BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 24(4), 387-394 (2016)

Trichostatin A Protects Liver against Septic Injury through Inhibiting Toll-Like Receptor Signaling

So-Jin Kim, Jin-Sook Park, Do-Won Lee and Sun-Mee Lee*

School of Pharmacy, Sungkyunkwan University, Suwon 16419, Republic of Korea

Abstract

Sepsis, a serious clinical problem, is characterized by a systemic inflammatory response to infection and leads to organ failure. Toll-like receptor (TLR) signaling is intimately implicated in hyper-inflammatory responses and tissue injury during sepsis. Histone deacetylase (HDAC) inhibitors have been reported to exhibit anti-inflammatory properties. The aim of this study was to investigate the hepatoprotective mechanisms of trichostatin A (TSA), a HDAC inhibitor, associated with TLR signaling pathway during sepsis. The anti-inflammatory properties of TSA were assayed in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Polymicrobial sepsis was induced in mice by cecal ligation and puncture (CLP), a clinically relevant model of sepsis. The mice were intraperitoneally received TSA (1, 2 or 5 mg/kg) 30 min before CLP. The serum and liver samples were collected 6 and 24-h after CLP. TSA inhibited the increased production of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in LPS-stimulated RAW264.7 cells. TSA improved sepsis-induced mortality, attenuated liver injury and decreased serum TNF-α and IL-6 levels. CLP increased the levels of TLR4, TLR2 and myeloid differentiation primary response protein 88 (MyD88) protein expression and association of MyD88 with TLR4 and TLR2, which were attenuated by TSA. CLP increased nuclear translocation of nuclear factor kappa B and decreased cytosolic inhibitor of kappa B (IκB) protein expression, which were attenuated by TSA. Moreover, CLP decreased acetylation of IκB kinase (IKK) and increased association of IKK with IκB and TSA attenuated these alterations. Our findings suggest that TSA attenuates liver injury by inhibiting TLR-mediated inflammatory response during sepsis.

Key Words: Histone deacetylase inhibitor, Nuclear factor kappa B, Sepsis, Toll-like receptor, Trichostatin A

INTRODUCTION

Sepsis, a systemic inflammatory response syndrome by infection, is life-threatening disease with high mortality and mobidity (Singhal *et al*., 2013). Severe sepsis is a leading cause of death in intensive care units and results in aggregate health care cost (Lagu *et al*., 2012). Despite continuous effort and research, effective pharmacologic agent for sepsis has not been developed. The complicated and ambiguous pathological mechanism of sepsis is one of the main causes of failure in management (Zhao *et al*., 2013). An uncontrolled hyper-inflammatory response and improper cytokine response during sepsis have been proposed as the cause of multiple organ failure, which is most lethal complication of sepsis (Riedemann *et al*., 2003). Given the large abundance of resident macrophages and their extensive endothelial surfaces, the liver plays a prominent role in the host immune response to infection (Chen *et al*., 1996). Moreover, the liver is believed to be a vulnerable organ responsible for the initiation of multiple organ dysfunction syndrome, which is the most lethal complication of sepsis (Bone, 1991).

Histone deacetylase (HDAC) regulates gene expression through chromatin remodeling (Wu and Grunstein, 2000). HDAC catalyzes the hydrolytic removal of the acetyl group of histone resulting in chromatin condensation and transcriptional silencing (Johnstone, 2002). Given the role in their potent efficacy in modulating the gene expression, HDAC inhibitors have been developed as anti-cancer agents (Pan *et al*., 2007). Accumulating evidences revealed that HDAC inhibitors possess anti-inflammatory properties (Kim *et al*., 2001; Tong *et al*., 2004). Trichostatin A (TSA), a representative HDAC inhibitor, improved the hepatic injury by decreasing interleukin (IL)-6 production in septic mice (Zhang *et al*., 2009).

Toll-like receptors (TLRs), crucial family of pattern recognition receptors, are the first line of defense system against microbial infection. Complex TLRs signaling and its associated

Open Access **http://dx.doi.org/10.4062/biomolther.2015.176**

Received Oct 30, 2015 **Revised** Nov 26, 2015 **Accepted** Dec 2, 2015
Published Online Jul 1, 2016

***Corresponding Author**
E-mail: sunmee@skku.edu
Tel: +82-31-290-7712, Fax: +82-31-292-8800

downstream regulators involved in pathogenesis and development of sepsis. In polymicrobial sepsis, hepatic *TLR4* and *TLR2* mRNA expression increased compared with that in sham-operated mice (Williams *et al*., 2003) and TAK-242, a TLR4 inhibitor, improved survival in a murine model of sepsis (Sha *et al*., 2011). Moreover, TLR protein expression upregulated in septic patients compared with healthy individuals. Activation of TLRs rapidly recruits adaptor molecules and triggers inflammatory gene expression through downstream pathways. Recently, HDAC inhibitors have been reported to regulate diverse signaling pathways through post-translational modification of non-histone proteins. TSA inhibited up-regulation of *myeloid differentiation primary response protein 88* (*MyD88*) gene expression in lipopolysaccharide (LPS)-induced bone marrow-derived dendritic cells (Roger *et al*., 2011) and suberoylanilide hydroxamic acid suppressed mitogen-activated protein kinase (MAPK) signaling pathway through acetylation of MAPK phosphatase-1 (Finkelstein *et al*., 2010).

Therefore, the present study was undertaken to elucidate the molecular mechanisms involved in the hepatoprotective effects of TSA, a HDAC inhibitor, in cecal ligation and puncture (CLP)-induced sepsis, particularly focusing on TLR signaling pathway.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin (10,000 U/mL and 10,000 μg/mL, respectively) were purchased from Gibco Life Technologies (Grand Island, NY, USA). LPS (*Escherichia coli* serotype O111:B4), tetrazolium bromide (MTT), and all other materials required for culturing cells were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used in this study were of reagent grade.

Cell viability

Mouse macrophage cell line RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). RAW264.7 cells were seeded in 96-well plates, incubated for 24-h in 10% FBS and 1% penicillin/streptomycin-contained DMEM. The cells were treated with vehicle (phosphate-buffered saline; PBS) or TSA (12.5, 25, 50, 100 or 200 nM) for 24-h, and then 100 μL of MTT solution (5 mg/mL in PBS) was added to each well. After 3-h incubation, the medium was removed, and subsequently 100 μL of dimethyl sulfoxide was added to each well to solubilize any deposited formazan. The optical density of each well was measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cell culture and treatment

RAW264.7 cells were cultured in 10% FBS and 1% penicillin/streptomycin contained-DMEM, and maintained at 37°C in an atmosphere of 5% CO_2. After 6-h incubation, the cells were starved with 0.5% FBS and 1% penicillin/streptomycin contained-DMEM. After 18-h starvation, the cells were treated with vehicle (PBS) or TSA (12.5, 25 or 50 nM) for another 1-h and then treated with LPS (1 μg/mL). After 24-h incubation, the culture media were harvested for assay.

Animals

Male C57BL/6 mice weighing 23-25 g (8 weeks old) were supplied by Orient Bio (Seongnam, Korea). The animals were housed in cages located in temperature-controlled rooms with a 12-h light-dark cycle and received water and food *ad libitum*. All animal procedures were approved by the Sungkyunkwan University Animal Care Committee and were performed in accordance with the guidelines of the National Institutes of Health (NIH publication No. 86-23, revised 1985).

CLP

Polymicrobial sepsis was induced by CLP as described by Chaudry *et al* (1979). Mice were anesthetized with an intramuscular injection of ketamine (100 mg/kg; Yuhan Corporation, Seoul, Korea) and xylazine (10 mg/kg; Boehringer Ingelheim, St. Joseph, MO, USA). Anesthestized mice were then opened with a 1-cm midline incision of the abdomen and the cecum was carefully exposed. The cecum was ligated just distal to the ileocecal valve to avoid intestinal obstruction and punctured twice using a 20-gauge needle. The punctured cecum was squeezed to extract feces and returned to the abdominal position. The abdominal incision was closed with two layers of running suture. After the operation, all animals subcutaneously received 1 mL normal saline injection for fluid resuscitation. Sham-operated mice were operated to laparotomy and intestinal manipulation, however, the cecum was neither ligated nor punctured.

Administration of TSA and experimental design

In survival experiments, mice intraperitoneally received vehicle (normal saline) or TSA (1, 2 or 5 mg/kg) 30 min before CLP. The dose and time point of TSA administration were selected based on a previously published report (Zhang *et al*., 2009) and our preliminary study. Animals were randomly divided to the following 6 groups (each group, n=6-10): (a) vehicle-treated sham (sham), (b) TSA (2 mg/kg)-treated sham (TSA), (c) vehicle-treated CLP (CLP), (d) TSA 1 mg/kg-treated CLP (TSA 1 mg/kg+CLP), (e) TSA 2 mg/kg-treated CLP (TSA 2 mg/kg+CLP) and (f) TSA 5 mg/kg-treated CLP (TSA 5 mg/kg+CLP). Mortality was recorded for up to 10 days after CLP and survivors were monitored for further 3 weeks to ensure that no late mortalities occurred (each group, n=10). On the basis of a survival test, a dose of 2 mg/kg was chosen for further biochemical studies. Under anesthesia, blood samples from the inferior vena cava and liver tissues were collected 6 and 24-h after CLP. The serum were isolated by centrifugation at 10,000×*g* for 10 min at 4°C.

Cytokine levels

The tumor necrosis factor (TNF)-α and IL-6 production in LPS-stimulated RAW264.7 cells and the serum levels of TNF-α and IL-6 were measured 6-h after CLP using commercially available enzyme-linked immunosorbent assay kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

Total RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from liver tissues using RNAiso Plus (Takara Bio Inc., Shiga, Japan) and the first strand of cDNA was synthesized via reverse transcription (EcoDry cDNA Synthesis Premix; Takara Bio, Inc.). The cDNA was am-

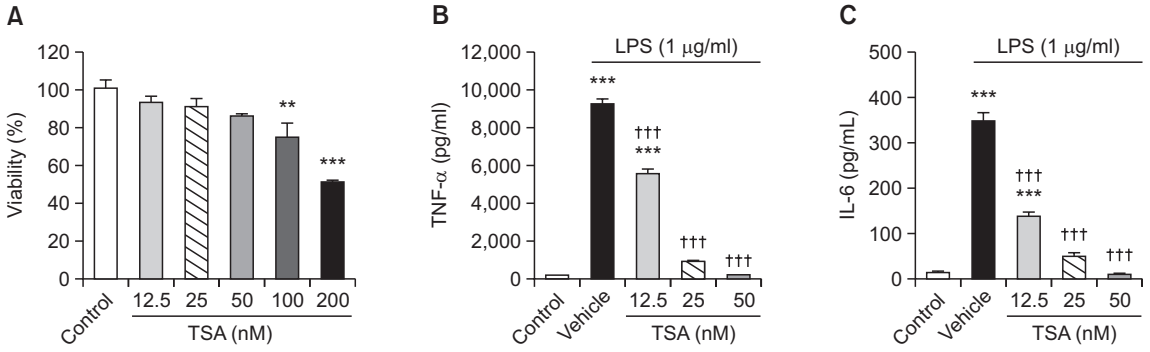

Fig. 1. Effect of TSA on TNF-α and IL-6 release in LPS-stimulated RAW264.7 cells. For the cellular toxicity assay, RAW264.7 cells were treated with vehicle (PBS) or various concentrations of TSA for 24-h (A). To measure cytokine release, RAW264.7 cells were treated with various concentrations of TSA for 1-h before LPS (1 μg/mL) stimulation. Twenty-four hours after LPS stimulation, release of TNF-α (B) and IL-6 (C) was measured. The results are presented as mean ± SEM of three independent experiments. ***p*<0.01, ****p*<0.001 versus control group. †††*p*<0.001 versus LPS group.

plified with primers and SYBR green (Roche Applied Science, Mannheim, Germany) using a thermocycler (Lightcycler Nano; Roche Applied Science). Gene specific primers used for cDNA amplification were 5′-AGCCCACGTCGTAGCAAACCACCAA-3′ (sense) and 5′-ACACCCATTCCCTTCACAGAGCAAT-3′ (antisense) for *TNF-α*; 5′-GAAAGTCAACTCCATCTGCC-3′ (sense) and 5′-CATAGCACACTACGTTTGCC-3′ (antiense) for *IL-6*; and 5′-GGCTGTATTCCCCTCCATCG-3′ (sense) and 5′-CCAGTTGGTAACAATGCCATGT-3′ (antisense) for *β-actin*. Real-time RT-PCR was performed with an specific amplification cycling conditions as follows: 35 cycles of 30 s at 94°C, 30 s at 65°C and 30 s at 72°C for *TNF-α*; 35 cycles of 30 s at 94°C, 45 s at 58°C and 30 s at 72°C for *IL-6*; and 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C for *β-actin*. The levels of mRNA expression were normalized to that of the *β-actin* mRNA expression and reported relative to the average of all Δcycle threshold (Ct)-values in each sample using the Ct method. All samples were carried out in duplicate to ensure amplification integrity.

Serum aminotransferase level

The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured 6 and 24-h after CLP with ChemiLab ALT and AST assay kits (IVDLab Co., Uiwang, Korea) using a Hitachi 7600 automatic analyzer (Hitachi, Tokyo, Japan).

Histological analysis

The liver tissue collected 24-h after CLP was fixed with 10% neutral buffered formalin. The sample was embedded in paraffin, sliced into 5-μm sections, and stained with hematoxylin and eosin (H&E). Histological changes were evaluated in random and nonconsecutive fields at x200 magnification (Olympus BX51/Olympus DP71, Tokyo, Japan).

Total, cytosolic and nuclear protein extraction

The liver tissue collected 6-h after CLP was homogenized in PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Seongnam, Korea) for total protein samples or in NE-PER® (Pierce Biotechnology, Rockford, IL, USA) for nuclear/cytosolic proteins according to the manufacturers' instructions. The levels of protein concentration were determined using BCA Protein Assay kit (Pierce Biotechnology).

Western blot analysis

Protein samples (16-20 μg) were loaded on 7.5-15% polyacrylamide gels, separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) using the Semi-Dry Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA). After the transfer, the membranes were blocked for 1-h with 5% (w/v) skim milk or bovine serum albumin powder in Tris-buffered saline with 0.1% Tween-20 at room temperature. Blots were incubated with primary antibodies overnight at 4°C. Primary antibodies against MyD88, TIR-domain-containing adapter-inducing interferon-β (TRIF), nuclear factor kappa B (NF-κB), inhibitor of kappa B (IκB)-α, acetylated-lysine (Ac-K; Cell Signaling Technology, Berverly, MA, USA), TLR4 and TLR2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. On the following day, the blots were incubated with appropriate secondary antibodies for 1-h at room temperature. Bands were detected using an ECL detection system (iNtRON Biotechnology) according to the manufacturer's instructions. The intensities of immunoreactive bands were evaluated using Total-Lab TL120 software (Nonlinear Dynamics, Newcastle, UK). Signals were standardized to that of β-actin (Sigma-Aldrich) and lamin B1 (Abcam, Cambridge, MA, USA) for whole/cytosolic lysate and nuclear fraction, respectively.

Immunoprecipitation

To assess the protein association, liver lysates were immunoprecipitated with anti-TLR4, anti-TLR2 and IκB kinase (IKK) antibodies (Santa Cruz Biotechnology). The mixture was incubated for 3-h at 4°C and beads were pelleted by centrifugation at 12,000×*g* for 30 s. The pelleted beads were washed five times in cold PBS, resuspended in loading buffer and heated at 95°C for 7 min before analysis by immunoblotting.

Statistical analysis

Survival data was prescribed by the Kaplan-Meier curve and analyzed by log-rank test. All other data were analyzed by the one-way analysis of the variance and the Bonferroni test was used for *post-hoc* comparisons. Statistical differences between the groups were considered significant at $p<0.05$.

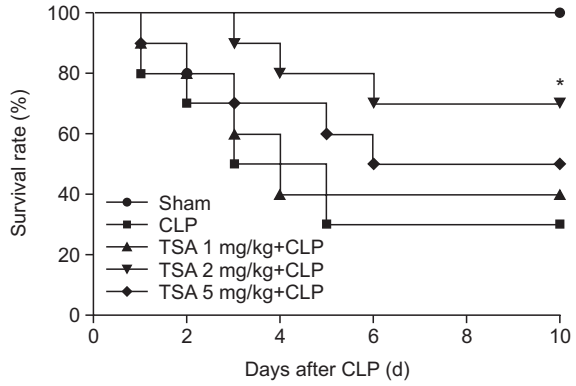

Fig. 2. Effect of TSA on CLP-induced lethality. Mice were intraperitoneally injected with vehicle (normal saline) or TSA (1, 2 or 5 mg/kg) 30 min before CLP (n=10 per group). Animals were monitored for 10 days after CLP. **p*<0.05 versus CLP group.

Results are presented as mean ± standard error of the mean (SEM).

RESULTS

TSA suppresses inflammatory cytokine release in LPS-stimulated RAW264.7 cells

To evaluate the anti-inflammatory properties of TSA, the levels of released cytokines were determined in LPS-stimulated RAW264.7 cells. TSA did not have any cytotoxicity on RAW264.7 cells at concentrations up to 50 nM (Fig. 1A). LPS significantly increased TNF-α and IL-6 release to 54.6- and 26.4-fold, respectively, than those in the control group. These increases were inhibited by TSA in a concentration-dependent manner (Fig. 1B, 1C).

TSA protects mice against sepsis-induced lethality

In the CLP group, the survival rate on the first day was 80% and stabilized at 30% on the fifth day after CLP. Log-rank analysis of 10-day survival demonstrated that 2 mg/kg TSA significantly improved survival rate compared with CLP group (p=0.0498; Fig. 2).

TSA attenuates serum levels and hepatic mRNA expressions of inflammatory cytokines in septic mice

The serum levels of TNF-α and IL-6 in the sham group were 186.0 ± 30.1 and 90.7 ± 3.2 pg/mL, respectively. CLP significantly increased TNF-α and IL-6 levels to 1.5- and 31.5-fold than those in the sham group, respectively, 6-h after CLP. These increases were attenuated by TSA (Fig. 3A, 3B). The hepatic *TNF-α* and *IL-6* mRNA expression exhibited a marked increase to 2.8- and 1.5-fold than those in the sham group, respectively. Consistent with observation of serum cytokine levels, TSA attenuated the increases in *TNF-α* and *IL-6* mRNA expression (Fig. 3C, 3D).

TSA attenuates hepatic injury in septic mice

The serum levels of ALT were 23.3 ± 0.9 and 28.5 ± 1.5 U/L, respectively, 6 and 24-h after sham operation. CLP significantly increased these values to 2.5- and 10.4-fold than those in the sham group, respectively. These increases were attenuated by TSA (Fig. 4A). The serum levels of AST were 43.7 ± 3.7 and 45.3 ± 3.3 U/L, respectively, 6 and 24-h after sham operation. CLP significantly increased these values to 3.3- and 7.4-fold than those in sham group, respectively, which were attenuated by TSA (Fig. 4B). According to histological analysis, there were marked pathological changes including inflammatory cell infiltration and necrosis in the liver that was evaluated at 24-h after CLP. These histological changes were ameliorated by TSA (Fig. 4C).

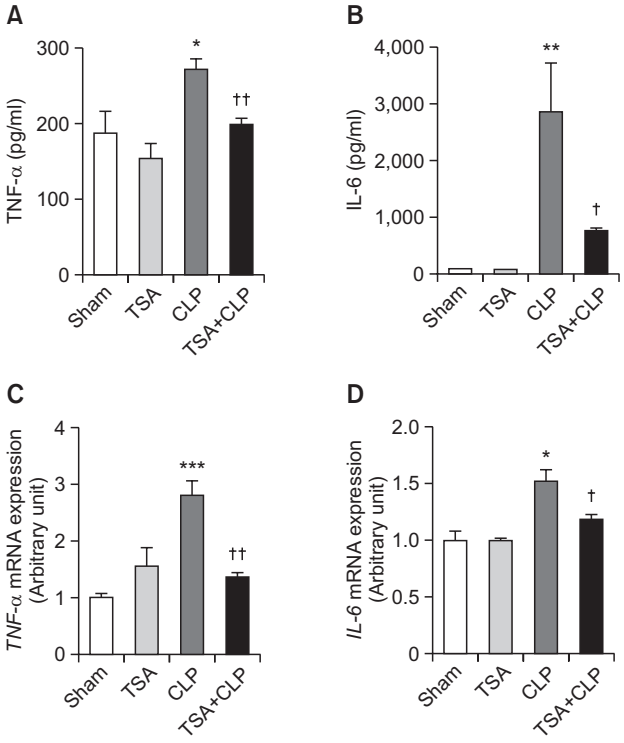

Fig. 3. Effect of TSA on serum levels and hepatic mRNA expressions of TNF-α and *IL-6* in sepsis. Mice were intraperitoneally administered vehicle (normal saline) or 2 mg/kg TSA 30 min before CLP. The blood samples were collected from the inferior vena cava 6-h after CLP and then serum levels of TNF-α (A) and IL-6 (B) were determined. The liver tissues were collected 6-h after CLP and then hepatic mRNA expressions of TNF-α (C) and IL-6 (D) were determined. The results are presented as mean ± SEM (n=6-8 per group). **p*<0.05, ***p*<0.01, ****p*<0.001 versus sham group. †*p*<0.05, ††*p*<0.01 versus CLP group.

TSA decreases TLR4 and TLR2 signaling pathway in septic mice

To determine the effect of TSA on TLR signaling pathway, we first determined the TLR4 and TLR2 protein expression. CLP significantly increased TLR4 and TLR2 protein expression to 1.9- and 1.8-fold, respectively, than those in the sham group 6-h after CLP. These increases were attenuated by TSA (Fig. 5A, 5B). CLP significantly increased MyD88 and TRIF protein expression to 1.7- and 1.5-fold, respectively, than those in the sham group 6-h after CLP. TSA attenuated increase in MyD88 protein expression, not TRIF (Fig. 5C, 5D). CLP significantly increased association of MyD88 with TLR4 and TLR2 to 1.5- and 1.6-fold than those in the sham group, respectively. These increases were attenuated by TSA (Fig.

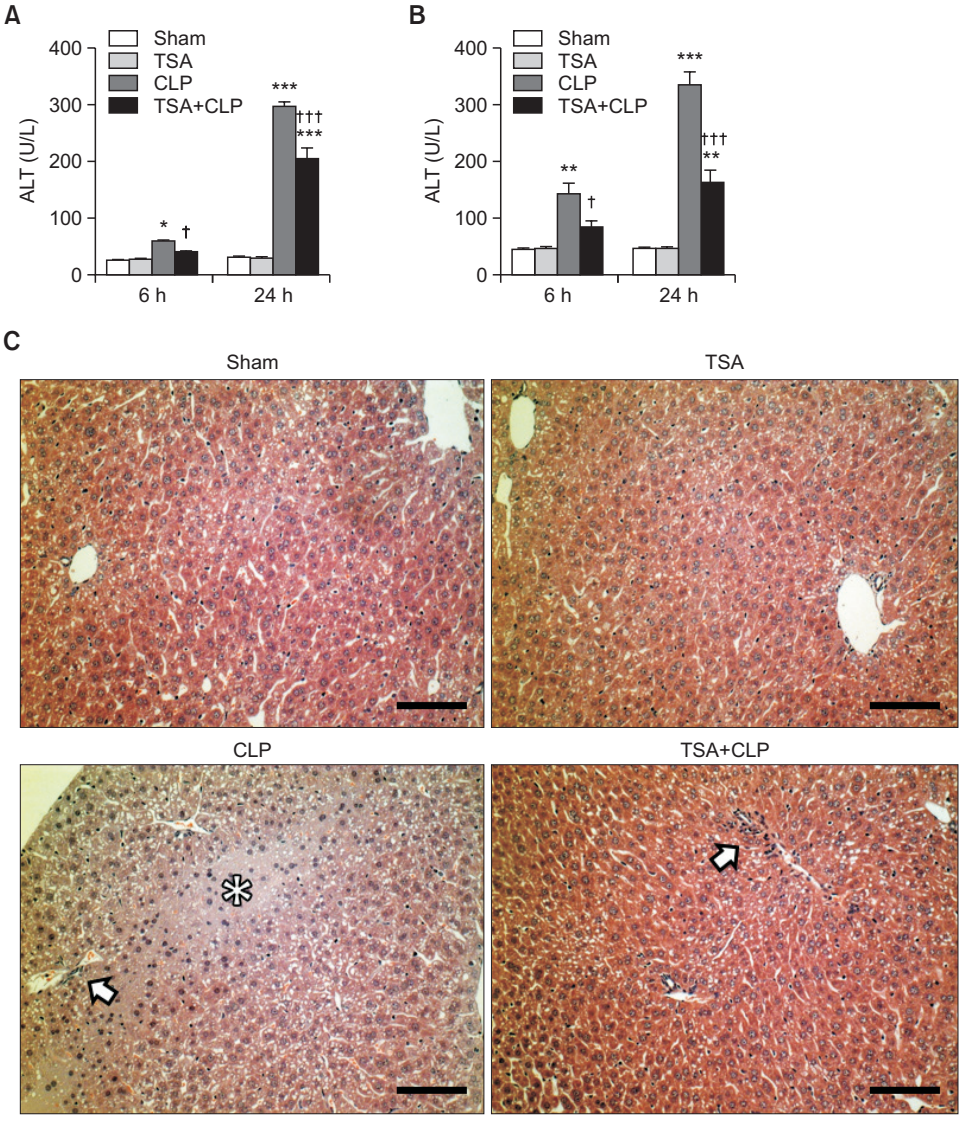

Fig. 4. Effect of TSA on hepatocellular damages in sepsis. Mice were intraperitoneally administered vehicle or 2 mg/kg TSA 30 min before CLP. The blood samples were collected from the inferior vena cava 6 and 24-h after CLP and then serum levels of ALT (A) and AST (B) were determined. Tissues were isolated and stained with H&E 24-h after CLP (magnification ×200). The arrow indicates neutrophilic infiltration and asterisk indicates necrosis. The results are presented as mean ± SEM (n=6-8 per group). *p<0.05, **p<0.01, ***p<0.001 versus sham group. $^{\dagger}p$<0.05, $^{\dagger\dagger\dagger}p$<0.001 versus CLP group.

5E, 5F). CLP significantly increased association of TRIF with TLR4 to 1.8-fold than that in the sham group. TSA did not affect this increase (Fig. 5G).

TSA decreases NF-κB signaling pathway in septic mice

To determine the effect of TSA on NF-κB signaling pathway, we first determined the NF-κB and IκB protein expression. CLP significantly increased nuclear NF-κB protein expression to 5.1-fold than that in the sham group 6-h after CLP. This increase was attenuated by TSA (Fig. 6A). CLP significantly decreased cytosolic IκB protein expression to 70% of that in the sham group, which was attenuated by TSA (Fig. 6B). CLP significantly decreased Ac-IKK protein expression to 38% of that in the sham group, which was attenuated by TSA (Fig. 6C). CLP significantly increased association of IκB with IKK protein expression to 2.0-fold than that in the sham group. This increase was attenuated by TSA (Fig. 6D).

DISCUSSION

Numerous evidences indicate that epigenetic modification is the master regulator for gene expression and plays an important role in inflammatory and host defense response (Roger *et al*., 2011). Acetylation is an essential epigenetic modification that determines the amplitude of immune system by controlling the chromatin structure, transcription factor activity and subsequent gene expression. Accumulating evidences imply that HDAC inhibitors possess anti-inflammatory properties. Inhibition of histone acetylation attenuates syno-

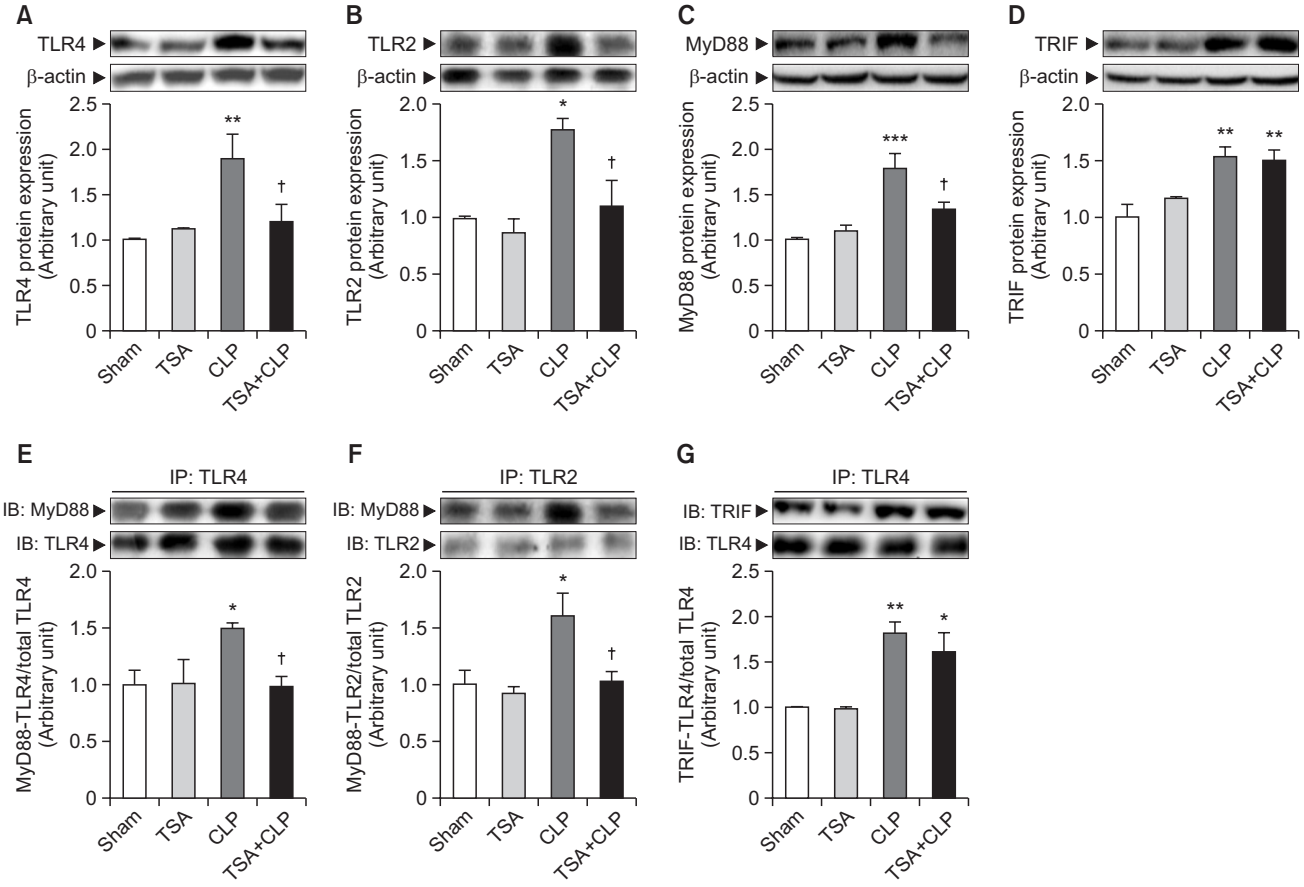

Fig. 5. Effect of TSA on TLR signaling pathway in septic liver. Mice were intraperitoneally administered vehicle or 2 mg/kg TSA 30 min before CLP. The liver tissues were collected 6-h after CLP and then TLR4 (A), TLR2 (B), MyD88 (C) and TRIF (D) protein expression were determined. Liver lysates were immunoprecipitated with anti-TLR4 and anti-TLR2 antibodies and precipitated proteins were immunoblotted using anti-MyD88 (E-F) and anti-TRIF (G) antibodies. The results are presented as mean ± SEM (n=6-8 per group) *p<0.05, **p<0.01, ***p<0.001 versus sham group. $^{\dagger}p$<0.05 versus CLP group.

vial inflammation in collagen-induced arthritis mouse model (Nasu *et al*., 2008) and allergen-induced airway inflammation in mouse asthma model (Choi *et al*., 2005). Moreover, HDAC inhibitors relate to sepsis that is caused by an uncontrolled hyper-inflammation. TSA suppressed myeloperoxidase activity in septic mice (Zhang *et al*., 2010) and attenuated endotoxemia-induced acute lung injury (Zhang *et al*., 2009). Moreover, it is reported that sepsis is characterized by upregulation of HDAC activity, which influences the acetylation status, and that the imbalance can be corrected by HDAC inhibitor (Li and Alam, 2011).

In the present study, administration of TSA improved CLP-induced mortality. A standardized septic animal model is critical to elucidate the pathophysiological process in sepsis. The CLP model has been extensively used in the research for sepsis and believed gold-standard model to closely resemble characteristics of human sepsis (Toscano *et al*., 2011). Severe sepsis causes lethal organ failure, which leads to septic shock and death. Among them, liver failure significantly relates to the high mortality in septic patients (Hebert *et al*., 1993). Liver performs important role through engaging in inflammation response. Interestingly, the liver also acts as a victim that suffers from the inflammatory responses (Nesseler *et al*., 2012). In the present study, CLP significantly increased the serum levels of ALT and AST. Importantly, histology analysis supported hepatic injury during sepsis. TSA not only decreased serum markers of liver damage but also attenuated pathological changes in liver. These results suggest that TSA improves hepatic injury during sepsis.

Excessive and uncontrolled inflammatory response contributes to pathophysiological progress of sepsis. Indeed, previous studies have demonstrated that inflammatory cytokines such as TNF-α and IL-6 peak at early phase of sepsis and are strongly associated with disease progress (Takala *et al*., 2002). In the present study, CLP significantly increased the serum levels of TNF-α and IL-6, which were attenuated by TSA. Moreover, TSA attenuated increases in hepatic *TNF-α* and *IL-6* mRNA expression in septic mice. This result suggests that TSA suppresses cytokine production at the transcriptional level.

TLRs play a critical role in the pathogenesis of sepsis as critical upstream mediators of excessive inflammation. In particular, TLR4 and TLR2 recognize gram-positive bacteria and gram-negative bacteria, respectively, which are main pathogens of sepsis. The levels of TLR4 and TLR2 protein expression increased in liver, lung and spleen in septic model and

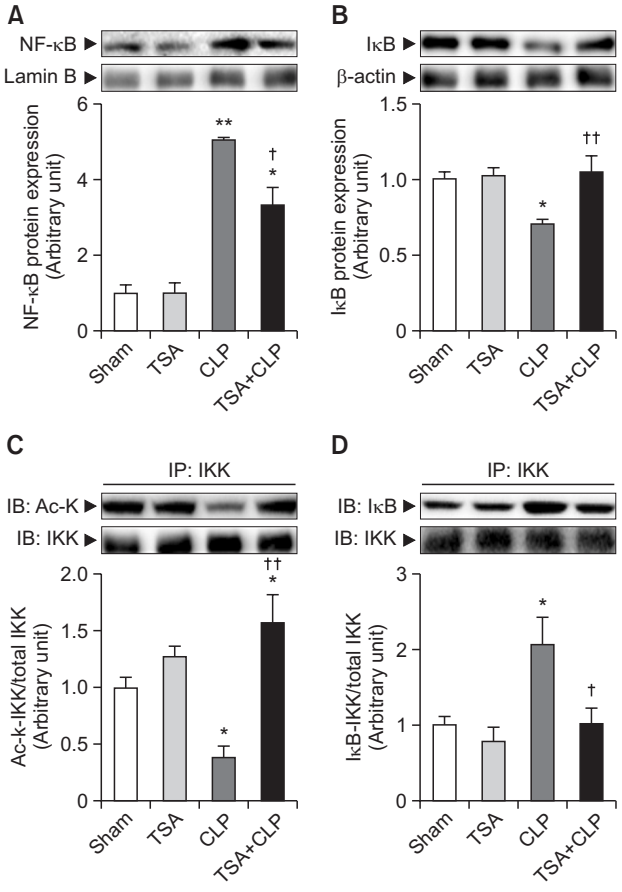

Fig. 6. Effect of TSA on NF-κB signaling pathway in septic liver. Mice were intraperitoneally administered vehicle or 2 mg/kg TSA 30 min before CLP. The liver tissues were collected 6-h after CLP and then nuclear NF-κB (A) and cytosolic IκB (B) protein expression were determined. Liver lysates were immunoprecipitated with anti-IKK antibody and precipitated proteins were immunoblotted using anti-Ac-K (C) and anti-IκB (D) antibodies. The results are presented as mean ± SEM (n=6-8 per group) *p<0.05, **p<0.01 versus sham group. $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01 versus CLP group.

these increases correlate with mortality (Williams *et al*., 2003). Previous studies showed that eritoran, a TLR4 antagonist, attenuated cardiac dysfunction via inhibiting cytokine expression in endotoxemia model (Ehrentraut *et al*., 2011). TLR2-deficient mice exhibited increased survival rates compared to wild type mice during sepsis (Bergt *et al*., 2013). Moreover, TSA inhibited the up-regulation of *TLR2* mRNA expression in LPS-stimulated bone marrow derived macrophage (Roger *et al*., 2011). In the present study, the hepatic TLR4 and TLR2 protein expression significantly increased after CLP and TSA attenuated these increases.

TLRs rapidly recruit adaptor molecules and trigger downstream signaling pathway after activation. Upon stimulation, adaptor molecules such as MyD88 and TRIF are recruited and subsequently lead to the activation of NF-κB signaling. Deficiency of MyD88 suppressed inflammatory cytokines and protected mice in polymicrobial sepsis (Kawai *et al*., 1999). Moreover, TAK-242 inhibited increases in serum cytokine levels by blocking interactions between TLR4 and its adaptor protein in endotoxemia model (Takashima *et al*., 2009). In the present study, CLP significantly increased MyD88 and TRIF protein expression. TSA attenuated increase in MyD88. Moreover, the association of MyD88 with TLR4 and TLR2, and TRIF with TLR4 increased after CLP. TSA attenuated the association of MyD88 with TLR4 and TLR2, not TRIF with TLR4. In previous report, TSA inhibited the up-regulation of *MyD88* mRNA expression in LPS-stimulated bone marrow derived macrophage but did not affect *TRIF* mRNA expression (Roger *et al*., 2011). Our result suggests that TSA suppresses MyD88-dependent TLR signaling pathway.

Activation of TLR leads to a multistep signaling process, which leads to NF-κB signaling activation. NF-κB plays a crucial role in pathophysiological process in sepsis via inducing the transcription of numerous inflammatory-related genes (Baldwin, 1996). Upon activation, IκB is phosphorylated by the IKK and subsequently degraded by the proteasome resulting in NF-κB activation (Beg *et al*., 1993). In septic patients, increased nuclear NF-κB expression is associated with higher rates of mortality and worse clinical outcome (Abraham, 2003). Recently, HDAC inhibitors have been reported to interfere with the activation of NF-κB signaling pathways. TSA inhibited the NF-κB signaling via suppressing proteosomal degradation of IκB (Chakravortty *et al*., 2000). Butyrate, a HDAC inhibitor, inhibited TNF-α-stimulated IκB phosphorylation and degradation in the adenocarcinoma cells (Lührs *et al*., 2001). Moreover, YopJ, a *Yeersinia* outer protein, suppressed kinase activity of IKK by acetylation of the active loop of IKK, resulting in suppression of IκB degradation (Mittal *et al*., 2006). It is interesting to note that in some instances, HDAC inhibitors have been reported to activate NF-κB pathway. Acetylation at lysine 221 of p65 subunit of NF-κB enhanced nuclear import of NF-κB (Chen *et al*., 2002). In our study, CLP increased nuclear translocation of NF-κB and TSA attenuated this increase. TSA attenuated decrease in cytosolic IκB protein expression in septic mice. Moreover, CLP decreased IKK acetylation and increased association of IKK with IκB. These alterations were attenuated by TSA. These results suggest that TSA inhibits translocation of NF-κB to nucleus via enhancing IKK acetylation.

In conclusion, TSA protects against sepsis-induced hepatic injury by inhibiting TLR signaling pathways. Thus, we propose that TSA might be useful as a potential therapeutic intervention for sepsis treatment.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

ACKNOWLEDGMENTS

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST; 2012R1A5A2A28671860). So-Jin Kim (2013034472; NRF-2013H1A2A1034472) received 'Global Ph.D. Fellowship Program' support from the NRF funded by the MEST.

REFERENCES

Abraham, E. (2003) Nuclear factor-κB and its role in sepsis-associated

organ failure. *J. Infect. Dis.* **187 Suppl 2**, S364-S369.
Baldwin, A. S. Jr. (1996) The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol.* **14**, 649-683.
Beg, A. A., Finco, T. S., Nantermet, P. V. and Baldwin, A. S. Jr. (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. *Mol. Cell. Biol.* **13**, 3301-3310.
Bergt, S., Wagner, N. M., Heidrich, M., Butschkau, A., Noldge-Schomburg, G. E., Vollmar, B. and Roesner, J. P. (2013) Hydrocortisone reduces the beneficial effects of toll-like receptor 2 deficiency on survival in a mouse model of polymicrobial sepsis. *Shock* **40**, 414-419.
Bone, R. C. (1991) The pathogenesis of sepsis. *Ann. Intern. Med.* **115**, 457-469.
Chakravortty, D., Koide, N., Kato, Y., Sugiyama, T., Mu, M. M., Yoshida, T. and Yokochi, T. (2000) The inhibitory action of butyrate on lipopolysaccharide-induced nitric oxide production in RAW 264.7 murine macrophage cells. *J. Endotoxin Res.* **6**, 243-247.
Chaudry, I. H., Wichterman, K. A. and Baue, A. E. (1979) Effect of sepsis on tissue adenine nucleotide levels. *Surgery* **85**, 205-211.
Chen, L. F., Mu, Y. and Greene, W. C. (2002) Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-κB. *EMBO J.* **21**, 6539-6548.
Chen, Y. R., Wang, X., Templeton, D., Davis, R. J. and Tan, T. H. (1996) The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and γ radiation. *J. Biol. Chem.* **271**, 31929-31936.
Choi, J. H., Oh, S. W., Kang, M. S., Kwon, H. J., Oh, G. T. and Kim, D. Y. (2005) Trichostatin A attenuates airway inflammation in mouse asthma model. *Clin. Exp. Allergy* **35**, 89-96.
Ehrentraut, S., Lohner, R., Schwederski, M., Ehrentraut, H., Boehm, O., Noga, S., Langhoff, P., Baumgarten, G., Meyer, R. and Knuefermann, P. (2011) *In vivo* Toll-like receptor 4 antagonism restores cardiac function during endotoxemia. *Shock* **36**, 613-620.
Finkelstein, R. A., Li, Y., Liu, B., Shuja, F., Fukudome, E., Velmahos, G. C., deMoya M. and Alam, H. B. (2010) Treatment with histone deacetylase inhibitor attenuates MAP kinase mediated liver injury in a lethal model of septic shock. *J. Surg. Res.* **163**, 146-154.
Hebert, P. C., Drummond, A. J., Singer, J., Bernard, G. R. and Russell, J. A. (1993) A simple multiple system organ failure scoring system predicts mortality of patients who have sepsis syndrome. *Chest* **104**, 230-235.
Johnstone, R. W. (2002) Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat. Rev. Drug Discov.* **1**, 287-299.
Kawai, T., Adachi, O., Ogawa, T., Takeda, K. and Akira, S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* **11**, 115-122.
Kim, M. S., Kwon, H. J., Lee, Y. M., Baek, J. H., Jang, J. E., Lee, S. W., Moon, E. J., Kim, H. S., Lee, S. K., Chung, H. Y., Kim, C. W. and Kim, K. W. (2001) Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat. Med.* **7**, 437-443.
Lagu, T., Rothberg, M. B., Shieh, M. S., Pekow, P. S., Steingrub, J. S. and Lindenauer, P. K. (2012) Hospitalizations, costs, and outcomes of severe sepsis in the United States 2003 to 2007. *Crit. Care Med.* **40**, 754-761.
Li, Y. and Alam, H. B. (2011) Modulation of acetylation: creating a pro-survival and anti-inflammatory phenotype in lethal hemorrhagic and septic shock. *J. Biomed. Biotechnol.* **2011**, 523481.
Lührs, H., Gerke, T., Schauber, J., Dusel, G., Melcher, R., Scheppach, W. and Menzel, T. (2001) Cytokine-activated degradation of inhibitory κB protein α is inhibited by the short-chain fatty acid butyrate. *Int. J. Colorectal Dis.* **16**, 195-201.
Mittal, R., Peak-Chew, S. Y. and McMahon, H. T. (2006) Acetylation of MEK2 and IκB kinase (IKK) activation loop residues by YopJ inhibits signaling. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 18574-18579.
Nasu, Y., Nishida, K., Miyazawa, S., Komiyama, T., Kadota, Y., Abe, N., Yoshida, A., Hirohata, S., Ohtsuka, A. and Ozaki, T. (2008) Trichostatin A, a histone deacetylase inhibitor, suppresses synovial inflammation and subsequent cartilage destruction in a collagen antibody-induced arthritis mouse model. *Osteoarthr. Cartil.* **16**, 723-732.
Nesseler, N., Launey, Y., Aninat, C., Morel, F., Mallédant, Y. and Seguin, P. (2012) Clinical review: The liver in sepsis. *Crit. Care* **16**, 235.
Pan, L. N., Lu, J. and Huang, B. (2007) HDAC inhibitors: a potential new category of anti-tumor agents. *Cell. Mol. Immunol.* **4**, 337-343.
Riedemann, N. C., Guo, R. F. and Ward, P. A. (2003) Novel strategies for the treatment of sepsis. *Nat. Med.* **9**, 517-524.
Roger, T., Lugrin, J., Le Roy, D., Goy, G., Mombelli, M., Koessler, T., Ding, X. C., Chanson, A. L., Reymond, M. K., Miconnet, I., Schrenzel, J., François, P. and Calandra, T. (2011) Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection. *Blood* **117**, 1205-1217.
Sha, T., Iizawa, Y. and Ii, M. (2011) Combination of imipenem and TAK-242, a Toll-like receptor 4 signal transduction inhibitor, improves survival in a murine model of polymicrobial sepsis. *Shock* **35**, 205-209.
Singhal, S., Alulen, M. W., McAnnally, J. R., Smith, K. S., Donnelly, J. P. and Wang, H. E. (2013) National estimates of emergency department visits for pediatric severe sepsis in the United States. *PeerJ* **1**, e79.
Takala, A., Nupponen, I., Kylänpää-Bäck, M. L. and Repo, H. (2002) Markers of inflammation in sepsis. *Ann. Med.* **34**, 614-623.
Takashima, K., Matsunaga, N., Yoshimatsu, M., Hazeki, K., Kaisho, T., Uekata, M., Hazeki, O., Akira, S., Iizawa, Y. and Ii M. (2009) Analysis of binding site for the novel small-molecule TLR4 signal transduction inhibitor TAK-242 and its therapeutic effect on mouse sepsis model. *Br. J. Pharmacol.* **157**, 1250-1262.
Tong, X., Yin, L. and Giardina, C. (2004) Butyrate suppresses Cox-2 activation in colon cancer cells through HDAC inhibition. *Biochem. Biophys. Res. Commun.* **317**, 463-471.
Toscano, M. G., Ganea, D. and Gamero, A. M. (2011) Cecal ligation puncture procedure. *J. Vis. Exp.* 2860.
Williams, D. L., Ha, T., Li, C., Kalbfleisch, J. H., Schweitzer, J., Vogt, W. and Browder, I. W. (2003) Modulation of tissue Toll-like receptor 2 and 4 during the early phases of polymicrobial sepsis correlates with mortality. *Crit. Care Med.* **31**, 1808-1818.
Wu, J. and Grunstein, M. (2000) 25 years after the nucleosome model: chromatin modifications. *Trends Biochem. Sci.* **25**, 619-623.
Zhang, L., Jin, S., Wang, C., Jiang, R. and Wan, J. (2010) Histone deacetylase inhibitors attenuate acute lung injury during cecal ligation and puncture-induced polymicrobial sepsis. *World J. Surg.* **34**, 1676-1683.
Zhang, L., Wan, J., Jiang, R., Wang, W., Deng, H., Shen, Y., Zheng, W. and Wang, Y. (2009) Protective effects of trichostatin A on liver injury in septic mice. *Hepatol. Res.* **39**, 931-938.
Zhao, T., Li, Y., Liu, B., Liu, Z., Chong, W., Duan, X., Deperalta, D. K., Velmahos, G. C. and Alam, H. B. (2013) Novel pharmacologic treatment attenuates septic shock and improves long-term survival. *Surgery* **154**, 206-213.