Available online at www.sciencedirect.com
SCIENCE DIRECT
JNMU
Journal of Nanjing Mecical University, 2007, 21(1): 36–41
www.elsevier.com/locate/jnmu
Research Paper

Role of myeloid differentiation factor 88 in HSP60 signal transduction in dendritic cells☆

Jiajun Chen[a], Zongquan Sun[a,*], Gang Su[a], Chao Liu[a], Jinping Liu[a], Yongzhi Deng[a], Jiawei Shi[a]
[a]*Department of Cardiovascular Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China.*

Received 28 August 2006

Abstract

Objective: To explore the role and mechanism of myeloid differentiation factor88 (MyD88) in HSP60 signal transduction in dendritic cells. **Methods**: Mouse DCs were cultured from murine bone marrow cells. The DC marker CD11c was detected by flow cytometry, then DCs were divided into control group, HSP60 groupand RNA interference group. Control group was cultured under normal condition, and HSP60 group was cultured with 10 μg/ml of HSP60. RNA interference group was first cultured with MyD88 siRNA for12 hours and then HSP60 was added into the culture mixture. All groups were cultured for 48 hours. Immunochemistry was used to detect the concentration of MyD88 and NF- κB. Western blot was used to detect the concentration of MyD88. Flow cytometry and mixed lymphocyte reaction (MLR) were used to detect the phenotype and functional properties of DCs. ELISA was used to detect the concentration of TNF-α, IFN-γ and IL-12 in the supernatant. **Results**: The expression of CD11c in murine bone marrow DCs was 88.76%. HSP60 stimulation increased the expression of CD80, CD86, MHC-Ⅱ in DCs and TNF-α, IFN-γ, IL-12 secretion in the supernatant. HSP60 stimulation also increased the level of MyD88 in the cytoplasm and promoted the shift of NF-κB to karyon and the proliferation of allogeneic T cells. MyD88 siRNA could decrease MyD88 and inhibit these effects induced by HSP60. **Conclusion**: HSP60 activates DCs through MyD88-dependent pathway. MyD88 plays a critical role in HSP60 signal transduction. Inhibition of MyD88 may be a novel way for treating disease correlated with HSP60.

Keywords: dendritic cells; MyD88; heat-shock proteins; signal transduction

INTRODUCTION

Dendritic cells are the professionally strongest antigen presenting cells which can activate T cells uniquely[1]. The function is linked with their condition of maturation. Toll-like receptors (TLRs) are transmembrane receptors expressed on the surface of DCs, mononuclear cells(MNCs), vascular endothelial cells (VECs) and other cells. TLRs play an important role in regulating maturation of DCs[2]. Many studies have been performed about its endogenous ligands and signal pathway, and consider that TLRs are critical proteins linking innate and acquired immunity. Heat shock protein 60 (HSP60) is a recently discovered endogenous ligand of Toll-like-receptor (TLR)4 which serves as a dangerous signal to the innate immune system and participates in other clinical pathological process such as autoimmune disease, diabetes and atherosclerosis[3]. Recent studies have suggested that autologous HSP60 can activate allograft rejection as an allogeneic antigen. Regulation of TLR signal pathway perhaps is a novel way to antagonize allograft rejection.

HSP60 is described to trigger inflammatory and cytokine responses including TNF and IL-12 p40 in macrophages through MyD88- dependent pathway[4]. However, there is no definitive conclusion in dendritic cells [5-6]. This study aimed to investigate the impact of MyD88 siRNA on the biological activity of dendritic cells (DCs) cultured from murine bone

☆This work was supported by National Natural Science Foundation of China(No.30471715).

*Corresponding author.
E-mail address: sunzong-quan@163.com

marrow and stimulated by HSP60, and explore the role and mechanism of MyD88 in HSP60 signal transduction in dendritic cells.

MATERIALS AND METHODS

Mice

Male BALB/C and male C57BL/6 mice, 7 week old, were purchased from Tongji Institute of Organ Transplantation, Huazhong University of Science an d Technology(China).

Reagents

The culture medium was RPMI 1640(Gibco company,USA) supplemented with10% FCS, 50 μM 2-ME, and 20 μg/ml gentamicin. Recombinant mice GM-CSF(rGM-CSF) were purchased from Peprotech company (USA).FITC-conjugated anti-mouse CD80 and CD86 were purchased from BioLegend company (USA). FITC-conjugated anti-mouse I-Ad was purchased from PharMingen company (USA). Anti-mouse TNF-α, IFN-γ and IL-12 ELISA kit were purchased from Jingmei biotech Co., Ltd (China). Anti-mouse MyD88 antibody was purchased from Lab Vision company (USA). siRNA and RNAi-mate were obtained from Shanghai GenePharma Co.,Ltd (China).

Bone marrow culture

After all muscl e tissues were removed from the femurs and tibias, the bones were washed twice with PBS. Both ends of the bones were cut with scissors, and then the marrow was flushed out using PBS. Single cell suspensions were treated with Tris-buffered ammonium chloride to lyse erythrocytes before culture. Bone marrow (BM) cells were cultured in 6-well culture plates at an initial density of 2×10^6/ml in 4 ml/well in 1 ml of medium supplemented with 20 ng/ml rGM-CSF [7]. Half of the medium was removed and replaced by the same volume of fresh RPMI 1640 containing the initial concentration of cytokine every 3 days. On the eighth day the DCs were collected and washed with PBS and divided into control group, HSP60 group and RNA interference group. Control group was cultured under normal condition, and HSP60 group was cultured with 10 μg/ml of HSP60. RNA interference group was first cultured with MyD88 siRNA for12 hours and then HSP60 was added into the culture mixture. All groups were cultured for 48 hours.

MyD88 siRNA transfection protocol

5 μg siRNA were diluted into serum-free medium in 500 μl. Then the siRNA solution was mixed with 15 μg RNAi-mate and Incubated for 30 minutes. The culture mixtures were added into 6-well plate containing DC in 2 ml. Fluorescence microscope was used to detect transfection efficacy.

Immunocytochemistry assay for MyD88 and NF-κB

The immunohistochemical detection for MyD88 and NF-κB was performed according to the method described by Yang DL et al[8].

Western blot analysis

The content of MyD88 was detected according to the method described by Verma S, et al[9]. It included extraction of protein, electrophoresis, membrane transfer, hybridization and colouration. The results were expressed by product of area and gray scale and the proportionality of experimental group to control group was calculated.

FACS analysis

Cells(5×10^5)were resuspended in 100 μl PBS and FITC-conjugated antibodies were added, then the cells were cultivated for 30 min at 4℃. The antibodies were anti- mouse CD80, CD86 and anti-mouse I-Ad. Then the cells were centrifuged and washed with ice cold PBS and resuspended in 500μl PBS . Flow cytometer (BD company,USA) and CellQuest software were used to detect the surface marker of cells.

Detection of cytokine

The supernatants were collected after 2 days' culture. Every group contained triple wells. The detection of cytokine was performed according to the description of the kit.

Allogeneic mixed lymphocyte reaction

T cells were purified from C57BL/6 spleen cells by lymphocytic separating medium. Triplicates of 5×10^5 enriched T cells were seeded into a 96-well flat bottom plate together with titrated numbers of Mitomycin C-treated(50 μg/ml, 20 min, 37℃)DCs. The proportion of DCs to T cells was graded as 1∶100, 1∶25 and1∶10. The mixed cells were cultured for 3 days, then 20 μl MTT were added into every well. After incubation in dark for 4 hours, the mixture was centrifuged and the supernatant was discarded. Then 100 μl DMSO was added, and the cells were placed in dark for 2 hours.Ultraviolet spectrophotometer was used to detect optical density (OD) (A570). Stimulation index (SI) was calculated by the equation∶SI =(OD of experimental group-OD of blank group)/(OD of control group-OD of blank

group).

Statistical analysis

Result data were expressed as mean ± standard deviation. SPSS 10.0 software was used for statistical analysis. ANOVA and LSD test were performed, $P < 0.05$ was considered as statistically significant.

RESULTS

FACS analysis for surface CD11c

The expression of CD11c in murine bone marrow DCs was 88.76%(***Fig. 1***).

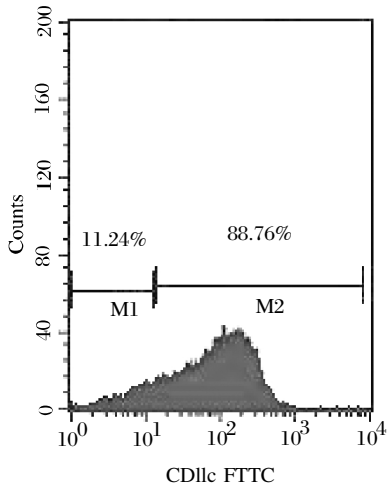


Fig. 1 The expression of CD11c in murine bone marrow DCs

Immunohistochemical detection of MyD88 and NF-κB

Immun ohistochemical detection showed that MyD88 was located in kytoplasm and there was strong positive staining in HSP60 group whereas weak positive staining in control group and RNA interference group. NF-κB was mainly located in kytoplasm in control group and RNA interference group. In HSP60 group, NF-κB was mainly located in karyon(***Fig. 2-5***).

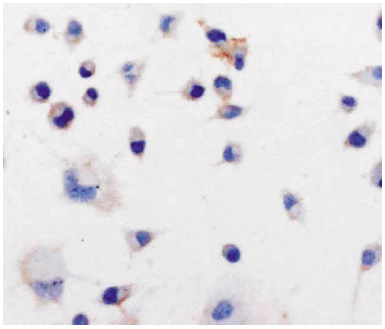


Fig. 2 Weak positive staining of MyD88 in RNA interference group. (DAB×400)

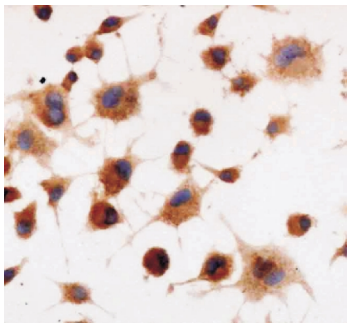


Fig. 3 Strong positive staining of MyD88 in HSP60 group. (DAB×400)

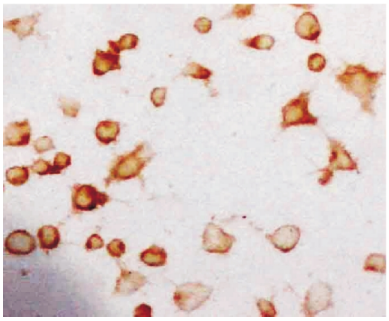


Fig. 4 NF-κB expression in kytoplasm in RNA interference group. (DAB×400)

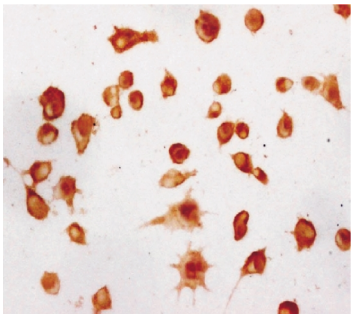


Fig. 5 NF-κB expression in karyon. in HSP60 group. (DAB×400)

Western blot analysis

The result showed that MyD88 increased dramatically in HSP60 group whereas greatly decreased in control group and RNA interference group. The ratio of content of MyD88 in RNA group to that in HSP60 group was 1:5.8(***Fig. 6***).

The effects of HSP60 on cell surface markers

The result of flow cytometry showed that the expression of CD80, CD86 and MHC-Ⅱ of HSP60 group were higher than those of control group and

RNA interference group (***Fig. 7***, ***A-F***).

The effects of HSP60 on cytokine

HSP60 promoted the excretion of TNF-α、IFN-γ and IL-12.There were significant deferernce in HSP60 group compared with control group and RNA interference group($P < 0.05$,***Fig. 8***).

Allogeneically mixed lymphocyte reaction

HSP60-treatment increased the proliferation of allogeneic T cells even at the density of 1 ∶ 100. However, DCs of RNA group and control group can not effectually stimulate proliferation of allogeneic T cells. There were significant diferernces ($P < 0.01$) between HSP60 group and RNA group or control group(***Table 1***).

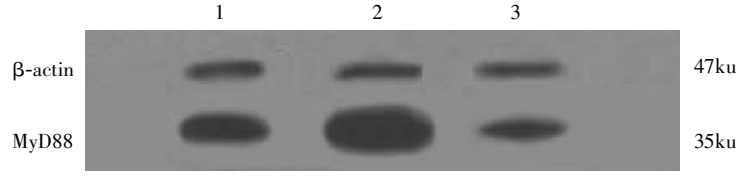
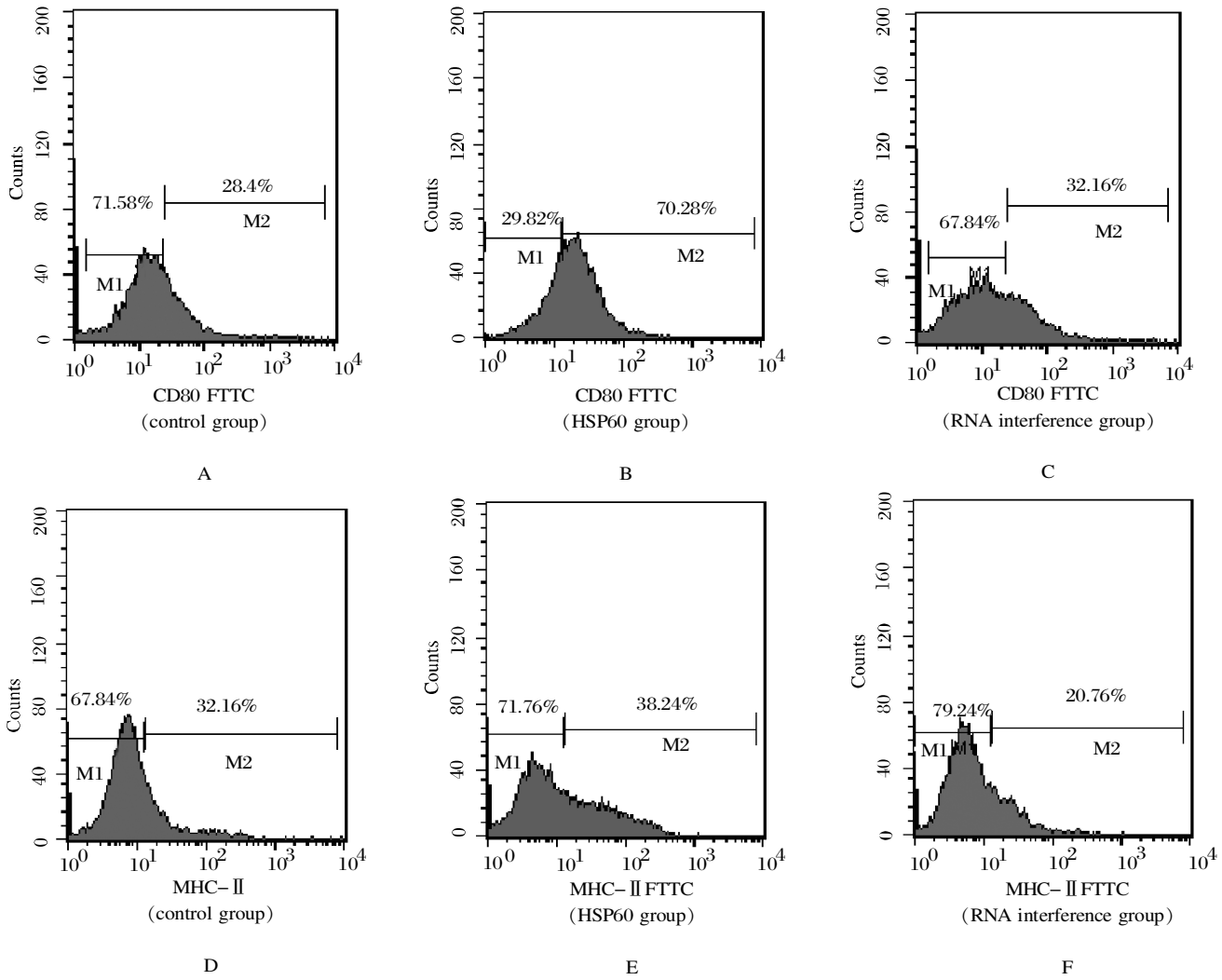


Fig. 6 Expression of MyD88. 1:control group;2:HSP60 group;3:RNA interference group



The expression of CD80 in three groups respectively were 28.42%(A) , 70.28%(B) and 32.16%(C), and the expression of MHC-Ⅱ in three groups respectively were 16.28%(D) , 38.24%(E) and 20.76%(F).

Fig. 7 The expression of cell surface markers in three groups

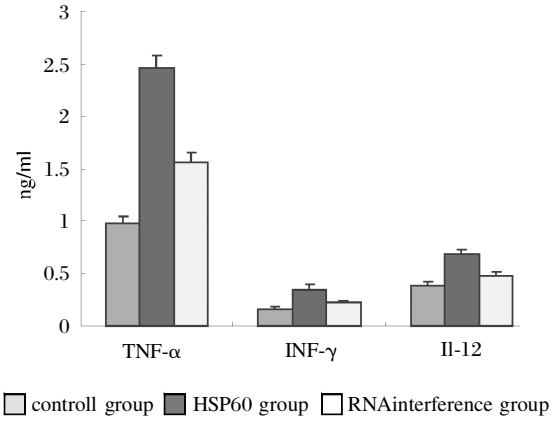


Fig. 8 Detection of cytokine in three groups

DISCUSSION

DCs are the sentinels of the adaptive immune system and have an important role not only in induction of immunity but also in maintenance of tolerance. DCs are involved in tumor, autoimmune disease, diabetes, atherosclerosis, and allograft rejection. The function of DCs depends on their degree of maturation. The recently discovered TLRs are germline-encoded, trans-membrane receptors that are critical for the detection of microbial pathogens. TLRs are expressed on the surfaces of mononuclear cells (MNCs), antigen presenting cells (APCs), vascular endothelial cells (VECs) and other cells which play important roles in regulating maturation of DCs and initiating both innate and adaptive immune responses. As a recently discovered new ligand of TLRs[10], HSP60 not only activates innate immunity but also plays a critical role in allograft rejection. So the signal pathway of TLRs activated by HSP60 attracted strong interest again.

Table 1 **Stimulation index of DCs in three groups to allogeneic T cell** ($n = 3$)

groups	the proportion of DCs to T cells		
	1∶10	1∶25	1∶10
Control group	1.13 ± 0.11*	1.21 ± 0.11*	1.35 ± 0.08*
HSP60 group	2.25 ± 0.22	2.86 ± 0.19*	3.14 ± 0.21
RNA interference group	1.21 ± 0.06*	1.24 ± 0.14*	1.35 ± 0.09*

Compared to HSP60 group,*$P < 0.01$

Many studies have shown that HSP60 activates MNCs and VECs through TLR4 in MyD88- dependent pathway .Once ligated, TLR4 initiate a signaling pathway via their universal signal adaptor protein, MyD88, inducing the activation of IL-1R associated kinase, TNF-α receptor association factor-6, mitogen-activated protein kinase and translocation of NF-κB, followed by initiating a maturation program consisting of increased expression of co-stimulatory molecules and release of pro-inflammatory cytokines [11]. MyD88 is an important adaptor protein of which defect can lead interruption of signal transduction[12].

In this study, we cultured DCs with HSP60 and treated with MyD88 siRNA, then observed the changes of biologic activity of DCs and explored the role of MyD88 in HSP60 signal trusduction. The results demonstrated that HSP60 stimulation increased the expression of CD80, CD86, MHC-Ⅱ in DCs and TNF-α, IFN-γ, IL-12 secretion in the supernatant. HSP60 stimulation also increased the level of MyD88 in the cytoplasm and promoted the shift of NF-κB to karyon and the proliferation of allogeneic T cells. It indicated that HSP60 promoted maturation of DCs and activated specific immunity reaction which were similar to those reported previously[13].In RNA interference group, western blot analysis showed that MyD88 was reduced effectivelly. Simultaneously, the activation of DCs was inhibited by MyD88siRNA which was proved by the decreased expression of CD80, CD86 and MHC-Ⅱ and TNF-α, IFN-γ, IL-12 secretion in the supernatant. The results proved that HSP60 activated DCs through MyD88- dependent pathway. MyD88 is a critical modulatory molecule in HSP60 signal trusduction.

RNA interference is a recently discovered novel genic technique which can inhibit genic expression at post-transcription level. The principle is that small interfering RNA directly triggers RNA interference and degrades homologous mRNA specifically and inhibits expression of target protein [14]. To aim directly at the key molecule, MyD88 in signal pathway, we composed 3 pairs of MyD88siRNA chemically and screened one high-performance pair of them of which sequence was

5-GAC UGA UUC CUA UUA AAU AdTdT-3-

5-UAU UUA AUA GGA AUC AGC CGdC-3-.

This provides a rationale of gene therapy for relative diseases aimed directly at DC.

DCs are mainly cells which link innate immunity

and acquired immunity. Different phenotype of DCs decide reaction of T cells to antigen. Mature DCs activate immune reaction while immature DCs induce immune tolerance [15]. The signal conduction of HSp60 in DCs and molecular mechanism are grave for controlling activity of DCs, treatment of autoimmune disease and inhibiting immunological rejection. Given that TLR4 plays a critical role in inflammatory signaling, and that HSP60 signals through TLR4, this study indicated that MyD88 was an essential component in HSP60 signaling. Inhibition production of MyD88 may be a novel way for treating disease correlated with HSP60.Our findings might have important implications for future treatment of immune disease aimed directly at TLRs and their signal pathway.

References

[1] Stuart LM, Lucas M, Simpson C,Lamb J,Savill J,Lacy-Hulbert A. Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. *J Immunol* 2002;168: 1627-35.

[2] Datta SK, Redecke V, Prilliman KR,Takabayashi K,Corr M, Talant T, *et al*. A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J Immunol* 2003; 170: 4102-10.

[3] Horvath L, Cervenak L,Oroszlan M,Prohaszka Z,Uray K, Hudecz F, *et al*. Antibodies against different epitopes of heat shock protein 60 in children with type 1 diabetes mellitus. *Immunol Lett*,2002; 80: 155-62.

[4] Zanin-Zhorov A, Nussbaum G, Franitza S,Cohen IR,Lider O. T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors. *FASEB J* 2003; 17:1567-9.

[5] Gobert AP, Bambou JC,Werts C,Balloy V,Chignard M, Moran AP,*et al*. Helicobacter pylori heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-in-dependent mechanism. *J Biol Chem* 2004; 279: 245-50.

[6] Chen K, Lu J,Wang L,Gan YH. Mycobacterial heat shock protein 65 enhances antigen cross-presentation in dendritic cells independent of Toll-like receptor 4 signaling. *J Leukoc Biol* 2004; 75:260-6.

[7] Lutz MB, Kukutsch N, Ogilvie AL,Rossner S,Koch F,komani N, *et al*. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 1999; 223:77-92.

[8] Yang DL, Xu YJ, Zhang ZX,Li CQ. Effect of nitric oxide on NF-κB activation induced by cigarette smoke extract in rat alveolar macrophage. *Chin J CellMol Immunol* 2003; 19: 7-9.

[9] Verma S, Wang CH, Li SH,Dumont AS,Fedak PW,Badiwala MV, *et al*. A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. *Circulation* 2002;106:913-9.

[10] Ohashi K, Burkart V, Flohe S,Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4complex. *J Immunol* 2000; 164: 558-61.

[11] Bulut Y,Faure E,Thomas L,Karahashi H,Michelsen KS,Equils O, *et al*. Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway . *J Immunol* 2002; 168:1435-40.

[12] Goldstein DR, Tesar BM, Akira S,Lakkis FG. Critical role of the Toll-like receptor signal adaptor protein MyD88 in acute allograft rejection. J *Clin Invest* 2003; 111:1571-8.

[13] Flohe SB, Bruggemann J, Lendemans S,Nikulina M,Meierhoff G,Flohe S,*et al*. Human heat shock protein 60 induces maturation of dendritic cells versus a TH1-promoting phenotype. *J Immunol* 2003; 170: 2340-48.

[14] Miyagishi M, Taira K. U6 promoter driven siRNAs with four uridine 3'overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat Biotechnol* 2002; 20 :497-500.

[15] Fairchid PJ, Waldmann H. Dendritic cells and prospects for transplantation tolerance. *Curr Opin Immunol* 2000; 12:528-35.