Article

Gene Delivery of SOCS3 Protects Mice from Lethal Endotoxic Shock

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Suppressor of cytokine signaling 3 (SOCS3) was reported as a feedback inhibitor of cytokine receptor signaling by inhibiting the JAK-STAT signal transduction pathway. We sought to test the anti-endotoxic septic shock effect of liposome mediated gene delivery of SOCS3 in a lethal endotoxic shock mouse model. BALB/c mice were injected intraperitoneally with 200 μg pcDNA3.1-SOCS3 cationic liposomes, while pcDNA3.1-IL-10 and empty vector as positive and negative control respectively. Forty-eight hours after gene delivery, mice were challenged with 4 μg of *E.coli* 0127:B8 LPS and 18 mg D-GalN administered *i.p.* 90 min later, serum TNF-α level was determined. Survival over the next 48 h was evaluated. Peritoneal macrophages from survival mice were stimulated *in vitro* with 1 μg/ml LPS for 18 h, and the supernatants were harvested for determination of the amount of TNF-α. We found that gene delivery of SOCS3 significantly increase the mouse survival rate from 27.8 ± 9.6% of control group to 61.1 ± 9.6% ($p < 0.01$). In comparison with control group (218 ± 13 pg/ml) and sham delivery group (219 ± 22 pg/ml), gene delivery of SOCS3 reduced the level of serum TNF-α (68 ± 9 pg/ml) significantly ($p < 0.01$). Furthermore, gene delivery of SOCS3 displayed the capacity of prevention of tolerance of peritoneal macrophages to LPS. These findings suggest that gene delivery of SOCS3 mediated by liposome is a promising approach for endotoxic septic shock treatment. *Cellular & Molecular Immunology*. 2005;2(5):373-377.

Key Words: gene delivery, SOCS3, endotoxic septic shock

Introduction

Endotoxic shock is a life-threatening disease caused by exposure to bacterial LPS. LPS triggers the release of pro-inflammatory cytokines that facilitate the development of endotoxic shock (1). Suppressor of cytokine signaling (SOCS) is a group of cytokine-induced proteins, functioning as a feedback inhibitor to control cytokine receptor signaling (2). The mammalian SOCS family contains eight members, including CIS (cytokine-induced SH2 containing protein) and SOCS1-SOCS7. All SOCS family members share a central SH2 domain, a conserved C-terminal motif called the SOCS box (3). It has been demonstrated that SOCS molecules negatively regulate cytokine receptor signaling by several mechanisms: 1) Direct inhibition of all four JAK kinases by SOCS1; 2) Indirect inhibition of JAKs due to the binding of SOCS to membrane proximal regions of receptor chains, resulting in steric hindrance of constitutive JAK binding to the receptor chains; 3) Competitive inhibition of STAT from binding to receptor chains by SOCS proteins; 4) Inhibition of downstream signaling pathways (2, 3). SOCS1 and SOCS3 are implicated in regulating innate immune responses and in attenuating signals delivered *via* pro-inflammatory cytokine receptors. Due to the innate immune system provides the first line of defense against microbial invasion, regulation of the innate immune response is critical, as excessive inflammatory reaction can be fatal. Upon recognition of pathogen-associated molecular patterns (PAMP) *via* toll-like receptors (TLR), innate immune cells such as monocytes and macrophages secrete massive amount of pro-inflammatory mediators, such as TNF-α, IL-6 and IL-1, resulting in septic shock. Bacterial LPS and CpG oligonucleotides, which signal *via* TLR4 and TLR9 respectively, strongly induce SOCS1 and

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Abbreviations: SOCS, suppressor of cytokine signaling; JAK-STAT, Janus kinase-signal transducers and activators of transcription; CIS, cytokine-induced SH2 containing protein; PAMP, pathogen-associated molecular patterns; TLR, toll-like receptors; D-GalN, D-Galactosamine; GAPDH, glyceraldehydes phosphate dehydrogenase.

SOCS3, and attenuate the ability of macrophages responding to subsequent stimulation by IFN-γ. It has been documented that SOCS3 can be induced by LPS, IL-6 and IL-10, and consequently SOCS3 plays a role in the negative regulation of LPS signaling (4, 5). Little is known, however, about the anti-sepsis effect of SOCS3 in murine septic shock model. In this study, using liposome mediated gene delivery, we tested whether gene delivery of SOCS3 can improve the survival in lethal endotoximia in the mouse. We found that gene delivery of SOCS3 was able to improved mouse survival with lethal endotoximia through modulation of TNF-α production and ameliorating peritoneal macrophages immune function.

Materials and Methods

Animal

Healthy female BALB/c mice (6-8 week-age) were obtained from animal facility at Tongji Medical College. Experiments were performed in accordance with the institute guidelines on animal use.

Plasmids

The pcDNA3.1-IL-10 expression plasmid containing CMV promoter linked to the open reading frame for the murine IL-10 cDNA was purchased from American Type Culture Collection (Rockville, MD). Plasmid pcDNA3.1 containing SOCS3 cDNA was kindly provided by Dr. Hilton DJ (the Walter and Eliza Hall Institute of Medical research, Australia). Plasmid pcDNA3.1 was used as control.

Plasmid preparations

The plasmids were transformed into competent DH5α *E.coli* cells and cultured to late log phase in LB medium containing ampicillin (50 μg/ml) as a selective marker (9). Plasmid DNA was isolated with an EndoFree plasmid isolation kit according to the protocol supplied by the manufacturer (Quiagen). LPS contamination in the plasmid preparations was less than 2 pg/μg DNA (endotoxin-specific chromogenic limulus test; Seikagaku Corporation, Tokyo, Japan).

In vivo gene delivery with liposome

BALB/c mice were injected intraperitoneally with 200 μg pcDNA3.1-IL-10, 200 μg pcDNA3.1-SOCS3 or pcDNA3.1 plasmid complexed with 100 nmol DDAB:DOPE liposomes (kindly provided by Dr. Rogy MA at University Vienna School of Medicine, Austria) as previously described (6).

Experimental endotoxic shock

At 48 h after the gene delivery of mIL-10, SOCS3, or pcDNA3.1, the animals were injected *i.p.* with 4 μg of *E.coli* 0127:B8 LPS and 18 mg D-GalN (Sigma Diagnostics, St. Louis, MO). After 90 min, mice were bled from the tail, and serum TNF-α level was determined. Survival over 48 h was evaluated. Experimental groups were designed as follows: 1) murine IL-10 gene delivery group as positive control; 2) murine SOCS3 gene delivery group; 3) murine IL-10 and SOCS3 genes co-delivery group; 4) pcDNA3.1 delivery group as negative control. Each group contains 6 mice, and all experiments were repeated 3 times.

In vitro stimulation of peritoneal macrophages with LPS

Forty-eight hours after the LPS/D-GalN challenge, the surviving mice were sacrificed and 15 ml ice-cold PBS and 2,000 IU heprin were administered *i.p.* The belly was massaged and 10 ml of fluid was recovered by aspiration. The macrophages were washed twice with RPMI-1640 (Gibco Laboratories) complete medium (containing 10% FCS, 2 mM L-glutamine and 100 U/ml penicillin and 100 U/ml streptomycin) and plated at a final concentration of 2×10^6 cells/ml in complete medium, into 6-well cell culture plate at 37°C in 5% CO_2. After 90 min, nonadherent cells were removed by gently washing the plates twice with complete medium. The adherent macrophages were then incubated with 1 μg/ml LPS. After 18 h, the supernatants were harvested and stored at -70°C for future cytokine assays.

Detection of the expression of gene delivery of SOCS3 by RT-PCR

At various time points mice were sacrificed by cervical dislocation and the organs were rapidly removed and frozen immediately in liquid nitrogen. Total cellular RNA was extracted from frozen organs by homogenization with Trizol reagent (Invitrogen), and cDNA was synthesized using Superscript II reverse transcriptase enzyme (Invitrogen) per instructions provided by the manufacturer. The PCR reaction was performed as follows: one cycle at 94°C for 5 min followed by 30 cycles at 94°C for 1 min, 60°C for 30 s and 72°C for 1 min; and finally one cycle at 72°C for 10 min. The PCR products were run on 1% agarose gel with 1 μg/ml ethidium bromide and visualized by UV. The primer sequences for amplification of a 420-bp SOCS3 fragment were 5'-CAC AGC AAG TTT CCC GCC GCC-3' and 5'-GTG CAC CAG CTT GAG AAG GCC GG-3'. The primers for GAPDH were: 5'-ACC ACC ATG GAG AAG GCC GG-3' and 5'-CTC AGT GTA GCC CAA GAT GC-3', which amplify a 500-bp fragment.

Determination of TNF-α with ELISA

Serum TNF-α and *in vitro* TNF-α production by peritoneal macrophages were determined using a mouse TNF-α ELISA kit (Genzyme, Cambridge, MA). The sensitivity of the assay is 2 pg/ml.

Statistical analysis

Data were presented as mean ± SEM. Group differences were analyzed with one-way ANOVA, and $p < 0.05$ was considered as significant difference.

Results

Expression of SOCS3 by liposome mediated gene delivery

To characterize the expression of gene delivery of SOCS3, RT-PCR was employed to detect the RNA of SOCS3 in

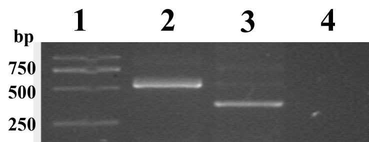

Figure 1. Detection of expression of gene delivery of SOCS3 by RT-PCR. Total RNA was isolated from spleens of mice transfected with SOCS3 and subjected to RT-PCR. The RT-PCR products were analyzed on 1% agarose gel electrophoresis. Lane 1, DNA molecular marker; Lane 2, house-keeping gene GAPDH; Lane 3, SOCS3; Lane 4, negative control for SOCS3 amplification from spleens of untreated mice.

different organs (e.g., liver, spleen) of animals treated with SOCS3. The SOCS3 mRNA was detected in various organs. As shown in Figure 1, for example, SOCS3 was expressed in the spleen of mice treated with SOCS3.

Gene delivery of SOCS3 improved the survival of mice with septic shock

As shown in Figure 2, treated with SOCS3 gene delivery, survival of mice challenged with LPS improved from 27.8 ± 9.6% of control group to 61.1 ± 9.6% ($p < 0.01$). Similarly, after gene delivery of IL-10, survival rate of mice increased to 72.2 ± 9.5% (in comparison with control, $p < 0.01$). In contrast, there is no significant survival difference between mock-delivery group and control group ($p > 0.05$).

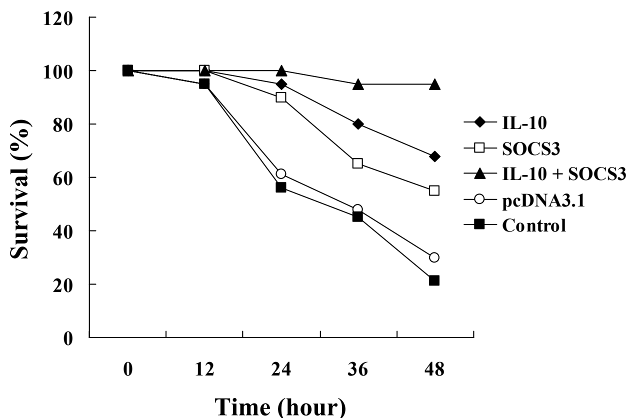

Figure 2. Forty-eight-hour survival of mice challenged with a lethal septic shock. Forty-eight hours prior to induction of septic shock, the mice were transfected *i.p.* with 200 μg pcDNA3.1-IL-10 plasmid, 200 μg pcDNA3.1-SOCS3 plasmid, 100 μg pcDNA3.1-IL-10 plus 100 μg pcDNA3.1-SOCS3 plasmid, 200 μg pcDNA3.1 plasmid complexed with 100 nmol cationic liposomes, respectively. The results represent the average survival rate of five different experiments with six mice per group in each experiment. Mice pretreated with IL-10, SOCS3 or both showed a significant improvement in survival (IL-10 group *vs* pcDNA3.1 group, $p < 0.01$; SOCS3 group *vs* pcDNA3.1 group, $p < 0.05$; IL-10 + SOCS3 group *vs* pcDNA3.1 group, $p < 0.01$).

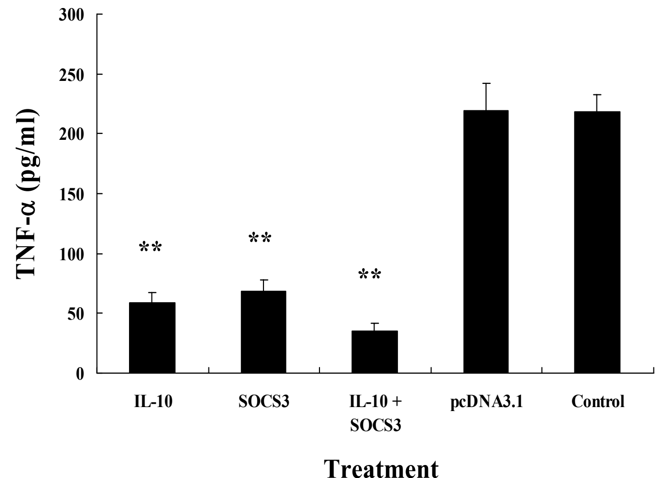

Figure 3. Serum TNF-α levels in mice challenged with LPS. Forty-eight hours prior to induction of septic shock, the mice were transfected *i.p.* with 200 μg pcDNA3.1-IL-10 plasmid, 200 μg pcDNA3.1-SOCS3 plasmid, 100 μg pcDNA3.1-IL-10 plus 100 μg pcDNA3.1-SOCS3 plasmid, or 200 μg pcDNA3.1 plasmid complexed with 100 nmol cationic liposomes. Thereafter, mice were challenged with lethal dose of LPS/D-GalN, and serum were sampled 90 min after LPS administration. TNF-α levels were determined by ELISA. Mice pretreated with SOCS3, IL-10 or SOCS3 plus IL-10 displayed a down-regulation of TNF-α production compared to mock-delivery group (IL-10 group *vs* pcDNA3.1 group, $^{**}p < 0.01$; SOCS3 group *vs* pcDNA3.1 group, $^{**}p < 0.01$; SOCS3 plus IL-10 group *vs* pcDNA3.1 group, $^{**}p < 0.01$).

Gene delivery with combined SOCS3 and IL-10 exhibited synergistic effect

It has been shown that the anti-inflammatory effect of IL-10 may be dependent on SOCS3. Therefore, we investigated whether there was a synergistic effect between SOCS3 and IL-10 in terms of survival. As shown in Figure 2, application of combined IL-10 and SOCS3 genes improved mouse survival rate to 94.4 ± 9.7%, which was significant higher than that of single gene delivery of SOCS3 or IL-10 ($p < 0.05$).

Gene delivery of SOCS3 significantly decreased serum TNF-α level

Because pro-inflammatory cytokine TNF-α plays a critical role in endotoxic septic shock. Next, we examined the down-regulating effect of gene delivery of SOCS3 and IL-10 on the serum TNF-α production induced by LPS. The TNF-α level in the plasma was determined 90 min after LPS-D-GalN administration and was found to be greatly reduced in both SOCS3 and IL-10 treatment groups ($p < 0.01$). As shown in Figure 3, TNF-α level in control group was 218 ± 13 pg/ml, pcDNA3.1 mock-delivery group 219 ± 22 pg/ml, SOCS3 group 68 ± 9 pg/ml, and IL-10 group 59 ± 8 pg/ml. Additionally, delivery of combined IL-10 and SOCS3 genes displayed more potent down-regulating effect on the serum

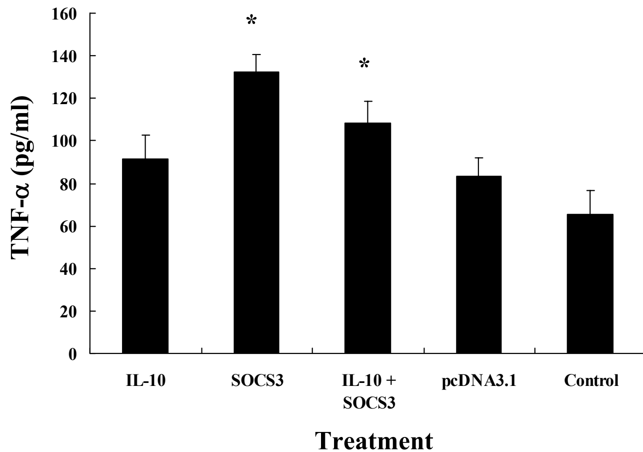

Figure 4. TNF-α production in the supernatant of peritoneal macrophages. Forty-eight hours prior to induction of septic shock, the mice were transfected *i.p.* with 200 μg pcDNA3.1-IL-10 plasmid, 200 μg pcDNA3.1-SOCS3 plasmid, 100 μg pcDNA3.1-IL-10 plus 100 μg pcDNA3.1-SOCS3 plasmid, or 200 μg pcDNA3.1 plasmid complexed with 100 nmol cationic liposomes. Forty-eight hours after LPS challenge, peritoneal macrophages were isolated from survivors. Macrophages (2×10^6 cells/ml in complete medium) were plated into 6-well plate at 37°C in 5% CO_2. After 90 min, nonadherent cells were removed by gently washing the plates twice with complete medium. The adherent macrophages were then incubated with 1 μg/ml LPS. After 18 h, the supernatants were harvested, and TNF-α productions were determined by ELISA. Surviving mice pretreated with SOCS3 responded more potently than control group (IL-10 group *vs* control group, $p > 0.05$; SOCS3 group *vs* pcDNA3.1 group, $^*p < 0.05$; SOCS3 group *vs* control group, $^*p < 0.05$; IL-10 + SOCS3 group *vs* control group, $p > 0.05$).

TNF-α production, compared with SOCS3 or IL-10 single gene delivery ($p < 0.05$).

Gene delivery of SOCS3 prevented the tolerance of peritoneal macrophages to LPS

As shown in Figure 4, the TNF-α production by peritoneal macrophages *in vitro* stimulated with LPS showed that, macrophages obtained from *in vivo* SOCS3 transfected animals produced higher level of TNF-α (132 ± 8 pg/ml) than those of control group (65 ± 7 pg/ml) and IL-10 treatment group (71 ± 6 pg/ml) ($p < 0.01$).

Discussion

In this study we demonstrated that gene delivery of SOCS3 resulted in reduced serum TNF-α production, and significantly improved the survival of mice with endotoxic shock. It was conceivable that there were two molecular mechanisms by which gene delivery of SOCS3 down-regulated TNF-α production and improved the survival in sepsis. One was that enhanced expression of SOCS3 negatively modulated the LPS signaling pathway *via* TLR4, the other was forced expression of SOCS3 attenuated autocrine signaling by pro-inflammatory cytokines liberated from activated macrophages by LPS. Berlato C et al. have demonstrated that murine macrophage cell line J774 transfected with SOCS3 can inhibit NO, TNF-α, IL-6 and GM-CSF production induced by LPS. Constitutive expression of SOCS3 led to down-regulation of NO synthesis enzyme and IL-6 mRNA expression (4). Additionally, similar to IL-10, SOCS3 transfected cells exhibited a feature of constitutive expression of IL-1R antagonist. Investigations into the role of SOCS3 on inflammatory responses using SOCS3 deficient macrophages, either derived from mice in which SOCS3 gene has been conditionally ablated in macrophages or generated from $SOCS3^{-/-}$ fetal liver cells, revealed remarkable specificity for SOCS3 in regulating STAT3 activation by IL-6 (3, 10). Although it can be speculated that modulation of STAT signaling by gene delivery of SOCS3 may play a major role for its anti-septic shock, we are aware that the limitation of this study is lack of direct experimental evidence to clarify whether or not gene delivery of SOCS3 mediated by liposome interfers STAT signaling pathways.

Rogy MA group's studies have shown that gene transfer of anti-inflammatory Th2 cytokines IL-4, IL-13 and IL-10 significantly improved the survival of septic shock in mice, and reduced serum TNF-α levels (6, 7). Herein, similar anti-septic shock effects have been achieved by gene delivery of SOCS3. Furthermore, in comparison with gene transfer of Th2 cytokines, we propose that there are several advantages by SOCS3 treatment: 1) SOCS3 directly inhibits cytokine signaling, thus exhibits more potent anti-inflammatory effects; 2) SOCS3 may prevent, at least in part, the activation of monocytes/macrophages by LPS through down-regulating TLR4 signaling; 3) SOCS3 plays double anti-inflammatory roles in both acute phase and recovery phase. In the acute phase of septic shock, SOCS3 inhibited the signaling pathway of massive produced inflammatory cytokines such as TNF-α, IL-1 and IL-6. After acute phase of septic shock, immune system was in a suppressive condition, displaying tolerance of monocytes/macrophages to LPS and increased anti-inflammatory cytokines production, including IL-10. At this phase, SOCS3 plays a role to ameliorate immune suppressive status. This idea is supported by report that SOCS3 can specifically down-regulate STAT3 induced by IL-6 and IL-10, wherein IL-6 and IL-10 play opposite pro-inflammatory and anti-inflammatory effects, respectively (2, 3).

Endotoxin tolerance has been characterized as diminished TNF-α expression after a second LPS stimulus (11). Abolishing the LPS tolerance is considered to be beneficial for the recovery of patients (12, 13). Our *in vitro* study showed that peritoneal macrophages from survival of mice transfected with SOCS3 exhibited increased TNF-α production compared with control groups after re-stimulation with LPS, suggesting that gene delivery of SOCS3 can break the LPS tolerance. Regarding the biological significance of breaking the LPS tolerance by gene delivery of SOCS3, it was observed that many pro-inflammatory genes responding

to bacterial LPS are persistently repressed during septicemia; this phenomenon of LPS tolerance is associated with immunosuppression and poor prognosis (12). Therefore, for patients with septic shock, the more rapidly the macrophages are able to respond, e.g., with the production of pro-inflammatory mediators, the sooner a recovery can be expected (13).

Consistent with our results presented here, forced expression of dominant negative (DN) STAT3 or wild type SOCS3, through periarticular adenovirus-mediated gene delivery, significantly reduced the arthritogenic process not only at the initiation but also during the progression of the disease (14). More recently, a recombinant cell-penetrating form of SOCS3 (CP-SOCS3) has been developed for intracellular delivery to counteract SEB-, LPS- and Con A-induced inflammation, and found that CP-SOCS3 protected animals from lethal effects of SEB and LPS by reducing production of inflammatory cytokines and attenuating liver apoptosis and hemorrhagic necrosis (15).

In conclusion, our study has clearly shown that gene delivery of SOCS3 was a promising approach for endotoxic septic shock treatment.

Acknowledgements

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References

1. Shirota H, Gursel I, Gursel M, Klinman DM. Suppressive oligodeoxynucleotides protect mice from lethal endotoxic shock. J Immunol. 2005;174:4579-4583.
2. Alexander WS. Suppressors of cytokine signaling (SOCS) in the immune system. Nat Rev Immunol. 2002;2:1-7.
3. Ilangumaran S, Ramanathan S, Rottapel R. Regulation of the immune system by SOCS family adaptor proteins. Semin Immunol. 2004;16:351-365.
4. Berlato C, Cassatella MA, Kinjyo I, et al. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. J Immunol. 2002;168:6404-6411.
5. Niemand C, Nimmesgern A, Haan S, et al. Activation of STAT3 by IL-6 and IL-10 in primary human macrophages is differentially modulated by suppressor of cytokine signaling 3. J Immunol. 2003;170:3263-3272.
6. Baumhofer JM, Beinhauer BG, Wang JE, et al. Gene transfer with IL-4 and IL-13 improves survival in lethal endotoxemia in the mouse and ameliorates peritoneal macrophages immune competence. Eur J Immunol. 1998;28:610-615.
7. Rogy MA, Auffenberg T, Espat NJ, et al. Human tumor necrosis factor receptor (p55) and interleukin-10 gene transfer in the mouse reduces mortality to lethal endotoxemia and also attenuates local inflammatory responses. J Exp Med. 1995;181: 2289-2293.
8. Sturlan S, Schneeberger A, Fang M, et al. Intraperitoneal administration of pMP6/liposome complexes inhibits the growth of co-localized colon-26 adenocarcinoma cells by inducing a tumor-specific immune response. Anticancer Res. 2003;23: 4843-4851.
9. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual (2nd ed), New York, NY: Cold Spring Harbor Laboratory Press, 1989.
10. Croker BA, Danielle L, Krebs DL, et al. SOCS3 negatively regulates IL-6 signaling *in vivo*. Nat Immunol. 2003;4:540-545.
11. Nimah M, Zhao B, Denenberg AG, et al. Contribution of MKP-1 regulation of p38 to endotoxin tolerance. Shock. 2005;23:80-87.
12. Chan C, Li L, McCall CE, Yoza BK. Endotoxin tolerance disrupts chromatin remodeling and NF-κB transactivation at the IL-1β promoter. J Immunol. 2005;175:461-468.
13. Rogy MA, Oldenburg HS, Coyle S, Trousdale R, Moldawer LL, Lowry SF. Correlation between APACHE III score and immunological parameters in critically ill septic patients with sepsis. Br J Surg. 1996;83:396-400.
14. Shouda T, Yoshida T, Hanada T, et al. Induction of the cytokine signal regulator SOCS3/CIS3 as a therapeutic strategy for treating inflammatory arthritis. J Clin Invest. 2001;108:1781-1788.
15. Jo D, Liu D, Yao S, Collins RD, Hawiger J. Intracellular protein therapy with SOCS3 inhibits inflammation and apoptosis. Nat Med. 2005;11:892-898.