


# SIRT1 suppresses burn injury-induced inflammatory response through activating autophagy in RAW264.7 macrophages

Fu Han, Zhenzhen Li, Shichao Han, Yanhui Jia, Lu Bai, Xiaoqiang Li, Dahai Hu 

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Department of Burns and Cutaneous Surgery, Fourth Military Medical University, Xi'an, Shaanxi, China

**Correspondence to** Dr Dahai Hu, Department of Burns and Cutaneous Surgery, Fourth Military Medical University, Xi'an 710032, Shaanxi, China; [dahaihu\\_123@163.com](mailto:dahaihu_123@163.com)

FH and ZL contributed equally.

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## ABSTRACT

The present study sought to investigate the association between silent information regulator 1 (SIRT1) and autophagy during systemic inflammatory response syndrome following burn injury. The experimental burn model in mice and macrophages were established. SIRT1 mRNA expression was quantified by quantitative real-time PCR. The protein levels of SIRT1 and the conversion of light chain 3 (LC3)-I to LC3-II were determined by western blot analysis. The formation of autophagosomes was assessed by green fluorescence protein-tagged LC3 fluorescence. The contents of inflammatory cytokines interleukin (IL)-1, IL-6, IL-10 and IL-18 were measured by ELISA. SIRT1 was highly expressed in burned tissues and RAW264.7 cells treated with serum obtained from mice with burn injuries. Moreover, SIRT1 overexpression augmented, whereas sirtinol, an inhibitor of SIRT1, attenuated burn injury-induced increasing number of autophagosomes and expression levels of LC3-II/LC3-I in RAW264.7 cells. Besides, sirtinol effectively prevented SIRT1-induced pro-inflammation during burn injury. Furthermore, autophagy inhibition by 3-methyladenine significantly attenuated SIRT1 overexpression-mediated pro-inflammatory cytokine production. SIRT1 abolished burn injury-induced inflammatory response by inducing autophagy.

## INTRODUCTION

Burn injury is a common type of trauma affecting adjacent organs.<sup>1</sup> Obvious local edema appears at the injured sites due to the increase of microvascular permeability, leading to varying degrees of systemic reactions, such as fever, leukocytosis and the synthesis of C reactive protein.<sup>2</sup> Inflammatory response following burns is essentially a defensive reaction, during which neutrophils and vascular endothelial cells are activated by the release of a large number of inflammatory mediators.<sup>3</sup> Systemic inflammatory response syndrome (SIRS) is defined as a massive inflammatory reaction triggered by surgical operation, trauma, burn injury, ischemia, pancreatitis and bacterial translocation, which can lead to multiple organ failure and even death. The balance of inflammatory mediators and immune cells determines the initiation of

## Significance of this study

### What is already known about this subject?

- Silent information regulator 1 (SIRT1) induces autophagy by deacetylation of autophagy-related pathway.
- Spinal SIRT1 expression attenuates pain induced by burn injury.
- SIRT1 exerts anti-inflammatory effects induced by burn injury.

### What are the new findings?

- SIRT1 expression is upregulated in burned tissues and cells.
- Inhibition of SIRT1 suppresses autophagy in burned RAW264.7 cells.
- Inhibition of SIRT1 promotes pro-inflammatory cytokine production.
- Inhibition of autophagy attenuates SIRT1-mediated inhibition of inflammation.

### How might these results change the focus of research or clinical practice?

- SIRT1 might be a valuable target for developing therapeutic strategy against burn injury.

SIRS.<sup>4</sup> However, the specific molecular mechanisms by which the pro-inflammatory SIRS response is induced by burn injury remain unclear.

Silent information regulator 1 (SIRT1) is a highly conserved nicotinamide adenine dinucleotide-dependent deacetylase that regulates stress responses, inflammation, cell senescence and apoptosis.<sup>5 6</sup> In recent years, several researches have proposed the anti-inflammatory effects of SIRT1 in the progression of burn injury. For instance, Bai *et al* demonstrated that SIRT1 could alleviate lung tissue damages induced by burn injury by downregulating pro-inflammatory cytokines and inhibiting the apoptosis of pulmonary microvascular endothelial cells.<sup>7</sup> Moreover, the administration of EX527, the specific antagonist of SIRT1, exacerbated severe burns-induced renal dysfunction by promoting the inflammatory response, oxidative stress and cell apoptosis in burned rats.<sup>8</sup> Cheng *et al* suggested that upregulation



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of spinal SIRT1 expression attenuated pain induced by burn injury.<sup>9</sup>

Several lines of evidence suggest that SIRT1 plays a pivotal role in the modulation of autophagy by regulating autophagy-related signaling pathway, such as forkhead transcription factor family,<sup>10</sup> peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 family,<sup>11</sup> Akt/mammalian target of rapamycin (mTOR) pathway<sup>12</sup> and mitogen-activated protein kinase family.<sup>13</sup> As mentioned above, these results suggested that SIRT1 might be crucial for modulating of burns-induced inflammatory response by inducing autophagy. To confirm this hypothesis, we examined the effect of SIRT1 on autophagosome formation and the production of inflammatory cytokines as well as the potential role of autophagy in regulating SIRT1 expression in burned macrophages.

## MATERIALS AND METHODS

### Establishment of burn injury model

Male C57BL/6J mice aged 6–8 weeks, obtained from Laboratory Animal Center of Xijing Hospital (the Fourth Military Medical University) were housed at room temperature on a 12:12 light:dark cycle. To establish a 20% total body surface area (TBSA) third-degree burn injury model, eight C57BL/6J mice were intraperitoneally anesthetized with 1% pentobarbital sodium (50 mg/kg; Sigma-Aldrich, St. Louis, Missouri, USA), and their backs were shaved. A metal block heated to approximately 400°C was placed on the back skin of mice for continuous 8 s. In addition, the control group (n=8) performed the identical procedure except burn injury. Mice were sacrificed by cervical dislocation at 48 hours after burn injury or anesthesia, and burned tissues and blood samples were all collected.

### Cell culture and treatment

The immortalized mouse RAW264.7 macrophages were purchased from American Type Culture Collection (Manassas, Virginia, USA), which were used for the following experiments. RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 IU/mL penicillin and 100 mg/mL streptomycin (all from Gibco, Carlsbad, California, USA) in a humidified incubator with 5% CO<sub>2</sub> at 37°C and then stimulated with mouse serum obtained from normal and burned mice for 48 hours.

### RNA extraction and quantitative real-time PCR analysis

The mRNA expression of SIRT1, light chain 3 (LC3)-I and LC3-II was detected by real-time PCR. Total RNAs of burned tissues and RAW264.7 cells were isolated by TRIzol reagent (Invitrogen, Carlsbad, California, USA) following its manufacturer's instructions. Complementary DNA was synthesized by the reverse transcription using the TianScript RT kit (Tiangen Biotech, Beijing, China). Real-time PCR was performed on ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA) using SYBR Premix Dimer Eraser kit (Takara, Shiga, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the control gene. The sequences of primers used were as follows: SIRT1: 5'-TCAGTGTCATGGTTCCTTTGC-3' (forward),

5'-AATCTGCTCCTTTGCCACT CT-3' (reverse); LC3-I: 5'-GACCGCTGTAAGGAGGTGC-3' (forward), 5'-CTTGACCAACTCGCTCATGTTA-3' (reverse); LC3-II: 5'-TTATAGAGCGATACAAGGGGGAG-3' (forward), 5'-CGCCGTCTGATTATCTTGATGAG-3' (reverse); GAPDH: 5'-CCCGTAGACAAAATGGTGAAGGTC-3' (forward), 5'-GCCAAAGTTGTTCATGGATGACC-3' (reverse). The 2<sup>- $\Delta\Delta$ CT</sup> method was performed to qualify the relative amount of mRNAs.

### Western blot analysis

The expression of SIRT1, LC3-I and LC3-II at protein levels was assessed by western blot analysis. The proteins extracted by radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich) were separated with a sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% gel) and then transferred into the polyvinylidene fluoride membranes, which were then incubated overnight with primary antibodies against SIRT1 (#8469), LC3-I (#4599), LC3-II (#3868) and GAPDH (#5174) as an internal control purchased from Cell Signaling Technology (Boston, Massachusetts, USA) at a dilution of 1:1000, followed by the secondary antibodies for 2 hours. The chemiluminescence kit (Thermo Scientific, Shanghai, China) was used to visualize the protein bands.

### Cell transfection and grouping

RAW264.7 cells treated with serum derived from burned mice were transfected with expression vectors encoding pcDNA3.1 SIRT1 or negative controls for 24 hours using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) following the manufacturer's instructions, prior to stimulation with 10 mM 3-methyladenine (3-MA; Sigma-Aldrich), an autophagy inhibitor or exposed to SIRT1 inhibitor sirtinol (25  $\mu$ M) for 1 hour.

### Immunofluorescence

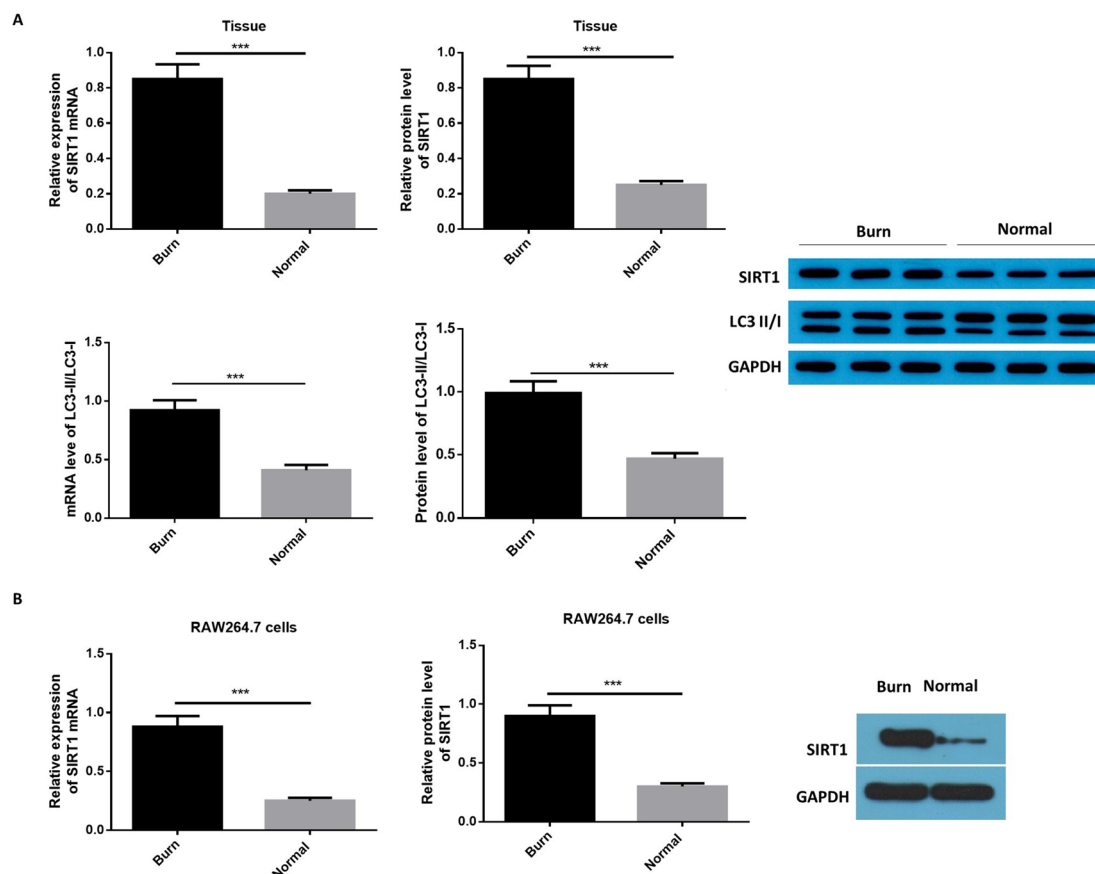
To determine autophagic flux, RAW264.7 cells were transiently transfected with green fluorescence protein (GFP)-tagged LC3 plasmid using Lipofectamine 2000. Transfected cells were subsequently fixed with 4% paraformaldehyde for 10 min, rinsed with phosphate-buffered saline for 3 times and stained with 4',6-diamidino-2-phenylindole for 15 min. The expression of LC3 was visualized with fluorescence microscope (Olympus, Tokyo, Japan).

### ELISA measurement of inflammatory cytokines

The levels of inflammatory cytokines including interleukin (IL)-1, IL-6, IL-10 and IL-18 in culture supernatants were determined by sandwich ELISA using ELISA kits (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instructions.

### Statistical analysis

Statistical analyses of all data were performed using SPSS V.19.0 (IBM, Chicago, Illinois, USA). All data were expressed as mean  $\pm$  SD. Student's t-test and one-way analysis of variance were used to assess the difference between groups.  $P < 0.05$  was considered to be statistically significant.



**Figure 1** Silent information regulator 1 (SIRT1) expression is upregulated in burned tissues and RAW264.7 cells. (A) The mRNA and protein levels of SIRT1, light chain 3 (LC3)-I and LC3-II in tissues obtained from mice suffering from burn injury and sham-operated mice (normal group) using quantitative real-time (qRT)-PCR and western blot analysis. (B) RAW264.7 cells were exposed to serum collected from burned or normal mice for 48 hours, and then the qRT-PCR and western blot analysis was applied to detect the expression of SIRT1 in these cells. \*\*\* $P < 0.001$ .

## RESULTS

### SIRT1 expression is upregulated in burned tissues and RAW264.7 cells

As shown in [figure 1A](#), the mRNA and protein levels of SIRT1 as well as the ratio of LC3-II/LC3-I were significantly upregulated in mice suffering from burn injury compared with the normal group. Likewise, RAW264.7 cells stimulated with the serum of burned mice exhibited higher SIRT1 expression at mRNA and protein levels relative to those treated with serum of normal mice ([figure 1B](#)). These data suggested that SIRT1 might be involved in the process of burn injury.

### Inhibition of SIRT1 suppresses autophagy in burned RAW264.7 cells

The in vitro burn injury resulted in the suppression of autophagy in RAW264.7 cells, evidenced by upregulated SIRT1 expression, decreased LC3-II/LC3-I ratio at mRNA and protein levels ([figure 2A](#)) and lower percentage of GFP-LC3-positive cells ([figure 2B](#)). However, the enforced expression of SIRT1 ameliorated burn injury-induced autophagy inhibition that was aggravated by treatment with sirtinol. Consistently, SIRT1 was silenced by small interfering RNA (siRNA) and its effects on autophagy and inflammation

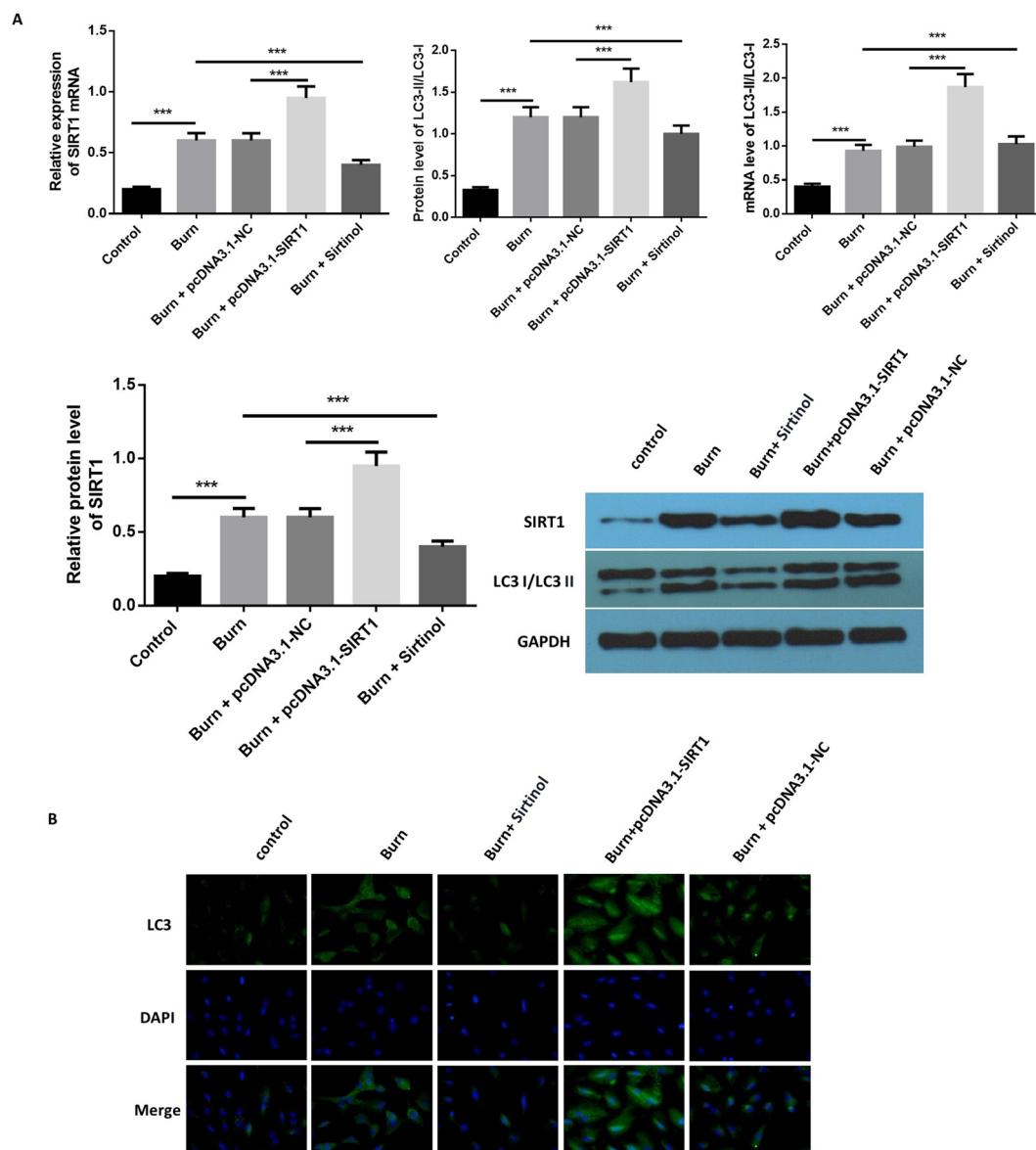
were then investigated. As shown in online supplemental figure S1a, the high transfection efficiency was achieved. Moreover, the knockdown of SIRT1 could effectively abrogate burn-induced upregulation of SIRT1 and LC3-II/LC3-I ratio (online supplemental figure S1b).

### Inhibition of SIRT1 promotes pro-inflammatory cytokine production in burned RAW264.7 cells

As demonstrated by ELISA test, the concentrations of pro-inflammatory cytokines, including IL-1, IL-6 and IL-18 were observably higher, while the secretion of anti-inflammatory cytokine IL-10 was inhibited in the burn group than those in the control group. On the contrary, ectopic expression of SIRT1 could markedly decrease the production of IL-1, IL-6 and IL-18, and upregulate IL-10 expression, while inhibition of SIRT1 by sirtinol led to a prominent increase in IL-1, IL-6 and IL-18 secretion and a significant decrease in IL-10 protein ([figure 3](#)). Conformably, the suppression of SIRT1 by siRNA partially overturned burn injury-mediated inflammatory cytokine expression (online supplemental figure S1c).

### Autophagy inhibition attenuates SIRT1-mediated inhibition of inflammation in burned RAW264.7 cells

We further determined whether autophagy could influence the effect of SIRT1 in burn injury. Further investigation



**Figure 2** Inhibition of silent information regulator 1 (SIRT1) suppresses autophagy in burned RAW264.7 cells. RAW264.7 cells were transfected with pcDNA3.1-SIRT1 or pcDNA3.1-NC following treatment with sirtinol before exposure to serum collected from burned or normal mice for 48 hours. The mRNA and protein levels of SIRT1, as well as the ratio of light chain 3 (LC3)-II/LC3-I at mRNA and protein levels (A) and GFP-LC3 puncta visualized by confocal microscopy (B) were then determined by quantitative real-time PCR, western blot analysis and immunofluorescence. \*\*\* $P < 0.001$ . NC, negative controls.

indicated that the blockade of autophagy with 3-MA, an autophagy inhibitor abrogated SIRT1 overexpression-induced conversion of LC3-I to LC3-II (figure 4A), augment of IL-10 levels and downregulation of IL-1, IL-6 and IL-18 levels (figure 4B).

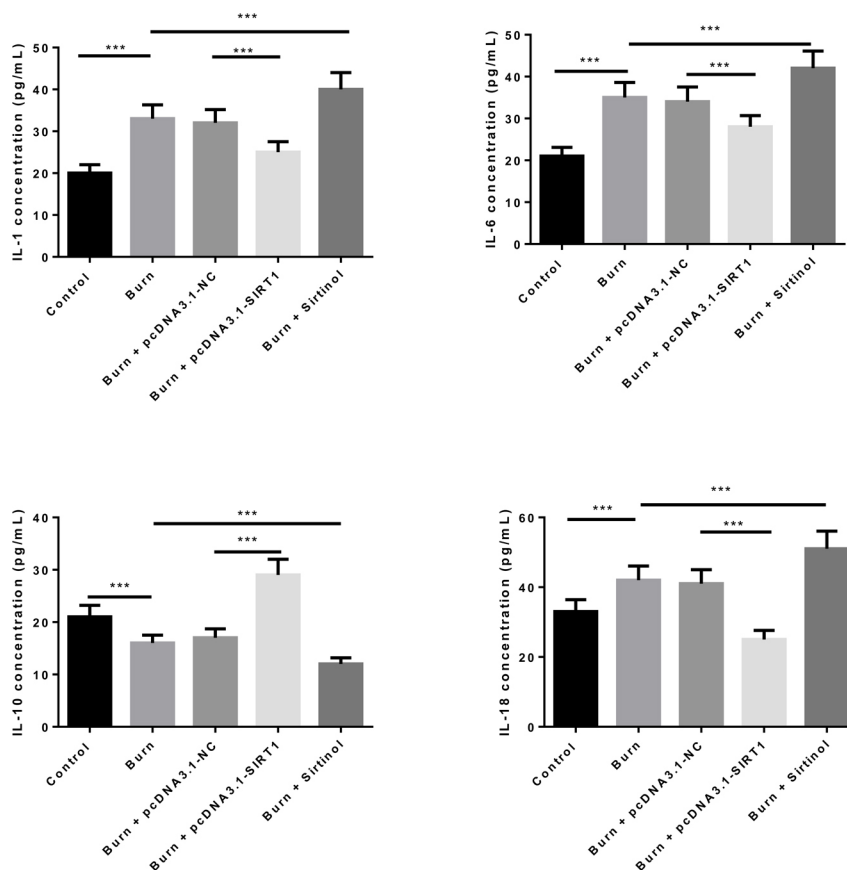
## DISCUSSION

SIRS often develops after burn injury, which is the common cause of morbidity and mortality.<sup>14</sup> The activation of SIRT1, the best characterized member of the sirtuin family, exhibits protective roles against severe burn-induced acute lung injury and acute lung injury.<sup>7,8</sup> Recently, Fan *et al* displayed that the mRNA and protein expression of SIRT1 in heart tissues were upregulated in the early stage of severely

burned rats as compared with the sham injury group.<sup>15</sup> A full-thickness burn injury comprising 30% TBSA resulted in S-nitrosylation of Sirt1 in mouse skeletal muscle.<sup>16</sup> In a 20% TBSA burn murine model of our study, the mRNA and protein levels of SIRT1 were significantly increased after severe burns. Additionally, mouse RAW264.7 macrophages stimulated by mouse burned serum for 48 hours also exhibited higher levels of SIRT1.

Autophagy has emerges as a stress-induced catabolic process implicated in a variety of pathological processes. There is increasing evidence that SIRT1 induces autophagy by deacetylation of autophagy-related pathway. For example, Ou *et al* revealed that SIRT1 facilitated autophagy and inhibited the mitochondrial damage and the



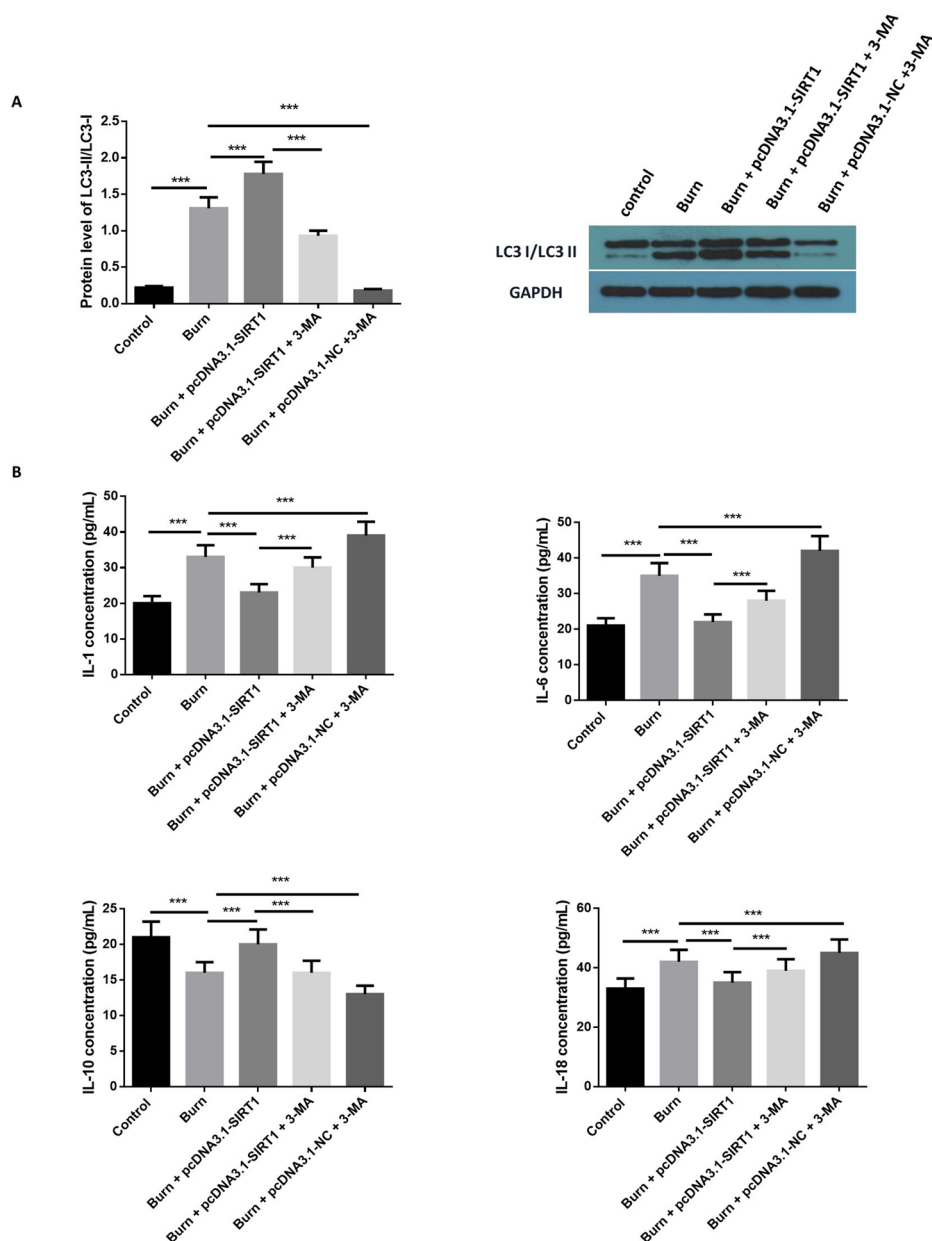


**Figure 3** Inhibition of silent information regulator 1 (SIRT1) promotes pro-inflammatory cytokine production in burned RAW264.7 cells. RAW264.7 cells were transfected with pcDNA3.1-SIRT1 or pcDNA3.1-NC following treatment with sirtinol before exposure to serum collected from burned or normal mice for 48 hours. The levels of interleukin (IL)-1, IL-6, IL-10 and IL-18 in culture supernatant in these cells were determined by ELISA. \*\*\*  $P < 0.001$ . NC, negative controls.

apoptosis of embryonic stem cells induced by oxidative stress through suppressing the mTOR pathway.<sup>17</sup> The SIRT1 inhibitor, EX-527, also has been shown to attenuate oxidized low-density lipoprotein-induced autophagy inhibition in macrophages by preventing the monocyte chemoattractant protein-1 production and lipid uptake.<sup>18</sup> In our in vitro assay, burned serum led to the downregulation of the autophagy markers LC3-II/LC3-I protein expression and autophagic flux confirmed by GFP-LC3 fluorescence assay in RAW264.7 macrophages. SIRT1 overexpression alleviated autophagy impairment induced by burns. In contrast, sirtinol, the specific inhibitor of SIRT1, effectively abolished pro-autophagy effects of SIRT1 overexpression in RAW264.7 macrophages following burn injury.

Recently, SIRT1 is also regarded as a critical immune regulator by suppressing macrophage activation during inflammation. A previous study reported that intraperitoneal injection with resveratrol, a SIRT1 activator could reduce the liver cell apoptosis and alleviate the inflammatory response by inhibiting the expressions of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and IL-1 $\beta$  and increasing the expression of IL-10 in kidney tissues in rats inflicted with 30% TBSA, full-thickness scald burn injury on the back.<sup>19</sup> Indeed, it has recently been shown that activation of SIRT1 by resveratrol alleviated myocardial damage through reducing apoptosis of cardiac myocytes and secretion

of IL-1 $\beta$  and TNF- $\alpha$  in rats with severe burns at early stage.<sup>15,20</sup> Tao *et al* indicated that intraperitoneal administration of resveratrol, by upregulating serum SIRT1 expression, inhibited the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 and increased the survival rate of severe burn with bacterial infections.<sup>21</sup> In RAW264.7 macrophages subjected to in vitro burn injury, the protein levels of IL-1, IL-6 and IL-18 were significantly enhanced in the present study. Conversely, the level of IL-10, an anti-inflammatory cytokine, was significantly suppressed in the burn group and recovered after SIRT1 overexpression. Nevertheless, knockdown of SIRT1 by siRNA led to the inhibition of autophagy and inflammatory factor release in burned RAW264.7 macrophages. Inhibition of SIRT1 by sirtinol could antagonize the anti-inflammatory effects of SIRT1 activation in burned RAW264.7 cells. As expected, suppression of autophagy with 3-MA increased the expression of pro-inflammatory cytokines by inhibiting SIRT1-dependent autophagy induction. Consistent with our results, Ren *et al* showed that autophagy inhibition by 3-MA pretreatment promoted the mRNA expressions of inflammatory cytokines including TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-12p40, and the autophagy induction by rapamycin pretreatment suppressed TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-12p40 in lipopolysaccharide (LPS)-induced macrophages, suggesting that autophagy can suppress the LPS-induced inflammatory



**Figure 4** Autophagy inhibition attenuates silent information regulator 1 (SIRT1)-mediated inhibition of inflammation in burned RAW264.7 cells. RAW264.7 cells were transfected with pcDNA3.1-SIRT1 or pcDNA3.1-NC following treatment with 3-methyladenine (3-MA) before exposure to serum collected from burned or normal mice for 48 hours. The ratio of light chain 3 (LC3)-II/LC3-I (A) and the concentration of interleukin (IL)-1, IL-6, IL-10 and IL-18 (B) were determined in these cells. \*\*\* $P < 0.001$ . NC, negative controls.

response in macrophages.<sup>22</sup> However, the present study is limited by its small sample size. Furthermore, this study is also limited to mice and cannot necessarily be generalized to larger species. There was no attempt to explore the in vivo effects of SIRT1 modulation on SIRS. Further study is needed to demonstrate if there is any cell-type specificity for burn injury-induced SIRT1 overexpression.

In conclusion, this study demonstrated that SIRT1 activation exerted anti-inflammatory effects against burn-induced injury through inducing autophagy in macrophages. These results illustrated that SIRT1 activation might be a potential therapeutic strategy for burn injury.

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#### ORCID iD

Dahai Hu <http://orcid.org/0000-0003-2287-8730>

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