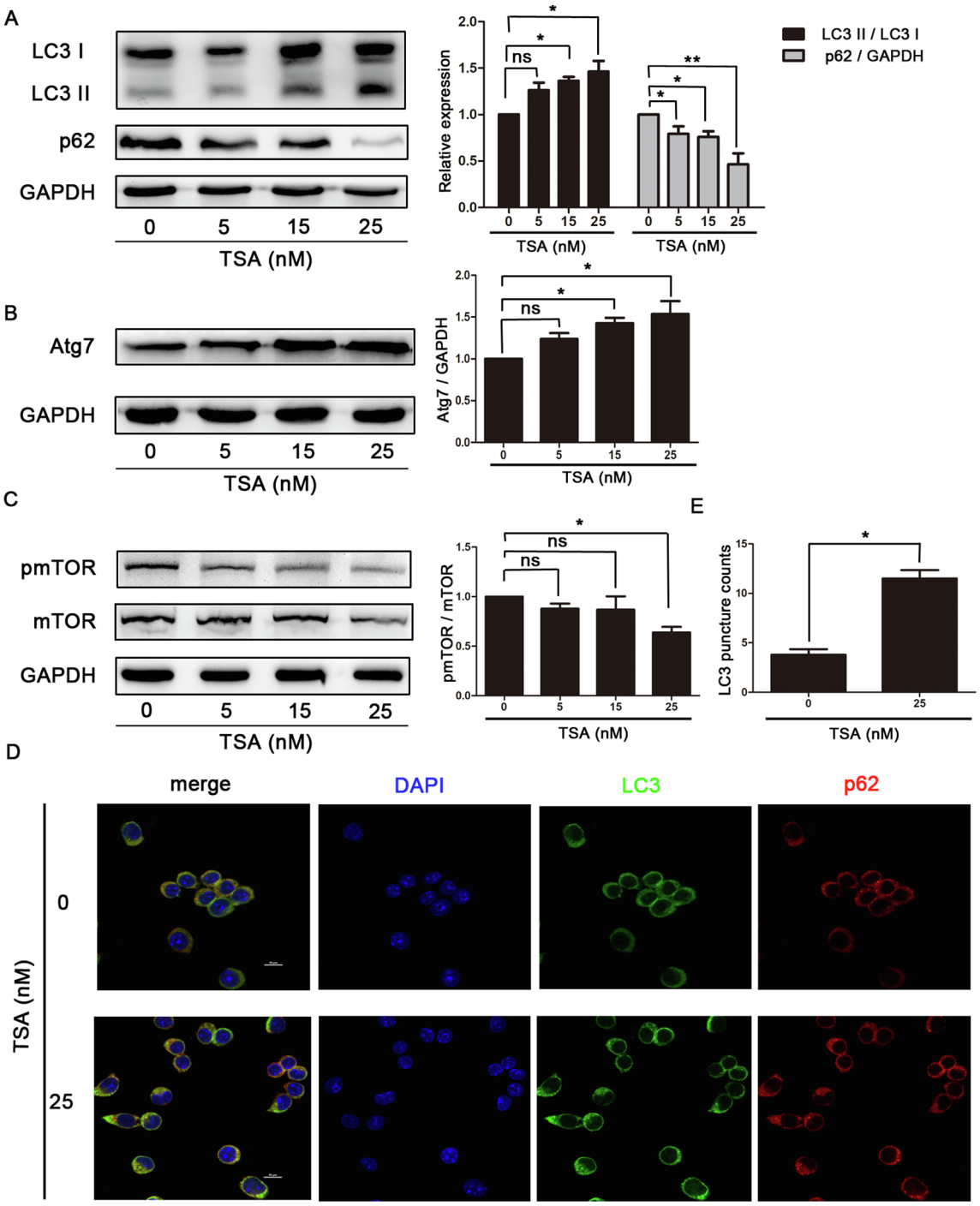

Fig. 2. TSA induces autophagy in RAW264.7 cells. Western blot was used to analyze the expression of autophagy-related proteins. A. Stimulation of RAW264.7 cells with up to 15 and 25 nM TSA elevated LC3 II levels and decreased p62 levels. B. The expression of Atg7 was upregulated by TSA in a dose-dependent manner. In addition, 25 nM TSA decreased the level of pmTOR (C). GAPDH was used as a loading control. The image shown is representative of four independent experiments. The data are expressed as the means ± s.e.m. of four replicated experiments. D. The expression of LC3 and p62 was also examined and photographed using a confocal microscope (scale bar: 10 μm). E. Puncture numbers in each cell were analyzed. The image shown is representative of three independent experiments. (*t* test was used in E. one-way ANOVA followed by Newman-Keuls post hoc test was used in A, B and C, $^{*}P < 0.05$, $^{**}P < 0.01$, and ns indicates no significance).

M2 macrophage polarization by enhancing autophagy in vitro. Furthermore, the administration of TSA modulates the peritoneal macrophage phenotype, resolves inflammation and improves the survival rate in septic mice in an autophagy-dependent manner.

Sepsis is a severe complication of infection in which the host develops an extreme immune response caused organ failure [1]. Immune dysfunction is considered to be a significant factor in the pathogenesis of sepsis [29]. Macrophages, an important immune cell type, play key roles in multiple stages of sepsis via their effects on inflammation and immune functions. The plasticity of M1 and M2 macrophages changes with the development of sepsis. M2 macrophages are involved in inflammation resolution and tissue repair, which are essential for the treatment of sepsis [5]. Studies have shown that macrophage gene expression in the inflammatory response causing M1 or M2 phenotypic

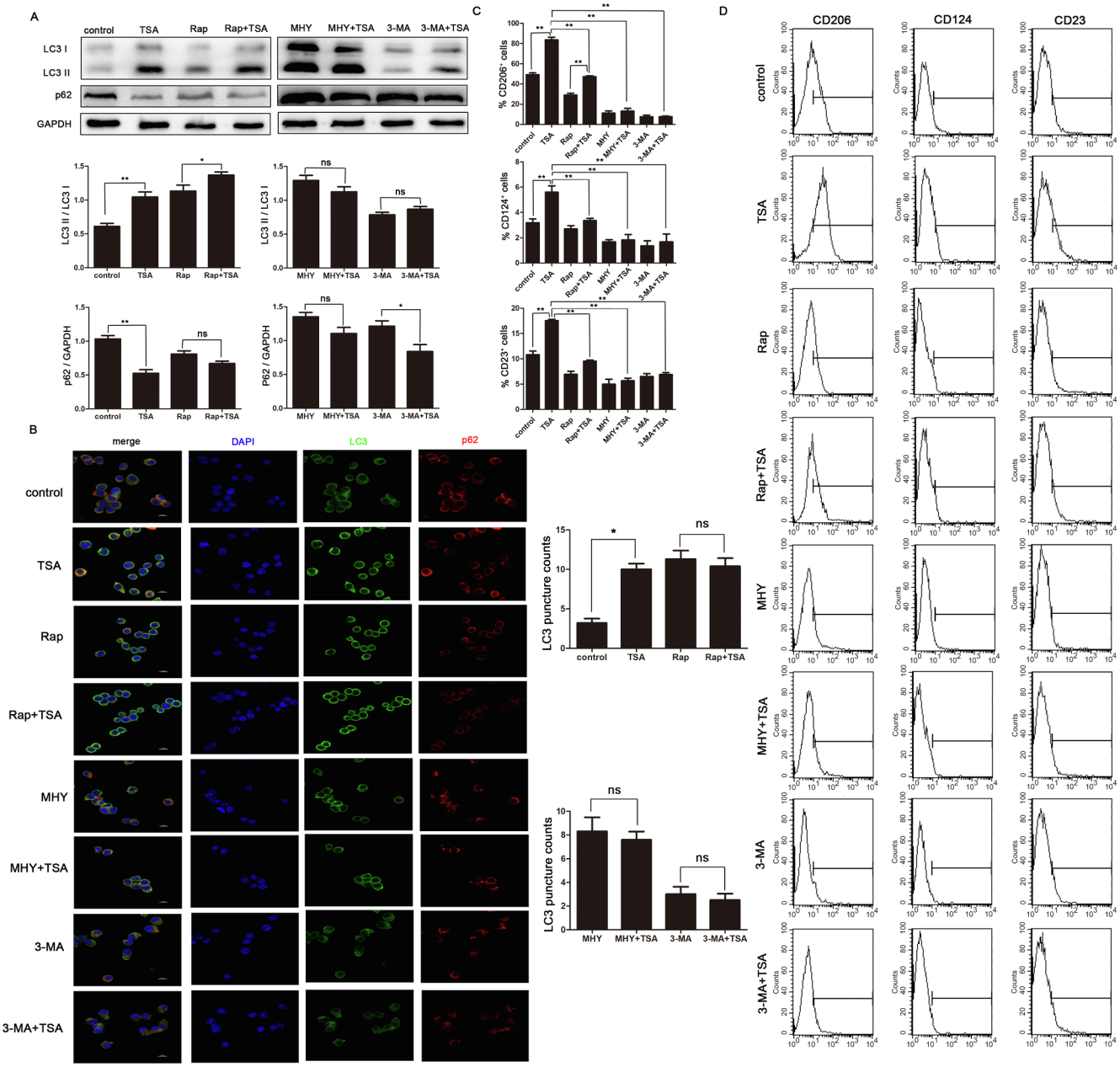

Fig. 3. Autophagy is essential for the induction of M2 polarization by TSA in RAW264.7 cells. RAW264.7 cells were stimulated with 25 nM TSA for 24 h after Rap (100 nM), MHY (100 nM) or 3-MA (4 mM) was added to the culture medium respectively for 2 h. The expression levels of LC3 and p62 were detected by western blot (A) or confocal microscopy (scale bar: 10 μm; B). GAPDH was used as a loading control. The image shown is representative of four (western blot) or three (confocal microscope) independent experiments. The data are expressed as the mean ± s.e.m. of four replicated experiments. Cells were labeled with CD206 (AF647), CD124 (PE) or CD23 (PE) respectively to determine M2 polarization using flow cytometry (D). The image shown is representative of four independent experiments. The proportion of M2 marker-positive macrophages was calculated (C). The data represent the mean ± s.e.m. (n = 4 per group). (one-way ANOVA; Newman-Keuls post hoc test, *P < 0.05, **P < 0.01, and ns indicates no significance).

activation can be regulated by HDAC inhibitors. For example, the HDAC inhibitor valproic acid has been shown to promote M2 macrophage polarization and thus to reduce inflammation in nitrogen mustard (NM)-induced lung injury [30]. Similarly, the inhibition of HDAC activity results in an increase in the recruitment of M2 macrophages and improves ventricular function and remodeling in a mouse model of acute myocardial infarction (MI) [31]. In this study, we investigated the effects of trichostatin A (TSA), a general histone deacetylase inhibitor, on the regulation of macrophage M1/M2 polarization in sepsis. Our data indicate that TSA increased the numbers of CD206-, CD124- and CD23-labeled macrophages in a dose-dependent manner in vitro. In vivo, administration of TSA induced peritoneal macrophage M2 polarization, which was identified as the proportion of $F4/80^+CD206^+$ or $F4/80^+CD124^+$ macrophages among $F4/80^+$ cells (total macrophages). These findings are consistent with those of a previous study showing that epigenetic modifiers could modulate the macrophage phenotype [32]. Further experiments are needed to confirm the mechanism of TSA-induced M2 macrophage polarization.

Autophagy, an intracellular process that delivers cytoplasmic constituents into the lysosome, has been verified to be involved in inflammatory innate immune responses [33]. Studies have revealed that autophagy is one of the factors that affects macrophage polarization

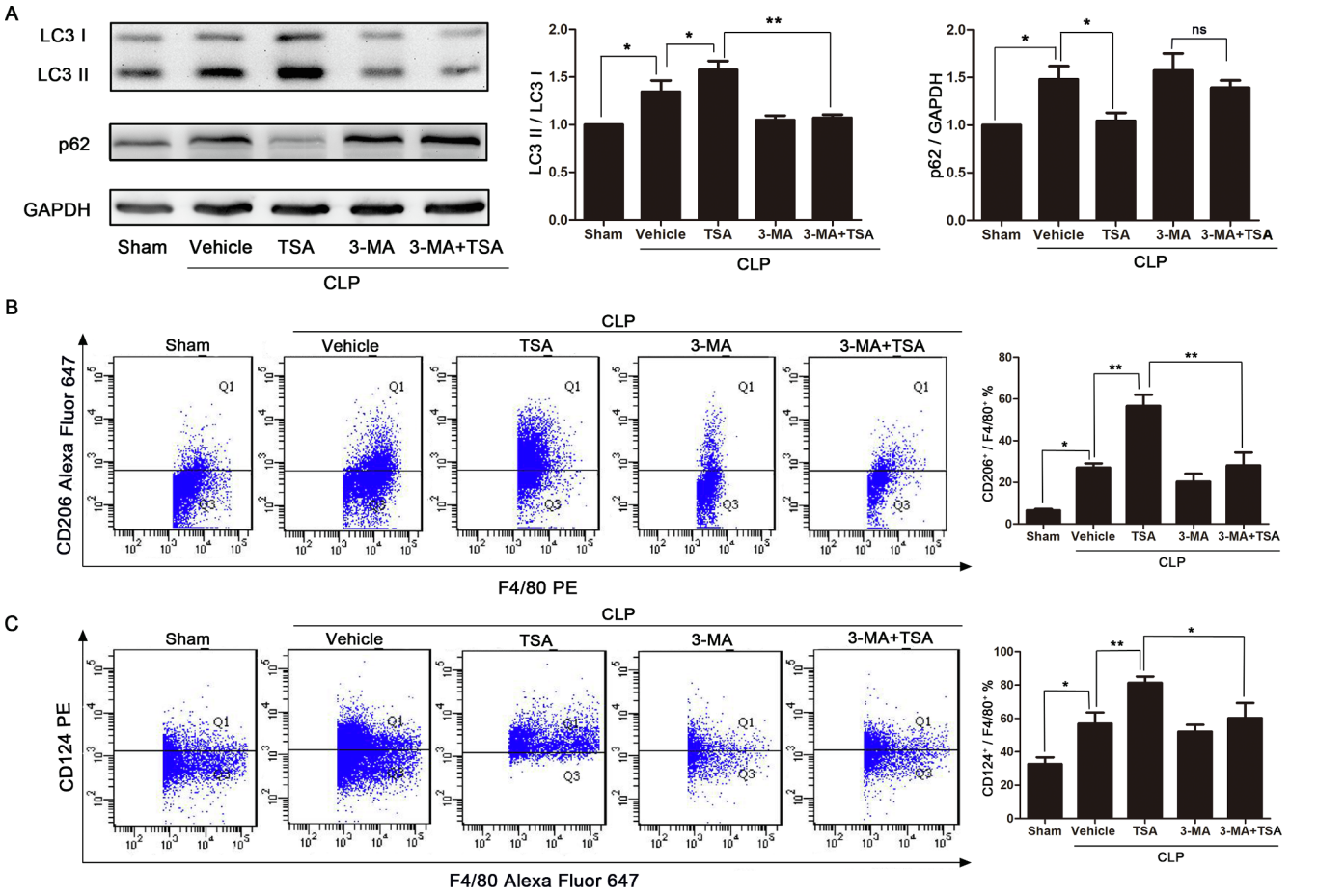

Fig. 4. Autophagy affects TSA-induced M2 polarization in the CLP model. Mice were intraperitoneally injected with 3-MA, an autophagy inhibitor (30 mg/kg) or PBS (0.15 ml/mouse) for 2 h. One hour after CLP, mice were treated with TSA (2 mg/kg, i.p.) or PBS (0.2 ml/mouse, i.p.). After 24 h, the mice were sacrificed, and PMs were harvested. A. Western blot analysis revealed an increase in autophagy in PMs in the TSA-treated group. A representative western blot is shown. The data are shown as the mean ± s.e.m. of four independent experiments. Flow cytometry was used to determine the number of M2 PMs. The F4/80-positive cells represent PMs, and CD206 (B) or CD124 (C) was used as an M2 marker to label M2 PMs. The proportions of M2 PMs were calculated. The images shown are representative of four independent experiments. The data are expressed as the mean ± s.e.m. (n = 4 per group). (one-way ANOVA; Newman-Keuls post hoc test, *P < 0.05, **P < 0.01, and ns indicates no significance).

[10,11,29]. In addition, TSA is an autophagy inducer that influences the proliferation and differentiation of tumor cells [18]. Therefore, we speculated that TSA may modulate the macrophage phenotype by affecting autophagy. As expected, TSA enhanced autophagy by increasing LC3 II expression and decreasing p62 expression both in RAW264.7 cells and in PMs. Next, we used the autophagy activator Rap, also known as a specific inhibitor of mTOR to stimulate cells in vitro. Interestingly, Rap unexpectedly decreased the proportions of M2 marker-labeled macrophages, but TSA reversed the Rap-induced decreases in CD206-labeled macrophages. This result is consistent with that of a previous study showing that inhibition of mTOR expression results in defective M2 polarization and enhanced M1 polarization [34]. In addition to modulating autophagy, mTOR also plays a major role in controlling cell metabolism and activation [35]. Previous data have shown that activation of mTOR is essential for macrophage M2 polarization by metabolic pathways [36]. Therefore, although we found that 25 nM TSA slightly decreased mTOR expression in macrophages, we speculated that mTOR is not a major factor influencing on TSA-induced autophagy in macrophage polarization. To further demonstrate the relationship between TSA-induced autophagy and macrophage polarization, we selected two autophagy inhibitors, MHY and 3-MA, to inhibit autophagy in vitro. MHY is an mTOR activator and inhibits the fusion between autophagosomes with lysosomes, leading to accumulation of LC3II and p62. 3-MA inhibited autophagosome formation, causing a reduction in LC3II and accumulation of p62. Our results reveal that regardless of the mTOR activation status, TSA cannot modulate the number of M2 macrophages if autophagy is inhibited. Likewise, when 3-MA was administered to septic mice to inhibit autophagy, TSA treatment did not induce peritoneal macrophage M2 polarization. All these data demonstrate that autophagy modulates the TSA-induced macrophage phenotype.

Organ dysfunction, uncontrolled inflammation and disordered inflammatory cell infiltration are major pathophysiological features of sepsis. Therefore, we next assessed the protective function of TSA and the correlation between autophagy and TSA treatment in septic mice. Our results revealed that TSA treatment attenuated the sepsis-induced major organ injury (lung, liver and kidney) caused by sepsis. Moreover, TSA treatment suppressed neutrophil accumulation and increased the number of macrophages in the peritoneum in septic mice. Treatment with TSA also attenuated sepsis-induced systemic inflammation by decreasing the levels of proinflammatory cytokines (TNF-α and IL-6) and increasing anti-inflammatory cytokine (IL-10) secretion. Through the above functions, TSA improved the survival rate of septic mice. These results are consistent with those of previous studies showing that epigenetic modifiers have beneficial anti-inflammatory effects in inflammatory diseases [16,32]. Like other previous studies, in which the blockage of autophagy exacerbated CLP-induced acute kidney injury [24], our data showed that when 3-MA was administered to inhibit autophagy two hours before sepsis, the protective function of TSA was dampened. The involvement of autophagy in modulation of the

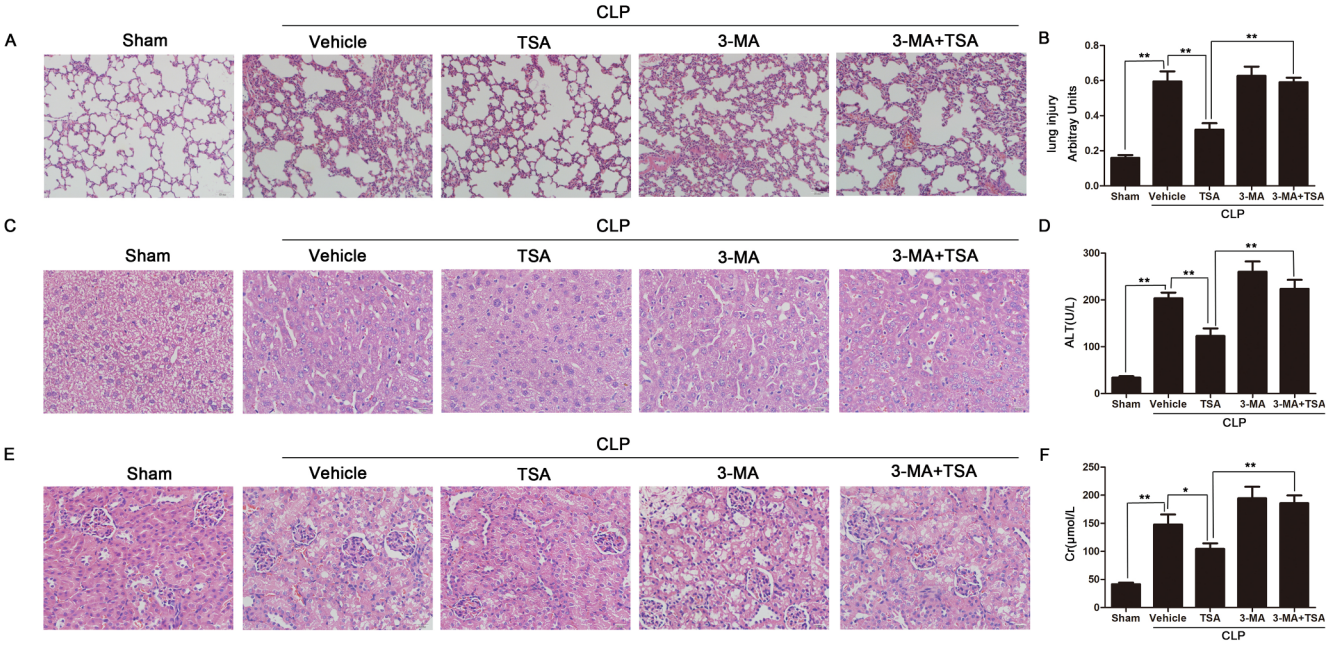

Fig. 5. 3-MA attenuates the protective functions of TSA in sepsis-induced multiple-organ injury. Mice were intraperitoneally injected with 3-MA (30 mg/kg) or PBS (0.15 ml/mouse) for 2 h. One hour after the CLP procedure, the mice were treated with TSA (2 mg/kg, i.p.) or PBS (0.2 ml/mouse, i.p.). After 24 h, the mice were sacrificed; the lungs, livers, kidneys and serum were harvested. Representative images of lung (scale bar: 50 μm; A), liver (scale bar: 20 μm; C) and kidney (scale bar: 20 μm; E) stained with hematoxylin and eosin. B. Lung injury scores. D. Level of ALT. F. Level of Cr. The data are expressed as the mean ± s.e.m. (n = 6–8 mice per group). (one-way ANOVA; Newman-Keuls post hoc test, *P < 0.05 and **P < 0.01).

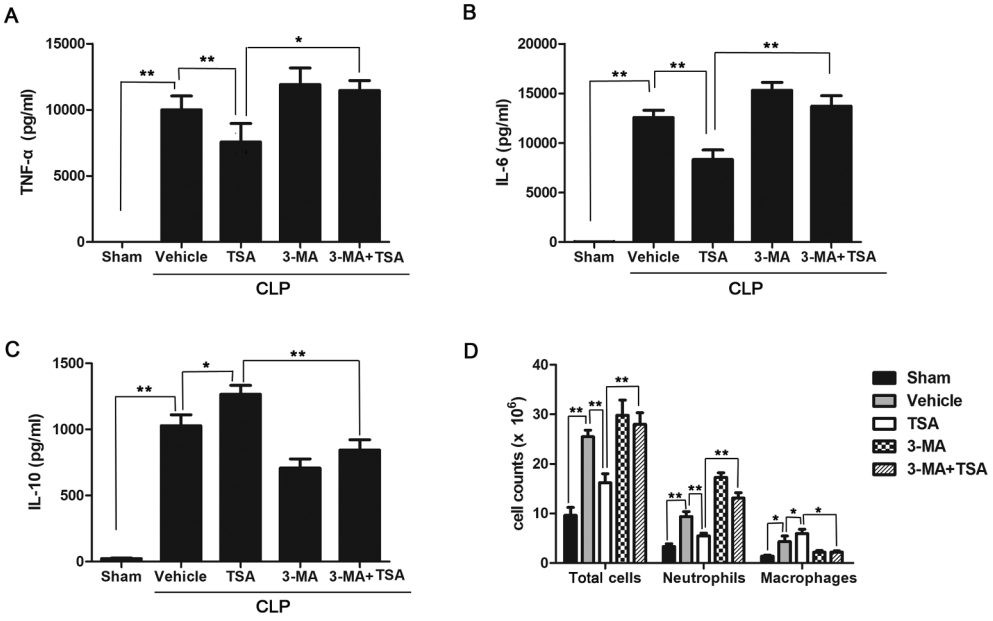

Fig. 6. Influence of 3-MA on the TSA-regulated inflammatory response in sepsis. Serum and PLF were harvested 24 h after CLP. ELISA was used to assess the levels of inflammatory cytokines. A. TNF-α. B. IL-6. C. IL-10. D. The number of total PLF cells, PLF neutrophils and PLF monocytes/macrophages. The data are expressed as the mean ± s.e.m. (n = 6–8 mice per group). (one-way ANOVA; Newman-Keuls post hoc test, *P < 0.05 and **P < 0.01).

inflammatory innate immune response has been widely studied [37]. Our results also provide evidence that autophagy plays a key role in modulating the inflammatory response by influencing innate immune cells in sepsis.

In conclusion, our data demonstrate a correlation between autophagy and the TSA-induced macrophage M2 phenotype. TSA enhanced autophagy and increased the numbers of M2 macrophages both in vitro and in vivo. Moreover, administration of TSA reduced systemic inflammation and improved the survival rate of polymicrobial septic mice. However, inhibition of autophagy abolished the above effects of TSA. This study presents the first evidence that TSA promotes the macrophage M2 phenotype by enhancing autophagy, consequently reducing inflammation during polymicrobial sepsis. We emphasize that targeting autophagy is a potential therapeutic strategy for inflammatory disorders.

Acknowledgments

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

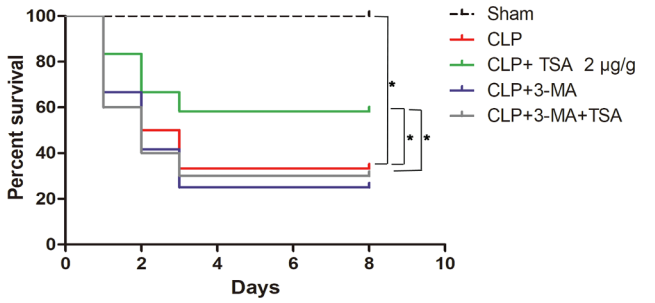

Fig. 7. 3-MA reduces survival rate in the TSA-treated septic mice. Kaplan-Meier survival curves and the log-rank (Mantel-Cox) test were used to detect survival rate in different groups. Mice treated with TSA (2 mg/kg) showed a higher survival rate than mice in the CLP group. But the administration of 3-MA decreased the survival rate of CLP mice even when treated with TSA (n = 10 mice per group). *P < 0.05.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105973.

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