Original Research

The deacetylation of Akt by SIRT1 inhibits inflammation in macrophages and protects against sepsis

Yanhui Jia*, Kuo Shen*, Jiaqi Liu*, Yan Li, Xiaozhi Bai, Yunshu Yang, Ting He, Yue Zhang, Lin Tong, Xiaowen Gao, Zhi Zhang, Hao Guan and Dahai Hu

Department of Burns and Cutaneous Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China
*These authors contributed equally to this paper.
Corresponding authors: Hao Guan. Email: guanhao@hotmail.com; Dahai Hu. Email: hudhai@fmmu.edu.cn

Impact Statement

Sepsis afflicts an alarming number of individuals and remains the leading cause of mortality worldwide. Much effort is needed to alleviate the high global burden and prevent disease progression. Most importantly, research on the mechanisms associated with sepsis is particularly important. This study is the first to illustrate Akt acetylation in macrophages. Akt deacetylation is necessary for its activation in macrophages. The administration of a drug that assists the regulation of macrophage polarization by targeting the Akt deacetylation may be a novel treatment for sepsis. SIRT1 mediated Akt deacetylation in macrophages stimulated with lipopolysaccharide. This work extends our understanding of inflammation regulation through SIRT1 in sepsis.

Abstract

Sepsis is characterized by uncontrolled inflammatory response and altered polarization of macrophages at the early phase. Akt is known to drive macrophage inflammatory response. However, how macrophage inflammatory response is fine-tuned by Akt is poorly understood. Here, we found that Lys14 and Lys20 of Akt is deacetylated by the histone deacetylase SIRT1 during macrophage activation to suppress macrophages inflammatory response. Mechanistically, SIRT1 promotes Akt deacetylation to inhibit the activation of NF-κB and pro-inflammatory cytokines. Loss of SIRT1 facilitates Akt acetylation and thus promotes inflammatory cytokines in mouse macrophages, potentially worsen the progression of sepsis in mice. By contrast, the upregulation of SIRT1 in macrophages further contributes to the inhibition of pro-inflammatory cytokines via Akt activation in sepsis. Taken together, our findings establish Akt deacetylation as an essential negative regulatory mechanism that curtails M1 polarization.

Keywords: Sepsis, acetylation, Akt, SIRT1, macrophages, inflammation

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Introduction

A complex illness called sepsis, which is linked to severe organ dysfunction and a high risk of dying, arises from an infection and a dysregulated host response to it.[1] As a complex illness, sepsis primarily manifested as an uncontrolled systemic synthesis of inflammatory mediators in response to microbial infection, or "cytokine storm."[2] The primary factor contributing to sepsis and septic mortality is unregulated inflammation.[3] The first line of defense against infection is innate immunity. Innate immunity is initially triggered by pattern-recognition receptors (PRRs) produced on immune cells to identify pathogen-related molecular patterns linked pathogens when an organism is infected by foreign pathogens.[4] Macrophages, as important innate immune cells, detect and respond to danger signals such as pathogens and tissue damage.[5] Different macrophage populations form as a result of macrophage plasticity, which is crucial to inflammation and its resolution to maintain host survival.[6,7] The two macrophage populations are referred to as traditionally activated macrophages M1 and alternatively activated macrophages M2 that secrete significant levels of anti-inflammatory cytokines and mediators, respectively.[8]

Akt, serine-threonine kinase, transmits signals that control many different cellular activities, including as metabolism, migration, and cell survival.[9–11] Akt signaling is constitutively active in macrophages and is crucial to their survival. Macrophages express three closely related, highly conserved homologs of Akt (Akt1, Akt2, Akt3), each of which differentially contributes to macrophage polarization.[12] Akt−/− mice are more susceptible to lipopolysaccharide (LPS)-induced endotoxin shock because Akt1 (commonly known as Akt) ablation increases M1 polarization of macrophages while Akt2 ablation results in their M2 polarization.[13,14] The main processes that trigger the activation of the Akt signaling pathway are phosphorylation and dephosphorylation. Other

modifications, including as ubiquitination, SUMOylation, and acetylation, have been discovered recently.[15,16] For the activation of Akt signaling, these modifications are just as crucial as phosphorylation. At lysine residues, SUMOylation and acetylation take place. To identify the Akt activation sites, 34 lysine residues have been examined by researchers.[17] Reversible acetylation regulates the activity of Akt according to research.[18,19] However, it is still unclear how Akt post-transcriptional alteration affects macrophage polarization and what it does.

SIRT1 functions as nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase and ADP-ribosyl transferase, which regulates a variety of processes mainly by deacetylating lysine residues on the target proteins (e.g. FOXO3,[20] p53,[21] and p65[22]). Melatonin upregulates SIRT1 expression in the testes to prevent LPS-induced testicular inflammation.[23] To prevent cognitive decline, baicalin modifies SIRT1's anti-inflammatory action in microglial cells.[24] SIRT1 reduces sepsis-induced acute kidney damage by causing Beclin1 deacetylation during autophagy.[25] The gathered research shows that SIRT1 is essential for regulating the inflammatory response and sepsis development.[26] An *in vitro* study showed SIRT1 could deacetylate and activate Akt acetylated at Lys^{14} and Lys^{20} in HEK293T cells by mass spectrometry.[18] SIRT1 deacetylates Akt to enhance its interaction with PIP3 and subsequent activation of Akt.[18] Increased PIP3 binding and Akt membrane localization are seen in the Akt acetylation-defective mutant K20R. Akt activity is elevated by the K20Q mutant, which mimics constitutive Akt acetylation. SIRT1 upregulation reduces Akt acetylation and restores activation of the Akt signaling pathway induced by ischemic postconditioning.[27] Nevertheless, the acetylation sites in Akt have not yet been identified in macrophages, and the biological consequences of modification in Akt remain elusive.

In this study, we identify that Akt is deacetylated at Lys14 and Lys20 by SIRT1 during macrophage activation. Rather than activating Akt activity, the acetylation of Akt dramatically suppresses its enzymatic activity and thus promotes macrophage M1 activation. Interestingly, Akt acetylation is found to be controlled by the SIRT1 which facilitates the binding of NF-κB to pro-inflammatory cytokines. Consequently, SIRT1 deficiency dramatically promotes Akt acetylation and thus elevated the M1 macrophages activation.

Materials and methods

Animal experiments

We strictly followed the ARRIVE instructions when conducting the animal research. The Fourth Military Medical University's Animal Experimental Ethics Committee awards funding for animal research. The male C57BL/6 mice used in the tests were 20–25 g in weight and 6–8 weeks old. Both lysMcre Cre− SIRT1flox/flox (wild-type) and lysMcre Cre+ SIRT1flox/flox (myeloid-specific SIRT1 knockout) mice have a C57BL/6 genetic background.

We dissolved SRT1720 (Selleck, Shanghai, China) at a concentration of 5 mg/mL in dimethyl sulfoxide (DMSO) (Invitrogen, Carlsbad, CA, USA). Akt inhibitor VIII (MCE, China) stock solution in DMSO was prepared (100 μM). The mice were intraperitoneally (i.p.) injected with either 16 mg/kg SRT1720 or 4 mL/kg DMSO at the designated time. A 1.5-mg/kg i.p. dosage of Akt inhibitor VIII in a saline solution was used *in vivo*. *In vitro*, Dulbecco's Modified Eagle Medium (DMEM) was used to dilute the Akt inhibitor VIII.

Model for LPS-induced sepsis: We administered 20 mg/kg of LPS from *Escherichia coli* serotype 0111: B4 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in sterile saline through intraperitoneal injection to the mice. Control mice received equivalent volumes of sterile saline.

Cell culture, plasmids, and transfection

The DMEM (Gibco) with 10% fetal bovine serum (FBS) was used to culture RAW264.7 cells (ATCC, Livingstone, MT, USA) (Gibco). The RPMI1640 medium, which contains 10% FBS and 100 IU/mL of penicillin/streptomycin, was used to sustain THP-1 cells (ATCC) (Beyotime). A 6-well plate containing nine mice's peritoneal macrophages (PMs) was created by peritoneal lavage with 5 mL of sterile phosphate-buffered saline (PBS). In DMEM with 10% FBS and 100 IU/mL penicillin/streptomycin, isolated PM were cultured. After 12 h, the culture medium will be changed. According to the manufacturer's recommendations, cells were grown in Opti-MEM (Invitrogen) and transfected with Lipofectamine 3000 (Invitrogen/life). From the Beijing AuGCT Biotehnology firm, pcDNA-3.1(+), pcDNA-3.1(+)-Akt, pcDNA-3.1(+)-K14R, and pcDNA-3.1(+)-K20R were obtained.

Antibodies and chemicals

Abcam was used to purchase anti-SIRT1 (ab110304), anti-acetyl-lys (ab80178), anti-p-Akt (ab81283), anti-p-p65 (ab16502), anti-p-p65 (ab28856), anti-acetyl-p65 (ab19870), and anti-p53 (ab131442) (Cambridge, MA, USA). Cell Signaling Technology supplied the anti-acetyl-p53 (2570). (Boston, MA, USA). We bought FITC-anti-mouse D86 and APC-anti-mouse CD206 from BD Biosciences (San Jose, CA, USA). We bought anti-actin and anti-ATPase Na+/K+ beta2 from Bioss Antibodies (Shenzhen, Guangdong, China). Selleck was paid for EX527 (Shanghai, China). We bought NAD+ from Cayman Chemical (Ann Arbor, MI, USA). We bought p300 inhibitor (CAS No. 328968-36-1) from MilliporeSigma (Pittsburgh, PA, USA).

Immunofluorescence

The cells were treated with 0.1% Triton X-100 diluted in phosphate buffered saline (PBS) for 20 min at room temperature after being fixed with 4% paraformaldehyde for 15 min. The cells were then blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature after being rinsed with PBS. The cells were then exposed to the p-Akt antibody for an overnight at 4°C. Goat anti-rabbit antibodies that were fluorescently tagged with Cy3 were applied to the cells after they had undergone four phosphate buffered saline with Tween20 (PBST) washes. The cells were treated with 4′,6-diamidino-2-phenylindole (DAPI) for 20 min at room temperature following four PBST washings. Images were taken using an Olympus IX71 light microscope (Olympus, Tokyo, Japan).

Fluorescence-activated cell sorting (FACS) analysis

PM was taken from mice with SIRT1 deficiency or with the wild-type genotype for the FACS analysis. Red Blood Cell Lysis Solution (Miltenyi Biotec, Germany) was used to remove red blood cells prior to antibody staining, and 10% BSA is used to block the cells. APC-anti-mouse CD206 and FITC-anti-mouse CD86 were used to stain the cells. We used Flowjo software to examine the data after performing FACS analysis on the FACS Aria III (BD Biosciences).

Real-time polymerase chain reaction (RT-PCR)

Using the PrimeScript RT Master Mix kit, we extracted the total ribonucleic acid (RNA) and converted the RNA into cDNA (TaKaRa, Shiga, Japan). Program temperature for reverse transcription is 37°C for 15 min and 85°C for 5 s. The SYBR qRT-PCR kit (TaKaRa) was used to do the real-time PCR, and the following procedure was used: 10 min at 95°C, then 40 cycles of 15 s at 95°C and 30 s at 60°C. Primers for SIRT1, IL-1β, IL-6, TNF-α, iNOS, Arg1, IL-10, Fizz1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and Ym-1 were purchased from TaKaRa Clontech. These primers sequences are as the follows:

SIRT1: Sense, 5′-CAGACCCTCAAGCCATGTTTGATA-3′;
Anti-sense, 5′-TTGGATTCCTGCAACCTGCTC-3′.
TNF-α: Sense, 5′-CGTCAGCCGATTTGCTATCT-3′;
Anti-sense, 5′-CGGACTCCGCAAAGTCTAAG-3′.
IL-1β: Sense, 5′-GCCCATCCTCTGTGACTCAT-3′;
Anti-sense, 5′-AGGCCACAGGTATTTTGTCG-3′.
GAPDH: Sense, 5′-TGTGTCCGTCGTGGATCTGA-3′;
Anti-sense, 5′-TTGCTGTTGAAGTCGCAGGAG-3′.
iNOS: Sense, 5′-CAAGCACATTTGGGAATGGAGA-3′;
Anti-sense, 5′-CAGAACTGAGGGTACATGCTGGAG-3′.
Arg1: Sense, 5′-AGCTCTGGGAATCTGATGG-3′;
Anti-sense, 5′- ATTACACGATGTCTTTGGCAGATA-3′.

The above genes were amplified.

Western blot

PBS was used to wash and scrape out the cells. Cell pellets were resuspended in 1-mM phenylmethanesulfonyl fluoride (PMSF)-containing radio immunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). After centrifuging the lysate, the supernatant was gathered. The protein samples were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) after being separated by an 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. 5% skimmed milk was used to block the membrane at room temperature for 1 h. The membranes were first treated with primary antibodies for an overnight at 4°C, and then with horse radish peroxidase (HRP)-conjugated secondary antibodies. The ECL Kit (Millipore, Billerica, MA, USA) was used to examine the protein expression.

NAD^+ concentration in cells

Using an NAD/NADH cell-based assay kit, we measured the NAD+ content in macrophages. RAW264.7 cells were treated with a permeabilization buffer for 30 min at room temperature after being rinsed with ice-cold PBS. After centrifuging the cell lysates at 1000*g* for 10 min, the supernatants were divided among 96 wells, and 100 μL of reaction buffer was added to each well. The absorbance of each well was assessed by a microplate reader following 1.5 h of incubation at room temperature on a plate shaker (Infinite 200 PRO, Tecan, Switzerland).

Immunoprecipitation

In week RIPA buffer (Beyotime) with 1 mM PMSF and a variety of protease inhibitors (Roche, Indianapolis, IN, USA), the cells were lysed. IgG, anti-acetyl-K, anti-SIRT1, or anti-Akt antibodies were all added to cell lysates. Protein A/G-agarose beads (Santa Cruz, CA, USA) were added to the protein-antibodies combination and incubated at 4°C for an additional hour after an overnight incubation. The complex-agarose beads were then thoroughly cleaned three times prior to being analyzed.

Enzyme-linked immunosorbent assay (ELISA)

TNF-α and IL-1β ELISA kit (RayBio, GA, USA) were used to detect the TNF-α or IL-1β secretion by macrophages. The concentrations of cytokines were calculated from the optical density (OD) values.

Genotyping of Sirt1 knockout mice analysis

The genome DNA was extracted from mice tail. Recombined allele was genotyped with the following primers:

Sirt1 primer15A: 5′TCCTTGCCACAGTCACTCAC3′;
Sirt1 primer 4A: 5′ACAGTCCCATTCCCATACC3′;
Cre primer N1: 5′CCGGTCGATGCAACGAGTGATGAGG3′;
Cre primer N2: 5′GCCTCCAGCTTGCATGATCTCCGG3′.

The PCR program was 95°C for 15 min, followed by 35 cycles at 94°C for 30 s, 63°C for 35 s, and 72°C for 45 s; 72°C for 3 min at last.

Histopathological analysis

The mice were killed by removing their necks after treatment, and we collected their liver, kidney, heart, and lung tissues. The tissues were embedded in paraffin after being treated in 4% paraformaldehyde. To demonstrate the degree of tissues destruction, hematoxylin and eosin stains were applied. Images were collected using an Olympus IX71 light microscope (Olympus, Tokyo, Japan).

Statistical analysis

We carried out the experiment at least three times. Using the statistical analysis program SPSS 17.0 (IBM, Armonk, NY, USA), all data were examined. To compare various treatment groups, a one-way analysis of variance (ANOVA) with Bonferroni or S-N-K's post hoc analysis was utilized. Comparisons with $P < 0.05$ were considered statistically significant.

Results

Akt activation requires Akt deacetylation in macrophages

To examine the role of acetylation in LPS-induced Akt activation, we treated macrophages with 1 μg/mL LPS[28] for 6 h and compared Akt acetylation with the formation of p-Akt[473]. LPS treatment resulted in the reduction of p-Akt and a global increase in protein acetylation (Figure 1(A)). Co-immunoprecipitation showed that LPS increased Akt acetylation in a time-dependent manner (Figure 1(B)). To examine whether deacetylation is required for p-Akt formation, macrophages were treated with LPS in the presence of SIRT1 activator or p300 inhibitor. The results demonstrate that both SRT1720, a more selective SIRT1 activator, and p300 inhibitor decreased Akt acetylation and significantly increased p-Akt formation. The SIRT1 inhibitor EX527 had the opposite effect on Akt acetylation and phosphorylation (Figure 1(C)).

Either iNOS upregulation or arginase 1 (Arg1) downregulation has been reported to be classical makers for M1 macrophage activation.[29] So, to determine whether deacetylation blocks M1 polarization, iNOS and Arg1 mRNA levels were measured. SRT1720 and p300 inhibitor had no impact on Akt mRNA (Figure S1) but totally reversed the LPS-induced increase in iNOS and decrease in Arg1 mRNA (Figure 1(D) and (E)). SRT1720 and p300 inhibitor also prevented the phosphorylation of STAT3 (Figure 1(F)), which is linked to gene transcripts for M1 macrophages.[29] These data show that deacetylation of Akt is essential for p-Akt formation in macrophages and p-Akt-dependent macrophage polarization.

LPS induces Akt acetylation by targeting SIRT1 in macrophages

Akt acetylation levels have been determined in primary cells (cardiac myocytes and fibroblasts) and cell lines (HeLa and human embryonic kidney [HEK] 293T) by SIRT1.[18] As the SIRT1 activator had an effect on Akt acetylation in macrophages, we focused on SIRT1 protein. In RAW264.7, LPS treatment lowers the levels of the protein and mRNA for SIRT1 (Figure 2(A) and (B)). LPS treatment of THP-1 cells also decreased the SIRT1 expression (Figure S2A). The peritoneal macrophages isolated from C57BL/6J mice were stimulated with LPS which was named M (LPS) or IL-4 which was named M (IL-4). The expression of SIRT1 in M (LPS) was lower than that in M (IL-4) (Figure 2(C) and (D)). We also used LPS or IL-4 to activate the THP-1 cells. The findings demonstrated that M (IL-4) had higher SIRT1 expression than did M (LPS) (Figure S2B). The research has demonstrated that SIRT1 activity has an effect on the acetylation status of p53.[30] The p53 acetylation level represents the SIRT1 activity. Therefore, consistent with these observations, LPS treatment resulted in a progressive decrease in SIRT1 activity (Figure 2(E)). This decreased activity occurred in spite of increased levels of the SIRT1 substrate NAD^+ (Figure 2(F)).

SIRT1 deacetylates Akt in macrophages

Numerous cellular processes that Akt regulates have also been revealed to be regulated by SIRT1,[31] and SIRT1 activators have been used to treat metabolic disorders with impaired Akt signaling.[32,33] So, we explored whether SIRT1 also deacetylates Akt in macrophages. The immunoprecipitation was carried out, and the outcomes demonstrated that these two proteins directly interact in macrophages (Figure 3(A)). This prompts us to investigate if Akt is deacetylated by SIRT1. Following LPS stimulation, the cells were pretreated with or without EX527. The acetylated Akt levels were tested by immunoprecipitation. The results showed that EX527 pretreatment increased the acetylation of Akt compared with no EX527 treatment following LPS stimulation (Figure 3(B)). As previously mentioned, Akt's membrane location and activation depend on its deacetylation. In order to determine whether SRT1720 pretreatment had any effect on the macrophages' Akt phosphorylation, immunofluorescence was used. The results revealed that SRT1720 reversed the reduced Akt phosphorylation caused by LPS in macrophages (Figure 3(C)). These findings imply that SIRT1 participates in macrophage Akt deacetylation.

Akt activation decreases the inflammatory response in macrophages

The studies have shown that Akts comprise three mammalian isoforms (Akt1, Akt2, and Akt3), which are similar in structure but distinctive in function. Akt1 is also generally named Akt.[34,35] Depending on cell type and conditions, Akt isoforms may have a variety of effects on Akt signaling. Therefore, it was investigated if Akt inhibition affected macrophage NF-B activation and inflammatory response. Inhibition of Akt1 increased p65's acetylation and phosphorylation. Otherwise, Akt2 inhibition downregulated p65 acetylation and phosphorylation. Furthermore, p65 acetylation and phosphorylation were unaffected by the inhibition of Akt3 (Figure 4(A)). In addition, pretreatment with an Akt1 inhibitor elevated the production of IL-1β, TNF-α, and iNOS, three pro-inflammatory genes, in LPS-induced macrophages. Akt2 inhibitor had the contrary effect on the pro-inflammatory genes compared with Akt1 inhibitor (Figure 4(B) and (D)). The anti-inflammatory genes Arg1 was decreased in macrophages pretreated with Akt1 inhibitor following LPS stimulation compared with Akt2 inhibitor following LPS stimulation. The expression of both pro-inflammatory and anti-inflammatory genes was not significantly altered by Akt3 inhibition (Figure 4(E)). The findings revealed that Akt, in fact, controlled inflammation in macrophages.

Akt deacetylation further decreases the LPS-induced inflammatory response in macrophages

The Akt molecule has been reported to undergo acetylation on Lys^{14} (K14) and Lys^{20} (K20). Deacetylation of these lysines is necessary for Akt membrane localization and activation.[18] Therefore, to investigate whether Akt acetylation plays a role in the inflammatory response of macrophages, Akt mutant expression plasmids were constructed. To conserve the net positive charge of the amino acid, Lys^{14} or Lys^{20} was substituted with Arg (the mutants are referred to as K14R or K20R in the following text). The RAW264.7 cells were transfected with Akt and Akt-mutant overexpression plasmids. The results showed that the Akt and Akt mutant plasmids

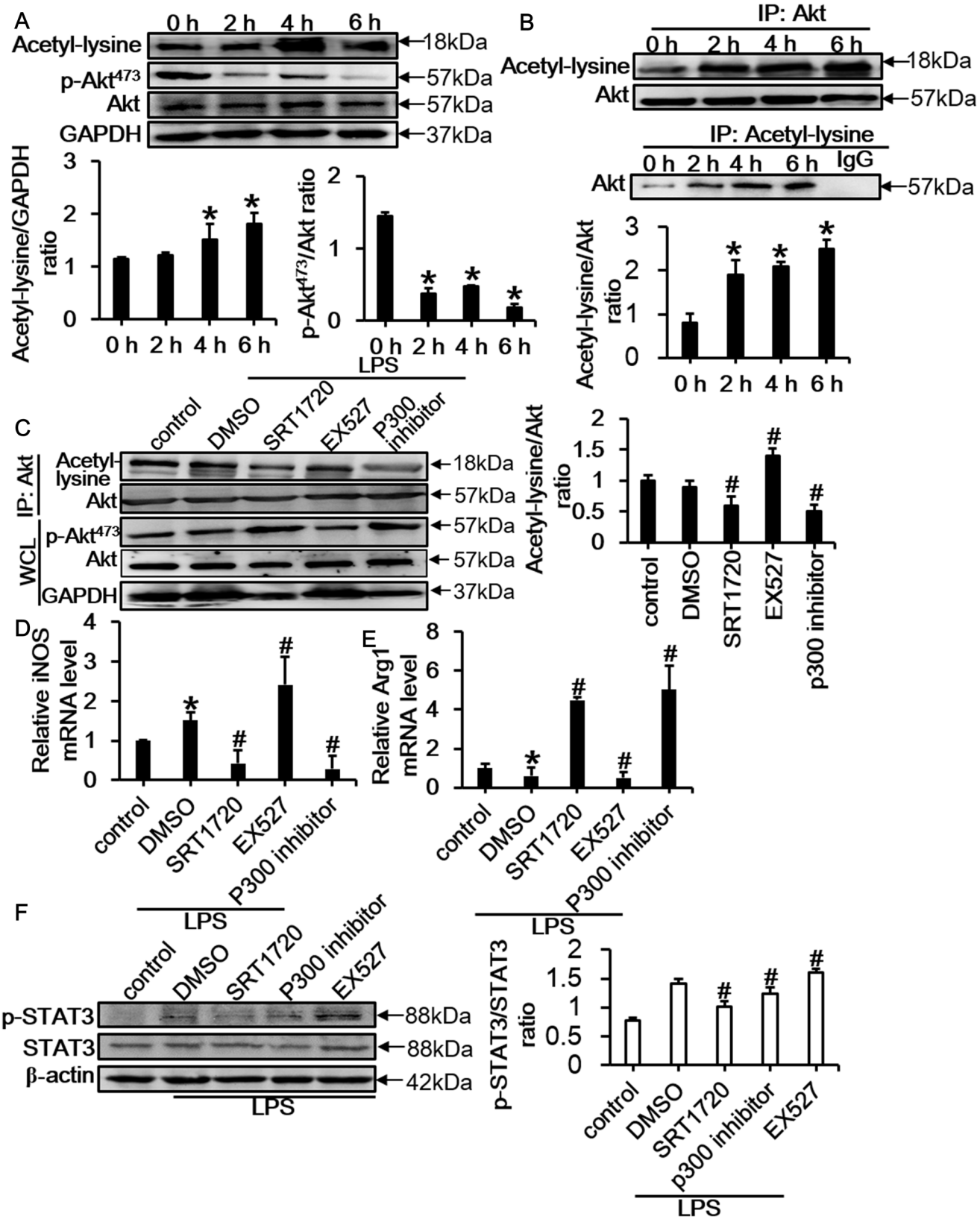

Figure 1. Akt deacetylation is essential for p-Akt expression in macrophages after LPS. (A) 1 μg/mL LPS was applied to RAW264.7 cells for 6 h before they were extracted at the scheduled time. Western blotting was used to examine the acetylation of Akt and p-Akt. (B) Immunoprecipitation (IP) analysis of Akt acetylation after 1 μg/mL LPS treatment at the indicated time. (C) Prior to receiving 1 g/mL LPS treatment, RAW264.7 cells were pretreated for 6 h with dimethyl sulfoxide (DMSO), SRT1720 (1 μM),[49] EX527 (10 μM),[50] and p300 inhibitor (10 μM).[51] Upper panel: IP experiment to assess Akt acetylation. Lower panel: Western blot analysis of the same cells' whole cell lysis (WCL). (D to F) Real-time RT-PCR examination of Arg1 (D) and iNOS (E) mRNA levels, as well as western blot analysis of p-STAT3 (F) in RAW264.7 cells that had been pretreated for 6 h with DMSO, SRT1720, EX527, or p300 inhibitor before being exposed for 12 h to 1 μg/mL LPS. Graphs display mean ± s.d. of three separate tests with technical duplicates. Values with various superscripts differ from one another significantly.

*$P < 0.05$ compared with 0 h.

#$P < 0.05$ compared with DMSO.

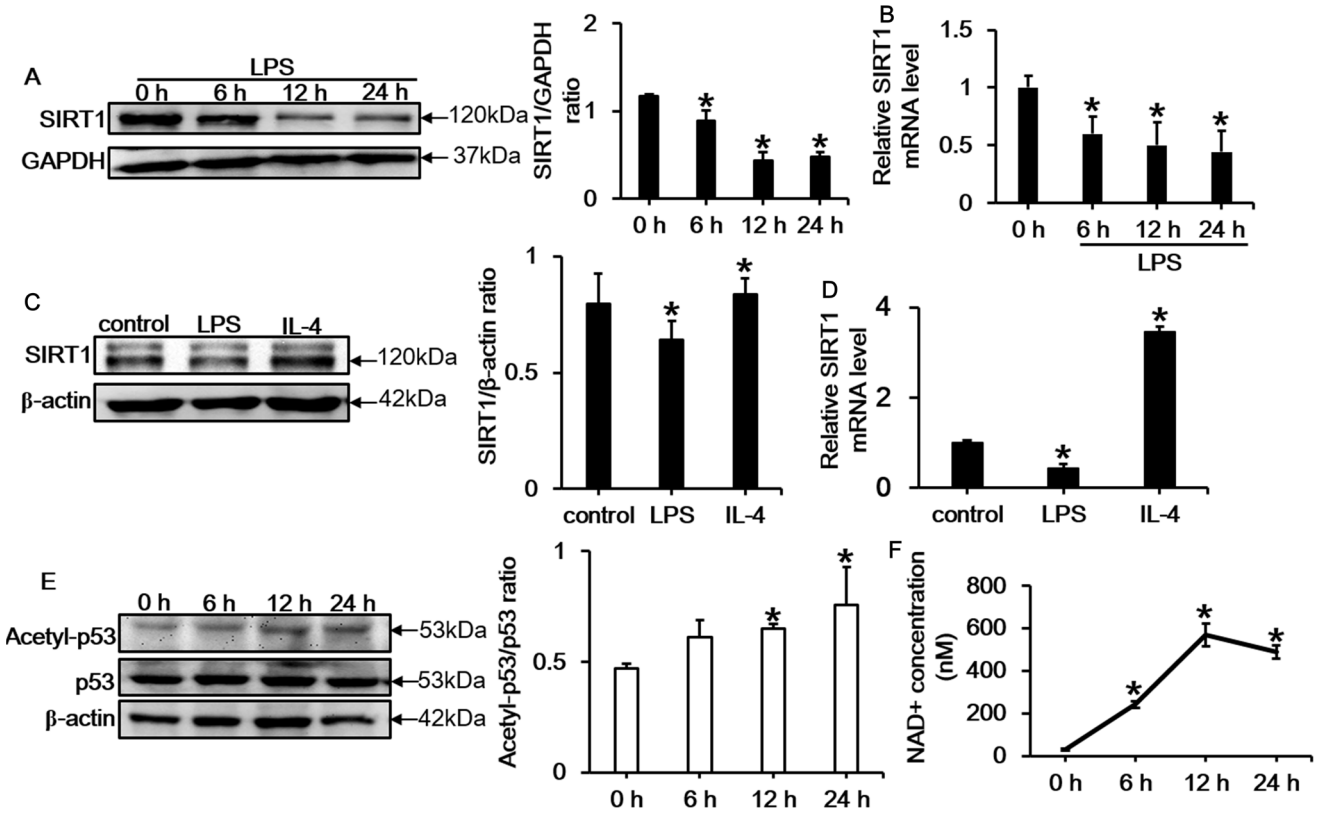

Figure 2. LPS induces downregulation of SIRT1 expression and activity. (A and B) RAW264.7 cells stimulated with 1 μg/mL LPS. Western blot analysis of SIRT1 protein levels (A). Analysis of SIRT1 mRNA levels using real-time PCR (B). (C and D) 1 μg/mL LPS or 100 ng/mL IL-4 were used to excite peritoneal macrophages for 24 h. Western blot analysis of SIRT1 protein levels (C). Analysis of SIRT1 mRNA levels using real-time PCR (D). (E and F) Acetylation of p53 (E) and NAD+ levels in cells treated with 1 μg/mL LPS for the specified durations or left untreated (0 h). The data represent the mean ± s.d. of three independent experiments with technical duplicates.
*$P < 0.05$ compared with 0 h or the control.

could be overexpressed efficiently in macrophages (Figure S3). Upon LPS stimulation, the Akt phosphorylation was decreased in macrophages transfected with the K14R or K20R mutant compared with that transfected with wild-type Akt but was higher than that in macrophages transfected with pcDNA3.1(+) (Figure 5(A)). The p65 acetylation and phosphorylation was examined to determine whether Akt activation has an impact on NF-B activity. The results showed that wild-type Akt, K14R, and K20R all decreased p65 acetylation and phosphorylation compared with pcDNA3.1(+) after LPS stimulation (Figure 5(B)). TNF-α and IL-1β mRNA levels reduced in response to wild-type Akt (Figure 5(C) and (D)). Wild-type Akt also enhanced the level of Arg1 mRNA (Figure 5(G)). However, K14R and K20R further decreased the mRNA levels of TNF-α and IL-1β compared with wild-type Akt (Figure 5(E) and (F)). These findings suggested that the inflammatory response in macrophages is brought on by acetylation-mediated Akt activation. The different acetylation sites of Akt may have diverse and important regulation on Akt activity.

SIRT1 regulates the Akt-mediated inflammatory response in macrophage

We thus concluded that SIRT1 might be involved in Akt's membrane localization. After being exposed to LPS, macrophages produced less Akt in the membrane fraction (Figure 6(A)), but SRT1720 rescued the decreased amount induced by LPS in RAW264.7 (Figure 6(B)) and THP-1 cells (Figure S4A), suggesting that SIRT1 promoted Akt membrane localization. The role of SIRT1 in Akt-mediated inflammation was then discussed. First, a wild-type Akt plasmid was transfected into macrophages. After that, either SRT1720 pretreatment or no pretreatment, LPS was used to activate the cells. The results showed that wild-type Akt decreased the mRNA levels of TNF-α, iNOS, and IL-1β in the presence of SRT1720 (Figure 6(C) to (E)). However, wild-type Akt enhanced the amounts of Arg1 mRNA (Figure 6(F)). IL-1β and TNF-α secretion were quantified using ELISA. The results reveal that the protein levels of IL-1β and TNF-α had a trend comparable with that of the mRNA levels (Figure S4B and C). According to these findings, SIRT1 participated in the Akt-mediated inflammatory response in macrophages.

SIRT1 regulates Akt-mediated inflammatory response *in vivo*

To further understand the functional role of SIRT1-mediated Akt activation, the effect of SIRT1 knockout on the tissues and Akt acetylation was analyzed in septic mice. The genotyping result of SIRT1 knockout mice was shown in the Figure S5A. The result showed that SIRT1 was effectively knockout. SIRT1 knockout resulted in increased inflammatory cell infiltration, diffuse macrovascular distribution, and the

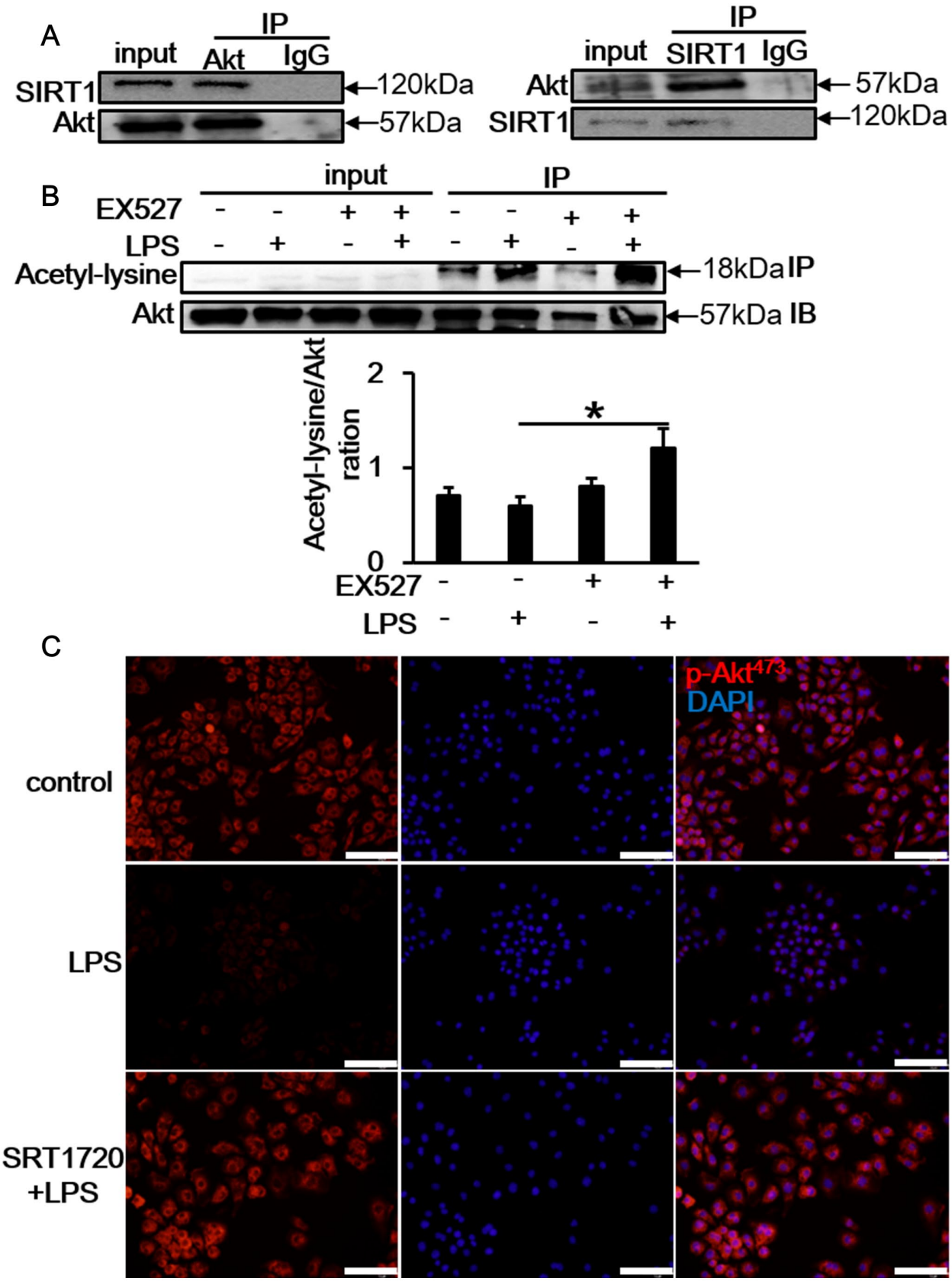

Figure 3. SIRT1 deacetylates Akt in macrophages. (A) Immunoprecipitation of endogenous SIRT1 or Akt from RAW264.7 cell lysates is shown. To determine whether Akt or SIRT1 are present in the input and immune complexes, Western blot analysis is used. (B) RAW264.7 cells were given a 6-h treatment with 10 μM EX527 before being stimulated with 1 μg/mL LPS. The cells were lysed. The cell extracts were immunoprecipitated with Akt antibodies. The total protein and acetylated Akt levels in the entire cell lysates were evaluated by western blotting. (C) Prior to being stimulated with 1 μg/mL LPS for 12 h, RAW264.7 cells were pretreated with or without 10 μM SRT1720 for 6 h. The process of immunofluorescence was employed to assess Akt phosphorylation. 50 μm is the scale bar. The data are representative of three independent experiments.
*$P < 0.05$ compared with LPS.

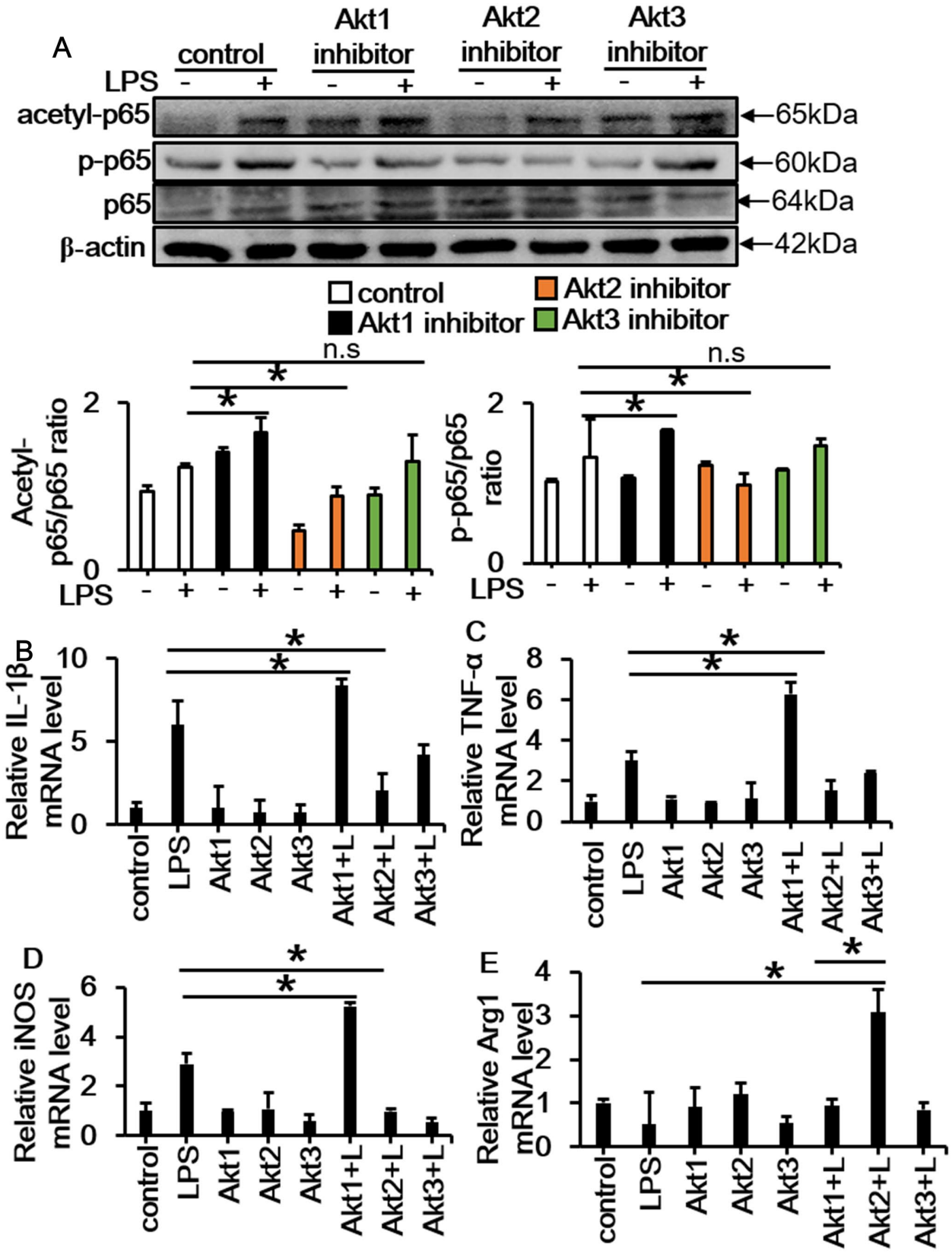

Figure 4. Akt mediates the inflammatory response in macrophages. (A) RAW264.7 cells were treated with 58 nM, 210 nM, and 2119 nM Akt inhibitor which inhibited Akt1 (generally named Akt), Akt2, and Akt3, respectively, for 30 min. (B to E) The cells were then either treated for 12 h with 1 μg/mL LPS or not. Western blot was used to evaluate the phosphorylation and acetylation of P65. Following stimulation with or without 1 μg/mL LPS, RAW264.7 cells pretreated with 58 nM, 210 nM, and 2119 nM Akt inhibitors were subjected to RT-PCR analyses to determine the amounts of I IL-1β (B), TNF-α (C), iNOS (D), and Arg1 (E) mRNA levels. Data represent mean ± s.d. of three independent experiments with technical duplicates.
*$P < 0.05$; n.s.: no significant difference.

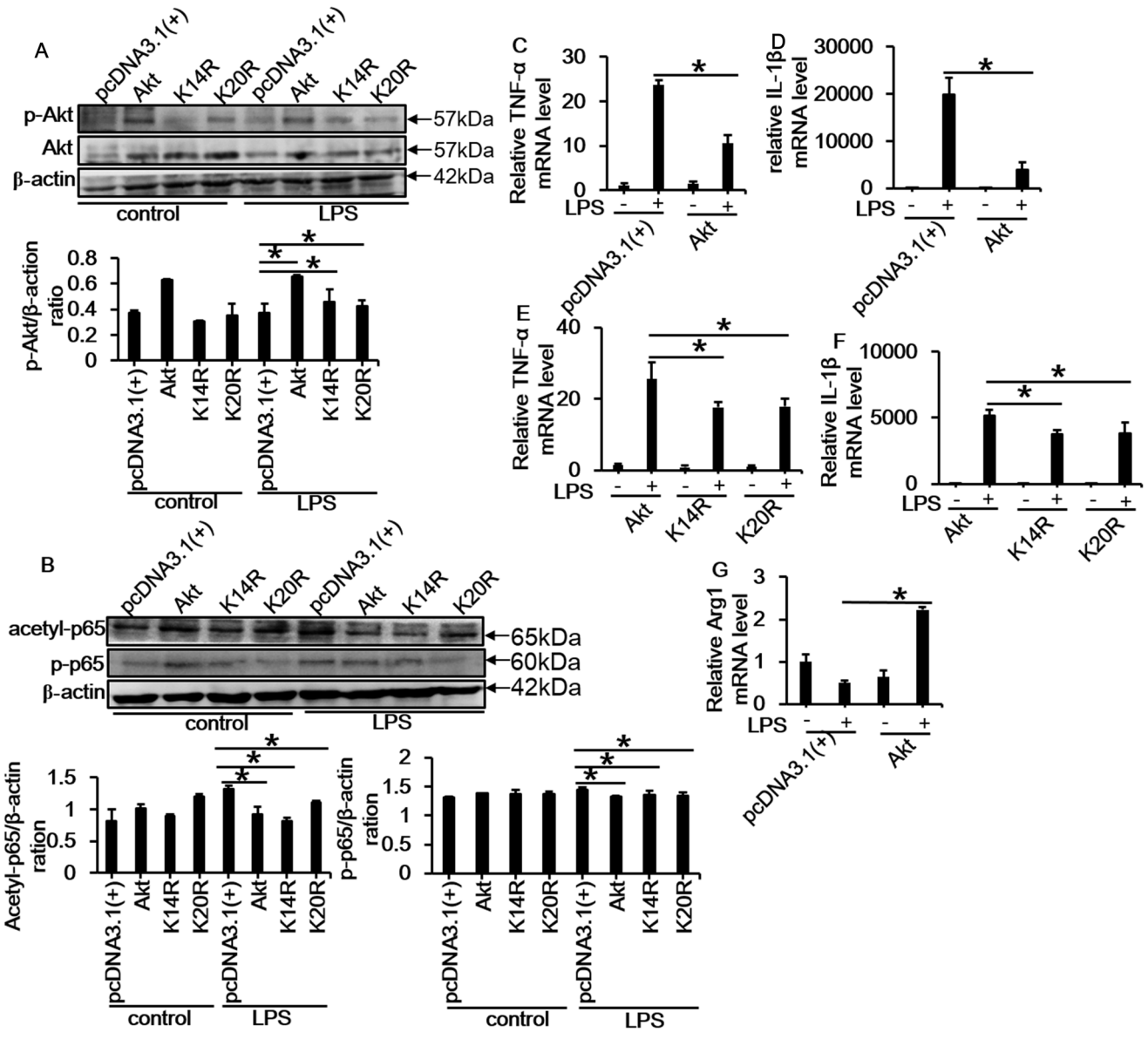

Figure 5. Deacetylated Akt decreases LPS-induced inflammatory cytokines in macrophages. (A and B) RAW264.7 cells were transfected with plasmids for 48 h. The cells were then either treated for 12 h with 1 μg/mL LPS or not. Western blot examination of p65 acetylation and phosphorylation (A) and Akt phosphorylation (B). TNF-α (C), IL-1β (D), and Arg1 (G) mRNA levels were examined using real-time PCR in RAW264.7 cells that had been transfected during 24 h with pcDNA3.1(+) or pcDNA3.1(+)-Akt and either received 1 μg/mL LPS or not. TNF-α (E) and IL-1β (F) mRNA levels were examined using real-time PCR in RAW264.7 cells transfected for 24 h with pcDNA3.1(+)-Akt, pcDNA3.1(+)-K14R, or pcDNA3.1(+)-K20R before being treated with 1 μg/mL LPS for 12 h or not. The graphs show the mean ± s.d. of three independent experiments.
*$P < 0.05$ compared to the corresponding control. n.s.: no significant difference. Akt: pcDNA3.1(+)-Akt; K14R: pcDNA3.1(+)-K14R; K20R: pcDNA3.1(+)-K20R.

displacement of the hepatocyte nucleus toward the periphery in the liver compared with those in wild-type septic mice (Figure 7(A)). SIRT1 knockout resulted in more inflammatory cell infiltration, necrosis, and degeneration in the heart and kidney than those in wild-type septic mice (Figure 7(A)). Inflammatory neutrophil and macrophage infiltration in the lung was more severe in the SIRT1 knockout sepsis group than that in the wild-type sepsis group (Figure 7(A)). We also inquired into the proportion of M1 to M2 in peritoneal macrophages (PMs). The results demonstrated that in septic SIRT1-knockout mice as opposed to wild-type mice, M1 macrophages were more active (Figure 7(B)). Compared to wild-type mice, PMs isolated from septic SIRT1-knockout mice had higher levels of Akt acetylation (Figure 7(C)). The results showed that SIRT1 knockout reduced the phosphorylation of Akt and increased the phosphorylation of NF-κB p65 in PMs isolated from septic mice (Figure 7(D)). To confirm that SIRT1 involved in tissue damage required Akt activation, healthy or septic mice were treated with SRT1720 in combination with the Akt inhibitor. Treatment with SRT1720 increased Akt activation, and the Akt inhibitor reduced it (Figure 7(E)). As a result, SRT1720 downregulated the expression of the pro-inflammatory cytokines iNOS and IL-1, whereas the Akt inhibitor enhanced it (Figure 7(F) and (G)). The serum levels of pro-inflammatory cytokines were additionally assessed using ELISA. The serum levels of TNF-α and IL-1β

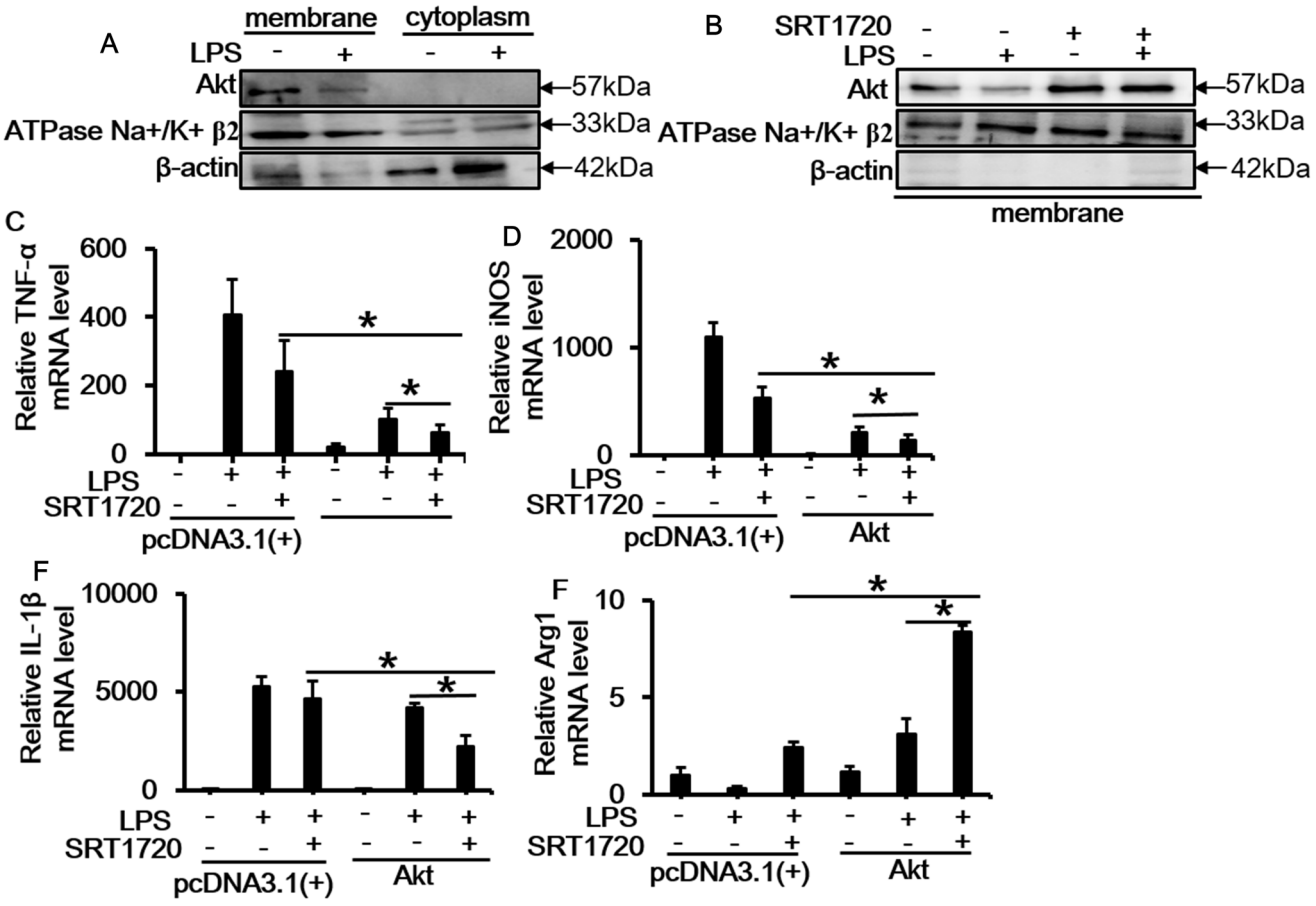

Figure 6. SIRT1-mediated deacetylation promotes Akt membrane localization and regulates the inflammatory response. (A) Western blot showed the LPS-induced localization of Akt to the membrane fraction. The cytoplasmic and membrane markers β-action and ATPase Na^+/K^+ β2 were also immunoblotted. (B) Western blot analysis of the localization of Akt to the membrane fraction in RAW264.7 cells treated with 10 μM SRT1720 for 6 h, followed by treatment with 1 μg/mL LPS for 12 h. (C-F) RAW264.7 cells were transfected with pcDNA3.1(+) or pcDNA3.1(+)-Akt for 24 h. Then, the cells were treated with 10 μM SRT1720 (6 h), 1 μg/mL LPS (12 h) or SRT1720 plus LPS. Real-time RT-PCR was used to analyze the mRNA levels of TNF-α (C), iNOS (D), IL-1β (E), and Arg1 (F). The graphs show the mean ± s.d. of three independent experiments.
*$P < 0.05$ compared to the corresponding control.

had no significant difference in the SIRT1−/− sepsis group compared with wild-type sepsis group after SRT1720 treatment (Figure S5B-C). TNF-α and IL-1β levels were markedly diminished in the SRT1720 group and SRT1720 plus the Akt inhibitor group in the context of sepsis, but SRT1720 had a more significant effect (Figure S5D-E). These results demonstrated that the interaction of SIRT1 and Akt regulated the tissues damage by affecting the inflammatory response during the sepsis.

Discussion

Macrophages are the first line of defense for innate immunity and are essential for the early initiation and control of inflammation. During glycolysis, the Toll-like receptor 4-ligand LPS triggers the production of IL-1 and other pro-inflammatory cytokines. Mannose reduces IL-1 gene expression to prevent LPS-induced macrophage activation.[36] Excessive activation of LPS has the potential to cause tissue damage and compromise host survival, as revealed in murine models of sepsis.[37] Activated Akt improved cardiac function and enhanced sepsis survival via mTOR inhibition.[38] Physiologic conditions precisely controlled the activation of Akt. The reports have evidenced that lysine modification is critical for Akt activity.[39] Down-regulating the inflammation and increasing Akt activation that is important for injured tissue repair following sepsis is a potential therapeutic strategy to improve clinical outcome.

In this study, we have shown that acetylation regulates Akt activation. Deacetylation preferentially triggers Akt translocation to the membrane. At baseline, Akt is maintained in a deacetylated form mainly through the activity of the deacetylase SIRT1. In RAW264.7 cells, LPS increased the acetylation of Akt and inhibited Akt membrane translocation. The phosphorylation of p65 increased NF-κB p65's acetylation, which in turn increased NF-κB overall transcriptional activity.[40] The activation and induction of M1 macrophage polarization by phosphorylated p65. The acetylation and phosphorylation of NF-κB p65 depend on the downregulation of Akt phosphorylation. In addition, elevated SIRT1 activity can deacetylate Akt and enhance Akt activation. *In vivo*, we observed that an SIRT1 activator attenuated Akt inhibitor-induced Akt phosphorylation, which prevented inflammatory cytokine release in PMs derived from septic mice.

We found that the degree of Akt acetylation affects Akt activation. Acetylated Akt is widely distributed in a variety

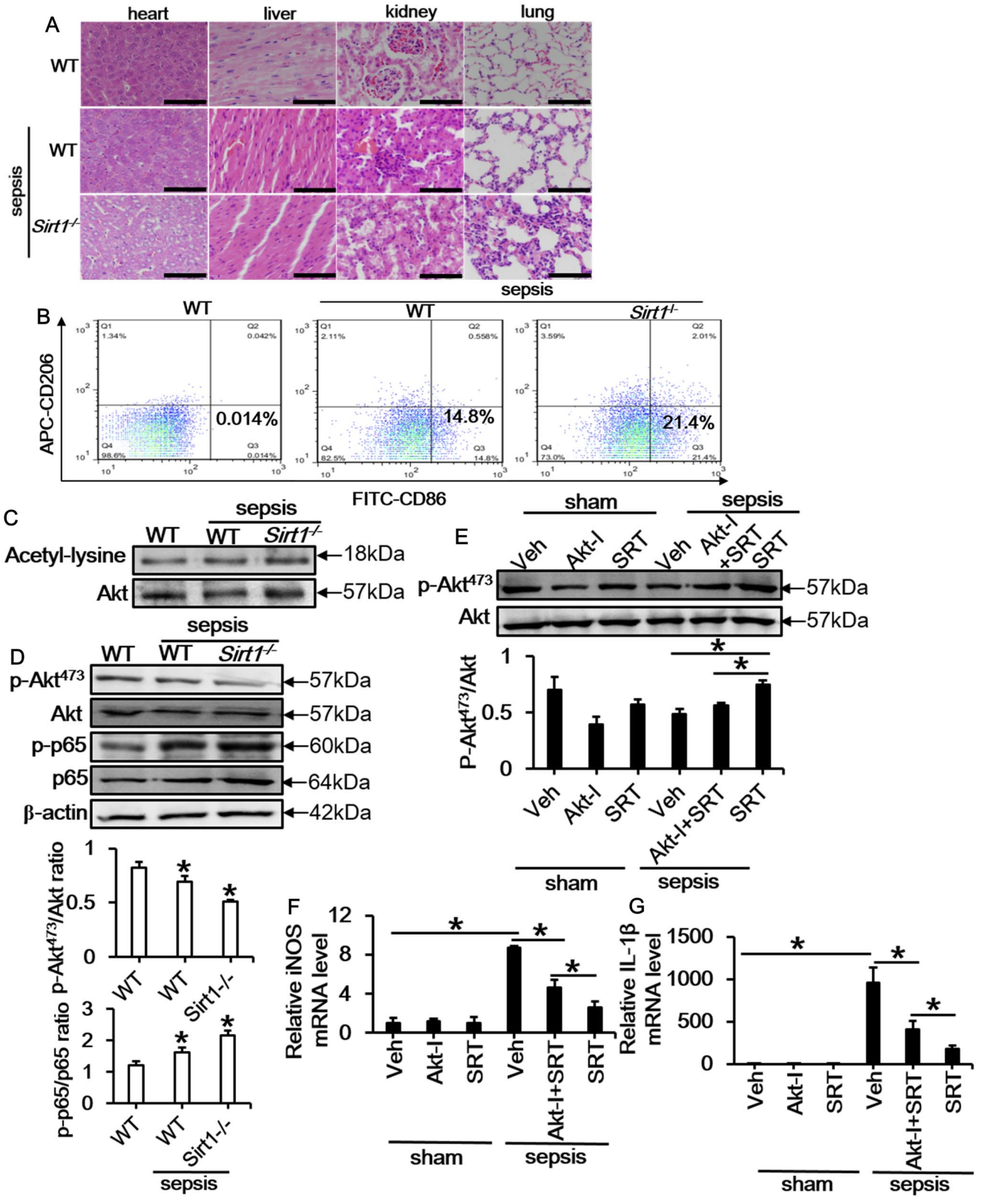

Figure 7. Akt modification by SIRT1 regulated the inflammatory response *in vivo*. (A) SIRT1 knockout (Sirt1−/−) and wild-type (WT) mice were given LPS treatment to cause sepsis. HE was used to stain the formalin-fixed, paraffin-embedded mouse liver, heart, kidney, and lung tissues. The scale bar is 100 μm. (B) The percentage of M1/ M2 in PMs from WT mice and *Sirt1*$^{-/-}$ were detected by FACS. (C) IP was used to analyze the acetylation of Akt in PM lysates from WT mice and *Sirt1*$^{-/-}$ septic mice. (D) Western blotting was used to analyze the phosphorylation of Akt and p65 in PM-isolated WT mice and *Sirt1*$^{-/-}$ septic mice. (E to G) Akt inhibitor (Akt-I), SRT1720, or vehicle (Veh) was injected into the mice to block endogenous Akt activity, enhance SIRT1 activity, and be the control, respectively. Western blot assessed phosphorylation of Akt (E). Real-time PCR was used to analyze the mRNA levels of iNOS (F) and IL-1β (G). The graphs show the mean ± s.d. of three independent experiments.
*$P < 0.05$ compared to the corresponding control.

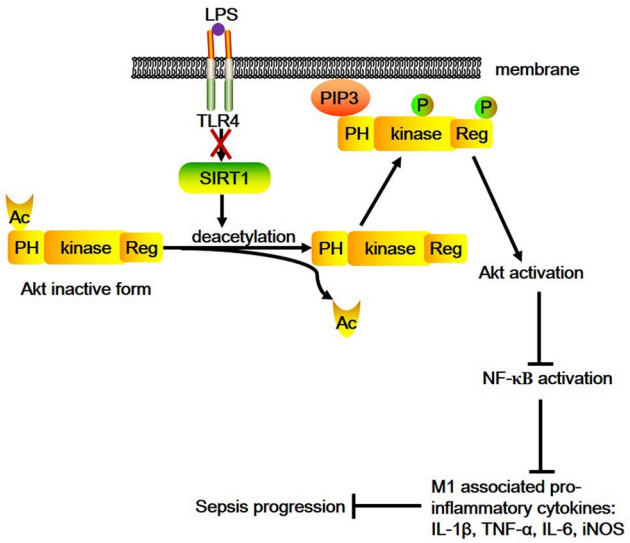

Figure 8. The schematic diagram illustrates the role of SIRT1 in regulating the deacetylation and activation of Akt. Akt affects M1 macrophage polarization. The PH domain of Akt is acetylated and thus inactive. SIRT1 can deacetylate Akt. Deacetylation enables the localization of Akt to the plasma membrane, thereby facilitating Akt phosphorylation and activation. Activated Akt prevents the progression of sepsis by downregulating pro-inflammatory cytokines. Ac: acetylation; Reg: regulatory domain.

of tissues, such as the heart, liver, brain, and skeletal muscle, and this modification inhibits Akt activity.[41] Nagalingam R *et al.* identified two acetylated lysine residues (Lys^{14} and Lys^{20}). The inhibition of membrane translocation by Akt acetylation also places restrictions on Akt's capacity to bind to PIP3.[18] We established that Akt can be bound to and deacetylated by SIRT1. SIRT1 knockout inhibited Akt translocation to the membrane. However, SRT1720 promotes increased membrane localization of Akt during LPS stimulation in macrophages. Lys^{14} is involved in the binding of PIP3 to Akt. Mutation of Lys^{14} will therefore eliminate the binding of Akt to PIP3.[18,42] These two lysine residues may function differently depending on the cell type and conditions. The findings of our investigation demonstrated that K14R and K20R both greatly reduced the acetylation of p65. Compared to the K20R mutant, the K14R mutant was more effective. Therefore, it is important to understand how Akt acetylation influences NF-κB activation. TNF-α and IL-1β mRNA levels were downregulated and Arg1 mRNA levels were increased by the overexpression of wild-type Akt. In contrast to wild-type Akt overexpression, the K14R and K20R mutants did not significantly affect the levels of Arg1 mRNA.

The mechanism by which Akt acetylation controls p-Akt formation in macrophages is not very clear. Deacetylation, on the other hand, promotes Akt membrane localization, which is required for S473 phosphorylation. M1- or M2-polarized active macrophages are both possible. High quantities of Arg1 are expressed by M2-polarized macrophages, which compete with iNOS for L-arginine, their shared substrate, reducing NO production and converting L-arginine to urea.[43] The data presented in this report showed that Akt inhibition resulted in M1 polarization because these cells expressed elevated IL-1β, TNF-α, and iNOS, which are markers of M1 polarization, and decreased Arg1 which is marker of M2 polarization. For NF-κB to be activated, which is a crucial transcription factor linked to the generation of inflammatory cytokines, its acetylation status must be present. We discovered that p65 was becoming more acetylated when Akt was inhibited.

Notably, SIRT1 is dynamically regulated by LPS. In macrophages stimulated with LPS, SIRT1 activity declines.[44,45] SIRT1 has a number of post-translational modification sites[46] that regulate the stability of proteins and the activity of enzymes.[47,48] Therefore, in the future, we should ascertain how the expression of the SIRT1 protein and enzymatic activity are related. The notion that this system can be altered therapeutically arises from SIRT1's capacity to control the Akt-dependent cell inflammatory response. SRT1720, a well-known SIRT1 activator, has been identified as an anti-inflammatory compound that has beneficial effects in some diseases. We found that SIRT1 knockout increased the acetylation of Akt and decreased the S473 phosphorylation of Akt in PMs isolated from septic mice. SRT1720 abrogated the reduction in Akt phosphorylation caused by the Akt inhibitor, thereby downregulating the mRNA levels of iNOS and IL-1β *in vivo*. In addition, there was a marked reduction in serum production of IL-1β and TNF-α.

Conclusions

The interdependence of Akt's post-translational modifications is shown by this work. We describe how phosphorylation and acetylation work together to regulate inflammatory reactions in macrophages (Figure 8). Understanding the interplay among SIRT1, Akt and NF-κB provides new insight into molecular mechanisms to regulate macrophage polarization, which is involved in the progression of sepsis.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. YHJ, KS, and JQL did the experiments, analyzed the data and wrote the manuscript. YL, XZB, and YSY assisted in design and editing. TH, YZ, LT, XWG, and ZZ assisted in animal experiment. HG and DH supervised the study and modified the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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ORCID ID

Yanhui Jia https://orcid.org/0000-0002-5831-9028

SUPPLEMENTAL MATERIAL

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