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Research Paper

IL-1RⅠ/MyD88-TIR mimic AS-1 inhibits the activation of MyD88-dependent signaling pathway induced by IL-1β in vitro

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Abstract

Objective: To test whether IL-1 RI/My088-TIR mimic AS-1 can work as a new compound that targeted at blocking MyD88-dependent signaling pathway, we investigated the physical structure and biological function of AS-1. **Methods:**The crystallographic structure of AS-1 was examined by ^{1}H nuclear magnetic resonance. The toxicity of AS-1 was measured with Methyl thiazolyl tetrazolium (MTT) assay. The effect of AS-1 on phosphorylation state of p38 MAPK and IRAK-1 was observed with Western blot. **Results:**The crystallographic details of AS-1 demonstrated that it was a tri-peptide sequence[(F/Y)-(V/L/I)-(P/G)] of the IL-1RⅠ-TIR domain BB-loop. No toxicity of AS-1 was shown to HEK 293A cells. The phosphorylation of p38 MAPK, induced by IL-1β significantly increased from those in the control group. AS-1 significantly reduced the phosphorylation of p38 MAPK induced by IL-1β. IL-1β increased the phosphorylation of IRAK-1 significantly, which was prevented by AS-1. **Conclusion:**AS-1 is a competitive mimic between IL-1RⅠ-TIR and MyD88-TIR domain, which most likely interferes with MyD88-dependent signaling pathway.

Key words: hydrocinnamoyl-L-valyl pyrrolidine(AS-1); MyD88; IRAK-1; mitogen-activated protein kinases; signaling pathway

INTRODUCTION

Interleukin-1 receptor(IL-1R) is one of the inflammatory molecules, which plays a key role in the activation of both innate and acquired immune response[1]. Bound with the ligands at its extracellular part, the receptor activates the signal transduction pathway through its intracellular part. The intracellular structure of IL-1R owns a conservative sequence as Toll-Interleukin-1 receptor domain(TIR) for the homology with the cytoplasmic portion of the Toll like receptors(TLRs)[2]. The TIR domain situates at the sequence of 625-784 amino acids and is the core element of the downstream signaling of IL-1R[3-4]. Serving as a scaffold for a series of protein-protein interactions, it leads to the activation of an intracellular signaling. When the extracellular part of IL-1R binding with ligands(IL-1α、IL-1β or IL-1Ra), TIR collects MyD88 to IL-1R and forms the TIRMyD88 activity complex. This complex triggers down-stream signaling pathway, activates mitogen-activated protein kinase(MAPK) and transcription factor nuclear factor-κB (NF-κB); which results in the expression of its target genes, including those in the immune response, inflammatory reaction, cell adhesion molecule secretion and cell growth and apoptosis, etc[5-7]. Bartfai T have shown previously that AS-1, a synthetic low molecular-weight IL-1RⅠ/MyD88-TIR mimic, disrupted the interaction of MyD88 with IL-1RⅠ at the TIR domain and prevented the serial reactions induced by IL-1β, by the imitation of a tri-peptide sequence[(F/Y)-(V/L/I)-(P/G)] of the TIR domain BB-loop[8]. In mouse thy-moma EL4 cells and spleen lymphocytes, Bartfai T performed coimmunoprecipitation and sandwich-ELISA assays to analyze IL-1RⅠ/MyD88 association by using antibodies specific for MyD88 and IL-1RⅠ and found that AS-1 prevented IL-1RⅠ/MyD88 association me-diated by IL-1β. Also, it was shown that AS-1 signifi-cantly inhibited the phosphorylation of p38 MAPK mediated by IL-1β. As a

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further proof of the effects on IL-1β signaling *in vivo*, researchers found that AS-1 significantly attenuated fever induced by IL-1β in mice. But it had not reported whether AS-1 had any cytotoxic effect, whether it prevented IL-1β signaling in other cells and changed other downstream signal molecules activation after preventing IL-1RⅠ/MyD88 association. Interleukin-1 receptor-associated kinase-1(IRAK-1) is an important adapter in the signaling complex of Toll/IL-1 receptor family. After IL-1RⅠ bound with corre-sponding ligand, its intracellular TIR domain combines with MyD88-TIR domain and forms IL-1 receptor complex, then it recruits IRAK-1 downstream. IRAK-1 is the first family member identified due to its association with the activated IL-1 receptor complex and its pro-nounced autophosphorylation[9]. So the activation and hyperphosphorylation of IRAK-1 can detect the acti-vation of MyD88-dependent signaling pathway after IL-1RⅠ being stimulated. MAPK is also an important downstream kinase of this signaling pathway, then its level of phosphorylation can reflect the activation of MyD88-dependent signaling pathway. With the coop-eration of College of Chemistry and Chemical Engi-neering of Hunan Normal University, we synthesized AS-1 under the guidance of the documents and analyzed its physical structure and biological function.

MATERIALS AND METHODS

Materials

Reagents

Boc-phe-osu, Boc-Val-Osu, IL-1β and β-actin antibody were purchased from Sigma(USA). MTT was obtained from Amresco(USA). Phosphorylated-p38 and p38 antibodies were products of Cell Signaling Technology(USA). Anti-IRAK-1 was purchased from Santa Cruz Biotechnology(USA).

Objects

HEK 293A cells were purchased from Shanghai cellular institution, preservation and passage by our laboratory. HEK 293A cells were maintained in Dulbecco's Modified Eagle Medium(DMEM) (Invitrogen corporation), supplemented with 10 U/ml Penicillin G, 10 μg/ml streptomycin, 2 mmol/L L-glutamine, and 10% fetal bovine serum at 37℃ and 5% CO_2 in humidified incubator.

Methods

Synthesis AS-1

AS-1 was prepared according to the listed methods in the document[8]. AS-1 was obtained as slight yellow oil, and then dried *in vacuo* for 24 h. Its structure was detected by nuclear magnetic resonance(1H NMR), which is consistent with the data of the reference[8].

Cytotoxicity experiment(MTT assay)

MTT was dissolved in DMEM at the concentration of 5 g/L, filtered for sterilization, and conserved away from light at 4℃. After the start of the experiment, 25 μl of MTT solution was added to each well containing cells (96-well microtiter plate). Then, the plate was incubated in a CO_2 incubator at 37℃ for 4 h. When the amethyst needle crystal was observed from the microscope, the media was removed, and 200 μl of dimethyl sulphoxide (DMSO) was instilled to each well, pipetted up, and down to dissolve crystals. Lastly, the plate was placed in the transfer to CliniBio-128 plate reader and the absorbance was measured at 492 nm. The cells were divided into six groups; control group served as blank contrast, IL-1β (10 ng/ml) stimulated group served as positive control and DMSO dissolvent group served as negative control. The absorbencies of different concentration of AS-1 solutions(0-100 μmol/L) were also measured.

Western blot analysis

Equal amounts of total cell protein(80 μg) were fractionated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE) and transferred onto Polyvinylidene difluoride(PVDF) membrane for immunodetection. The membranes were blocked in 5% nonfat milk in TBS containing 0.05% Tween-20 for 2 h at room temperature, then incubated with anti-p-p38, anti-p38, IRAK-1 or anti-β-actin at 4℃ overnight and followed by peroxidase-conjugated secondary antibodies at room temperature for 1h. The immune complex was visualized by enhanced chemiluminescent reagent (ECL) method, and then exposed to X-ray film. There were five groups, including the control group(neither IL-1β nor AS-1), IL-1β stimulated group and different concentration of AS-1(0-100 μmol/L)with IL-1β groups.

Statistical analysis

Data was presented as mean±SEM. For tests of difference between the groups, one-way ANOVA was performed. P values less than 0.05 were defined as statistically significant. All experiments were routinely performed at least four times with comparable results.

RESULTS

The crystallization structure of AS-1 was detected by NMR

AS-1 that we had synthesized was slight yellow oil, and its structure was detected by ^{1}H NMR:(400 MHz, $CDCl_3$) δ :7.34-7.17(m, 5 H), 6.24 (d, J = 8.4 Hz, 1H), 4.60, 4.58(dd, J = 6.8 Hz, 7.2 Hz, 1H) 3.74-3.70(m, 1H), 3.49-3.40(m, 3H), 2.99-2.92(m, 3H), 2.53(t, J =

8.0 Hz, 1H), 1.97-1.83(m, 5H), 0.90(d, J = 6.8 Hz, 3H), 0.82(d, J = 6.8 Hz, 3H); LRMS(EI, 70 eV) m/z(%): 302 (M^+, 5), 260(2), 204(14), 154(5), 127(4), 105(8), 91 (14), 72(100). The crystallographic details of AS-1 coincided with those of the reference[8]. The chemical molecular constitution of AS-1 was shown in ***Fig 1***.

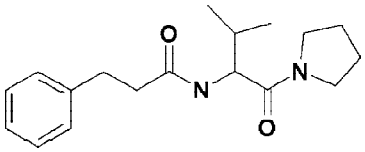
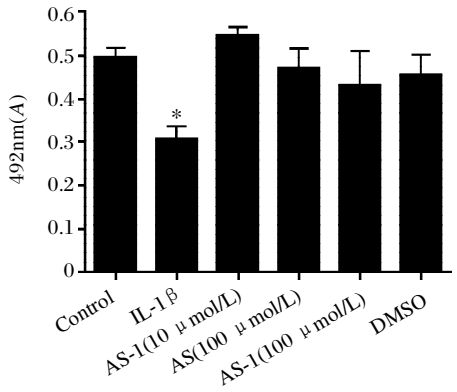


Fig 1 Chemical molecular constitution of AS-1

MTT assay found that AS-1 has no cytotoxicity

To detect if AS-1 had any cytotoxicity, MTT assay was taken on HEK 293A cells. MTT can permeate plasma membrane(the amber dehydrogenase in the mitochondria of living cell deoxidize exogenous MTT) to create indissolvable royal purple needles of formazan crystal which are found deposited in cells. The crystal can be dissolved by DMSO. The quantity of living cells can be detected indirectly by using CliniBio-128 plate reader and measured absorbance at 492 nm[10]. This empirical method can detect whether AS-1 has cytotoxicity or it reduces quantity of living cells. Our result demonstrated that IL-1β stimulated groups had cytotoxicity reaction and those 492 nm A figures were decreased by 37.90% compared with control(0.50 ± 0.02 Vs 0.31 ± 0.03, P < 0.05). No toxicity of AS-1 were shown to HEK 293A cells at all the concentrations of 10 μmol/L, 50 μmol/L, 100 μmol/L and DMSO group. The 492 nm A figures of AS-1 groups were not significantly different compared with control(P > 0.05, ***Fig 2***).



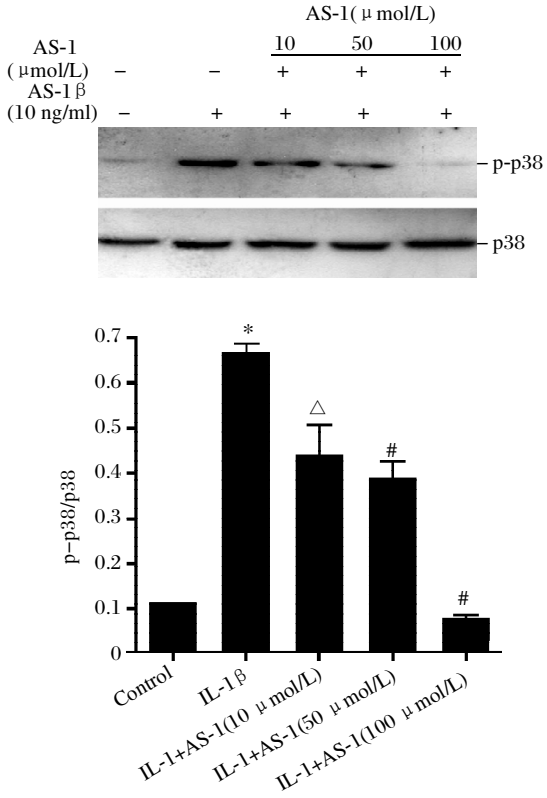
Different concentrations of AS-1(0-100 μmol/L) were added to cells for MTT assay. IL-1β (10 ng/ml) stimulated group served as positive control. It was shown that IL-1β stimulated group had cytotoxicity reaction. No toxicity of AS-1 were shown to HEK 293A cells at all the concentrations of 10 μmol/L, 50 μmol/L, 100 μmol/L and DMSO groups(P > 0.05). Compared with control, $^*P$ < 0.05.

Fig 2 The results of MTT assay signed AS-1 has no oxicity to HEK 293 cells

AS-1 attenuated activation of p38 MAPK mediated by IL-1

The degree of the phosphorylation of p38 MAPK induced by IL-1β was analyzed by Western Blot to detect whether AS-1 can efficiently block the association of IL-1R-TIR and MyD88-TIR and the downstream of signal transduction.

It was shown that the phosphorylation of p38 MAPK induced by IL-1β (10 ng/ml) significantly increased by 50.90%,(0.11 ± 0.01 Vs 0.67 ± 0.02, P < 0.05) compared with control group; Compared with IL-1β stimulated groups, the phosphorylation of p38 MAPK induced by preoccupation of AS-1(10 μmol/L) decreased by 34.32%(0.67 ± 0.02 Vs 0.44 ± 0.07, P < 0.05), that AS-1(50 μmol/L) decreased by 41.79% (0.67 ± 0.02 Vs 0.39 ± 0.04, P < 0.01), and AS-1 (100 μmol/L) decreased by 89.55% (0.67 ± 0.02 Vs 0.07 ± 0.01, P < 0.01, ***Fig 3***).



HEK 293A cells were incubated with different concentrations of 0-100 μol/L for 15 min, each group was added IL-1β (10 ng/ml) to stimulate for 24 h, then extracted total protein of cells for Western Blot. Control had added neither AS-1 nor IL-1β. Compared with control group, $^*P$ < 0.05; compared with IL-1β stimulated group, $^\triangle P$ < 0.05; compared with IL-1β stimulated group, $^\#P$ < 0.01.

Fig 3 AS-1 attenuated the activation of p38 MAPK mediated by IL-1β

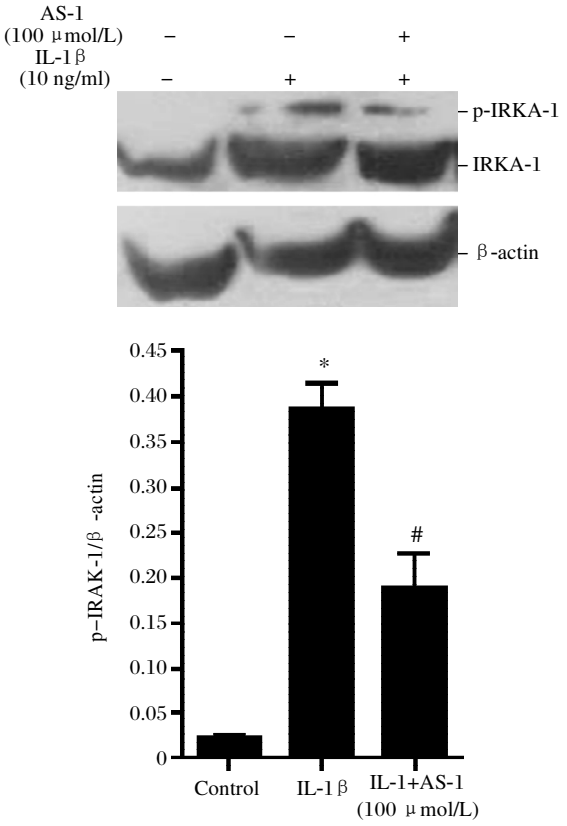
AS-1 attenuated the activation of IRAK-1 mediated by IL-1 β

We had described that IRAK-1 was an important downstream signal molecular of MyD88-dependent pathway[9], so the degree of phosphorylation of IRAK-1 was performed by using Western Blot to measure the inhibitory action of AS-1.

Western Blot showed that IL-1β caused IRAK-1 activation, and the phosphorylation of IRAK-1 in IL-1β stimulated group significantly increased by 157.83% (0.02 ± 0.01 Vs 0.39 ± 0.03, $P < 0.05$) compared with control group. IL-1β stimulated group plus AS-1 evidently decreased the phosphorylation of IRAK-1, by 51.28%(0.39 ± 0.03 Vs 0.19 ± 0.04, $P < 0.05$), than that of the IL-1β stimulated group(***Fig 4***).

DISCUSSION

It was demonstrated that, the low molecular-weight MyD88 mimetic AS-1 we synthesized had no cytotoxicity to HEK 293A cells through MTT assay, and it attenuated the phosphorylation of p38 MAPK and IRAK-1 mediated by IL-1β .



HEK 293A cells were incubated with AS-1(100 μmol/L) for 15min, then added IL-1β (10 ng/ml) to stimulate for 24 h. Control had added neither AS-1 nor IL-1β . Compared with the control group, $^{*}P < 0.05$; compared with IL-1β stimulated group, $^{\#}P < 0.05$.

Fig 4 AS-1 attenuated the activation of IRAK-1 mediated by IL-1 β

The Toll/IL-1 receptor family plays an important role in inflammation and regulation of immune responses[2]. These receptors are characterized by the TIR domain, which locates the C-terminal homology motif [11]. A principal function of the TIR domain is mediating homotypic protein-protein interaction in the signal transduction pathway. After the extracellular part of IL-1R binding with ligands, MyD88 bound to IL-1R via homotypic interactions mediated by TIR domains to form TIR-MyD88 activity complex, swhich in turn recruited IRAK-1, thereby allowing the association with death domain(DD) of MyD88[3]. When the cells were in quiescent condition, the N-terminal of IRAK-1(DD) combined with Toll-interacting protein(Tollip), which inhibited IRAK-1 to form dimer. But when IL-1R was activated, Tollip/IRAK-1 complex shifted to the MyD88-TIR complex, then Tollip dissociated from the complex, which made the association of DDs of MyD88 and IRAK-1 and then formed IRAK-1 dimer, leading IRAK-1 autophosphorylation/cross phosphorylation[12]. Kollewe C had reported that IRAK-1 was phosphory-lated at Thr^{209} and resulted in a conformational change of the kinase domain, permitted further phosphoryla-tions to take place[13]. For this reason, when MyD88-dependent signaling pathway was activated mediated by IL-1β , the total protein of IRAK-1 in cells would not increase. But there was more IRAK-1 dimer and phos-phorylation of IRAK-1 was increased. AS-1 blocked the associa–tion of TIR of IL-1R and MyD88, and then prevented MyD88 from recruiting and activating down-stream IRAK-1, so the phosphorylation of IRAK-1 decreased.

After forming IRAK-1 dimer, IRAK-4(another member of IRAK family) combined with MyD88-TIR-IRAK-1 complex and then self phosphorylated, thereby allowing the activation of tumor necrosis factor receptor associated factor 6(TRAF6), which made TRAF family form homologous or heterologous oligomerization[14]. Meanwhile, IRAK-1 made TAK1- Binding Protein 2(TAB2) bind with TRAF6-Transforming growth factor beta-activated kinase 1(TAK1)-TAB1 complex. This led to the ubiquitylation of TRAF6, which induced the activation of TAK1. TAK1, in turn, phosphorylated both MAPK and the IKK complex(inhibitor of nuclear factor-κB(I κ B)-kinase complex). Then it activated NF-κB/AP-1 and induced the expression of its target genes[4,15].

MAPKs were found include the extracellular signal-regulated kinases(ERKs), the stress-activated/c-Jun NH2-terminal kinases(SAPK/JNKs), p38-MAPK. Involvement of all three classical MAPK pathways had been implicated in the mechanisms of immune response[16-17]. These cascades differ in their upstream activation sequence and in downstream substrate specify.

As an important downstream kinase of MyD88-dependent signaling pathway, the phosphorylation of p38MAPK could be used as sign of the activation of this signaling pathway[18]. According with the describing in reference[8], AS-1 inhibited IL-1 β-induced the phosphorylation of p38MAPK in EL4 thymoma cells, we also demonstrated AS-1 attenuated the phosphorylation of p38MAPK mediated by IL-1 β in HEK 293A cells.

MyD88-dependent signaling pathway plays a crucial role in the activation of both innate and acquired immune responses. Recently, researchers also found that it participated in the regulation of varied heart diseases, such as myocardial infarction, ischemia/reperfusion (I/R), cardiac ventricle remodeling and so on[19-21]. According to our research results, AS-1 inhibited efficiently the phosphorylation of IRAK-1 and p38 MAPK induced by IL-1β , then prevented MyD88 dependent signaling pathway, and it had no cytotoxicity according to MTT assay. Therefore, AS-1 hoped to become a new drug that targeted at blocking the association of IL-1R Ⅰ and MyD88 and further interfere MyD88-dependent signaling pathway.

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References

[1] Kimbrell DA, Beutler B. The evolution and genetics of innate immunity[J]. *Nat Rev Genet* 2001;2(4):256-67.

[2] Slack JL, Schooley K, Bonnert TP, Mitcham JL, Qwarnstrom EE, Sims JE, et al. Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. *J Biol Chem* 2000; 275 (7):4670-8.

[3] Martin MU, Wesche H. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1receptor family. *Biochem Biophys Acta* 2002;1592(3):265-80.

[4] Roeder A, Kirschning CJ, Rupec RA, Schaller M, Weindl G, Korting HC. Toll-like receptors as key mediators in innate antifungal immunity. *Med Mycol* 2004;42(6):485-98.

[5] Boraschi D, Taqliabue A. The interleukin-1 receptor family. *Vitam Horm* 2006;74 (5):229-54.

[6] Beutler B, Hoebe K, Georqel P, Tabeta K, Du X. Genetic analysis of innate immunity: identification and function of the TIR adapter proteins. *Adv Exp Med Biol* 2005;560 (9):29-39.

[7] Yamamoto M, Akira S. TIR domain-containing adaptors regulate TLR-mediated signaling pathways. *Nippon Rinsho* 2004;62(12): 2197-203.

[8] Bartfai T, Behrens MM, Gaidarova S, Pemberton J, Shivanyuk A, Rebek JJ. A low molecular weight mimic of the Toll/IL-1 receptor/ resistance domain inhibits IL-1 receptor-mediated responses. *Proc Natl Acad Sci USA* 2003;100(13):7971-6.

[9] Gan L, Li L. Regulations and roles of the interleukin-1 receptor associated kinases(IRAKs) in innate and adaptive immunity. *Immunol Res* 2006;35(3):295-302.

[10] Smythe GM, Hodgetts SI, Grounds MD. Immunobiology and the future of myoblast transfer therapy. *Mol Ther* 2000;1(4):304-13.

[11] Radons J, Dove S, Neumann D, Altmann R, Botzki A, Martin MU, et al. The Interleukin 1(IL-1) receptor accessory protein Toll/IL-1 receptor domain: analysis of putative interaction sites in vitro mutagenesis and molecular modeling. *J Bio Chem* 2003;278(49): 49145-53.

[12] Huang YS, Misior A, Li LW. Novel role and regulation of the interleukin-1 receptor associated kinases(IRAK) family proteins. *Cell Mol Immunol* 2005;2(1):36-9.

[13] Kollewe C, Mackensen AC, Neumann D, Knop J, Cao P, Li S, et al. Sequential autophosphorylation steps in the interleukin-1 recptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. *J Biol Chem* 2004;279(7):5227-36.

[14] Akira S, Takeda K. Toll-like receptor signaling. *Nat Rev Immunol* 2004;4(7):499-511.

[15] West AP, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. *Annu Rev Cell Dev Biol* 2006;17(5):527-33.

[16] Dickinson RJ, Keyse SM. Diverse physiological functions for dual-specificity MAP kinase phosphatases. *J Cell Sci* 2006;119(22):4607-15.

[17] Liu Y, Shepherd EG, Nelin LD. MAPK phosphatases-regulating the immune response. *Nat Rev Immunol* 2007;7(3):202-12.

[18] Jo EK, Yang CS, Choi CH, Harding CV. Intracellular signalling cascades regulating innate immune responses to Mycobacteria: branching out from Toll-like receptors. *Cell Microbiol* 2007;9(5): 1087-98.

[19] Min XY, Qian B, Liu Li, Zhang XJ, Cheng YL. The time correlation with the activation of molecule targets in Toll like receptor 4 signaling parthway in mice myocardium activated by LPS. *Acta Universitatis Medicinalis Nanjing(in Chinese)* 2007;27(6):538-41.

[20] Cristofaro P, Opal SM. Role of Toll-like receptors in infection and immunity: clinical implications. *Drugs* 2006;66(1):15-29.

[21] Ha T, Hua F, Li Y, Ma J, Gao X, Kelley J, et al. Blockade of MyD88 attenuates cardiac hypertrophy and decreases cardiac myocyte apoptosis in pressure overload-induced cardiac hypertrophy *in vivo*. *Am J Physiol Heart Circ Physiol* 2006;290(3):H985-94.