DOI:10.3724/SP.J.1008.2012.00233

· Original article ·

A critical amino acid in Toll-like receptor 7 TIR domain impacting its binding with MyD88

WEI Wei, ZHANG Yi-liang, GUO Ying-jun, SUN Shu-han*
Department of Medical Genetics, College of Basic Medical Sciences, Second Military Medical University, Shanghai 200433, China

[**Abstract**] **Objective** To study the mechanism of Toll-like receptor 7 (TLR7) signal transduction by identifying the critical amino acid in TLR7 Toll/Interleukin-1 receptor (TIR) domain. **Methods** We carried out a series of truncating mutagenesis and site-direct mutagenesis to obtain TLR7 mutants. These TLR7 mutants were transfected into HEK293T cells. NF-κB signal pathway activation was determined by the reporter gene assay. Immunoprecipitation and immunoblotting were employed to determine the binding between TLR7 and MyD88. **Results** We identified a RXR signal motif with two arginines at position 1004 and 1006, which plays a significant role in TLR7 signal transduction. We further demonstrated that only the arginine at position 1004 could affect the binding between TLR7 and MyD88. **Conclusion** We conclude that the arginine at position 1004 of human TLR7 is critical for its binding with MyD88 in signal transduction.

[**Key words**] Toll-like receptor 7; innate immunity; signal transduction
[Acad J Sec Mil Med Univ, 2012, 33(3): 233-239]

Innate immunity is a central component of the immune system where it functions as a block to the growth and the dissemination of pathogens at early stage of infection[1]. Cells of innate immune system express a number of receptors such as TLRs, RLRs and NLRs[2]. TLRs are type Ⅰ transmembrane proteins which recognize pathogen-associated molecular patterns (PAMPs). TLRs selectively recognize specific components of pathogens. For example, TLR3 recognizes double-stranded RNA of virus, TLR4 recognizes lipopolysaccharide of gram-negative bacteria, and TLR7 and TLR8 recognize single-stranded RNA from virus[2-3]. The TLRs identify PAMPs and then trigger the intracellular network signal pathways, which results in the development or acceleration of inflammatory reactions and autoimmune diseases.

TLRs are composed of extracellular domain, transmembrane domain and cytoplasmic domain[4]. The extracellular domain consists of 19-25 leucine-rich repeats (LRRs). These LRRs mediate the specific interaction of ligand-receptor[5-6]. The cytoplasmic domain is mainly composed of Toll/Interleukin-1 receptor (TIR), which mediates the intracellular signal transduction. TIR domain recruitment of an adapter protein called myeloid differentiation factor 88 (MyD88). MyD88 is the initiator of intracellular signal pathways for most TLRs. MyD88 recruits IRAK4, and IRAK4 recruits IRAK6 directly or indirectly, and then the transcription factor NF-κB is activated by the signaling cascades via IRAK6, resulting in the production of cytokines, such as IL1-β, IL-6, TNF-α, and type Ⅰ interferon(IFN)[2].

TLR7 is located in the endosomal compartments[7]. The ligands of TLR7 were originally identified as imiquimod, resquimod (R848) and guanine analogs such as loxoribine[8]. TLR7 also recognizes single strand RNAs which originate from viruses such as HIV, HCV and influenza virus. Studies have indicated that TLR7 recognizes the synthetic poly(U) RNAs and some small interfering RNAs as well. The correlation between TLR7 and autoimmune diseases, such as systemic lupus

[**Received**] 2011-11-13 [**Accepted**] 2012-03-20
[**Biography**] WEI Wei, master. E-mail: vikerpp@yahoo.com.cn
* Corresponding author. Tel: 021-81871053, E-mail: smmushsun@sina.com

erythematosus, has been confirmed because TLR7 is involved in the activation of dendritic cells and autoreactive B cells through recognizing endogenous single strand RNAs[9]. TLR7 recruits MyD88 through its TIR domain after activation, and then IRAK4 and IRAK6 are recruited. Finally the transcription factor NF-κB is activated, which results in the production of cytokines and type Ⅰ IFN[2]. Although the signal pathway has been substantiated by many studies, the mechanism by which TIR domain recruits MyD88 is unknown. In this study, we constructed various TLR7 mutants and used the dual luciferase reporter assay system to indentify the critical site involved in TLR7 signal transduction. By using the co-immunoprecipitation assay, we demonstrated that arginine at 1004 was critical for human TLR7 activation by affecting the binding between TLR7 and MyD88.

1　Materials and methods

1.1　Reagents and antibodies　PrimeStar DNA polymerase and restriction enzymes were purchased from TaKaRa Biotechnology Co. Ltd. PCR primers were synthesized by Sangon. Lipofectamine™ 2000 was purchased from Invitrogen. R848 was purchased from Alex. The Dual-Luciferase Reporter Assay System was purchased from Promega. Anti-human TLR7 and MyD88 antibodies were purchased from Abcam.

1.2　Construction of expression vector and TLR7 truncating mutants　Human TLR7 sequence was generated by PCR and ligated into the cloning site of the expression vector pEGFP-N1. A series of truncating reverse primers were designed and used to generate various TLR7 truncating mutants, which were also ligated to pEGFP-N1. MyD88 was cloned from cDNA of THP-1 and ligated into the expression vector pCMV-C-MYC, whose C-terminus would express a Myc tag. NF-κB was cloned into firely luciferase reporter plasmid pGL3 (Promega). The Renilla lucierase reporter plasmid pRL-TK was also purchased from Promega.

1.3　Cell culture　Human embryonic kidney (HEK) 293T cells were cultured in DMEM cell culture medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Gibco) and penicillin/streptomycin antibiotics (Invitrogen).

1.4　Site-directed mutagenesis　QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used for site-directed mutation. Mutants were made as the kit's instruction described. Sequences of the oligonucleotides were designed available upon request. All mutants were confirmed by sequencing.

1.5　Reporter gene assay　HEK293T cells (2×10^5 cells/well) cultured in 24-well plates were transfected with expression vectors wild-type TLR7, TLR7 truncating mutants or site-direct mutants, empty pEGFP-N1 vector (0.2 μg/well) together with the firefly luciferase reporter plasmid, NF-κB-luc (0.2 μg/well), and a Renilla luciferase internal control vector, pRL-TK (20 ng/well), using Lipofectamine™ 2000 reagent. Twenty-four hours after transfection, cells were stimulated with R848 (2 μg/well). After stimulation for 8 hours, cells were lysed in lysis buffer (Promega). By using Dual-Luciferase Reporter Assay System (Promega), firefly luciferase and Renilla luciferase were measured. Relative luciferase activity was calculated by the ratio of firefly luciferase activity to Renilla luciferase activity.

1.6　Immunoprecipitation and immunoblotting　HEK293T cells were cultured in 6-well plates and were transfected with expression vectors wild-type TLR7, TLR7 truncating mutants, or site-direct mutants (4 μg/well). After 24 h, the cells were lysed and collected with lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 10 mmol/L EDTA, 1% Nonidet P40, 25 mmol/L iodoacetamide, 2 mmol/L phenylmethylsulfonyl fluoride), which was supplemented with Complete Protease Inhibitor (Roche Applied Science). The homogenates were centrifuged at 13 000×g for 30 min at

4℃, and the supernatants were mixed with 10 μl anti-c-Myc affinity agarose (Thermo) and incubated overnight at 4℃. The immunocomplexes were washed three times with TBS-T buffer (TBS plus 0.05% Tween-20). Then the mixtures were eluted into 30 μl Elution Buffer (Thermo). Samples and cell lysintes were analyzed by SDS-PAGE (8% or 12%) under reducing conditions followed by immunoblotting with anti-TLR7 or anti-MyD88 antibody.

2 Results

2.1 TLR7 truncating mutants analysis The TIR domain of TLR7 consists of about 160 amino acids. As there are no certain sorting signal motifs in the cytoplasmic tail of TLR7, we firstly constructed two truncation mutants of TLR7 which contain the whole TIR region deletion (TLR7ΔTIR) and half TIR region deletion (about 80 amino acids, TLR7Δ80) of TLR7. To detect the function of two mutants, HEK293T cells were transfected with TLR7ΔTIR, TLR7Δ80, wild-type TLR7 or empty vector. After transfection cells were stimulated by R848, using NF-κB transcription activity as an indicator, we tested the relative luciferase activity by reporter gene assay (Fig. 1). Both TLR7ΔTIR and TLR7Δ80 were insensitive to R848 stimuli, which indicated that the critical region for TLR7 signal transduction may locate in the second half of TIR domain.

To identify the accurate region for TLR7 signal transduction, we generated three more truncation mutants in second half of TIR domain (Fig. 2). Approximately 20 more amino acids were deleted in each mutant compared with the previous one. These mutants were named as TLR7Δ20, TLR7Δ40 and TLR7Δ60. As described previously, these mutants were transfected into HEK293T cells and then were measured by reporter gene assay (Fig. 3). The mutant named TLR7Δ60 inhibited TLR7-mediated NF-κB signal pathway activation. These results suggested that the crucial region between TLR7Δ40 and TLR7Δ60 would be essential for signal transduction of TLR7.

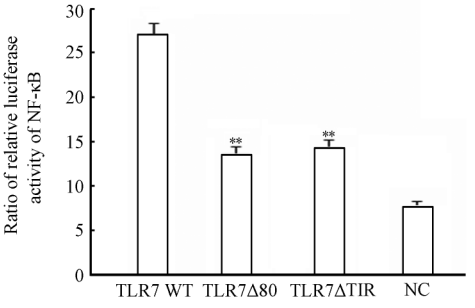

Fig 1 Activation of NF-κB by TLR7 whole or half TIR region truncating mutants in HEK293T cells

HEK293T cells in 48-well plates were transfected with 400 μg expression plasmids encoding empty vector, wild-type TLR7 (TLR7 WT), TLR7ΔTIR or TLR7Δ80 truncating mutants by using Lipofectamine 2000. After 42 h, cells were stimulated with R848 (500 ng/ml) for 6 h. Then cells were harvested and lysed in passive lysis buffer, and luciferase reporter gene activity was measured. NC: Negative control. ** $P<0.01$ vs TLR7 WT. $n=3$, $\bar{x}\pm s$

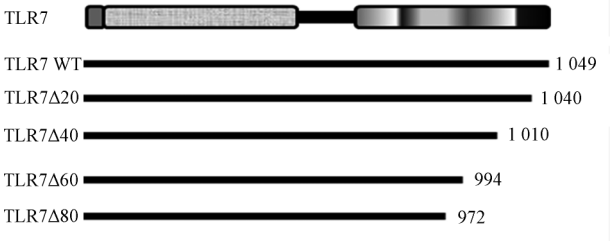

Fig 2 Schematic diagram of TLR7 truncation mutants used in this study

TLR7Δ20 signifies the mutant truncated approximate 20 amino acids. Others are named in the same manner. TLR7 WT: Wild-type TLR7

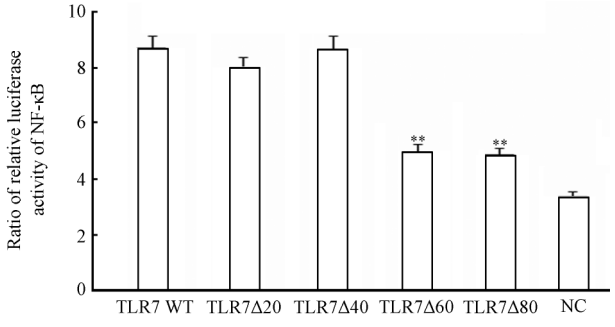

Fig 3 Activation of NF-κB by TLR7 truncating mutants in HEK293T cells

HEK293T cells were transfected with empty vector, wild-type TLR7 (TLR7 WT) or TLR7 truncating mutants as in Fig 1. After 42 h, cells were stimulated with R848 for 6 h, and luciferase reporter gene activity was measured. NC: Negative control. ** $P<0.01$ vs TLR7 WT. $n=3$, $\bar{x}\pm s$

2.2　RXR signal motif in crucial region impaired TLR7 signal transduction　The crucial region between TLR7Δ40 and TLR7Δ60 consists of 16 amino acids. Although the certain sorting signal motif in TLR7 cytoplasmic region is not clear, some typical motifs have been reported in many other molecules. Di-arginine motif RR or RXR, as an endoplasmic reticulum (ER) processing signal, has been identified just in the crucial region. Hence, to locate the critical amino acid sequence in this region, we replaced two conservative arginines at 1004 and 1006 by glycins. DNA sequences were confirmed by sequencing.

To identify the function of RXR motif in the crucial region, wild-type TLR7 and the mutant (TLR7 R1004GR1006G) were transfected into HEK293T cells respectively and NF-κB relative luciferase activity was measured after R848 stimulated (Fig. 4). The relative luciferase activity was reduced by half compared with wild-type TLR7. These results suggested that RXR signal motif is essential for signal transduction of TLR7.

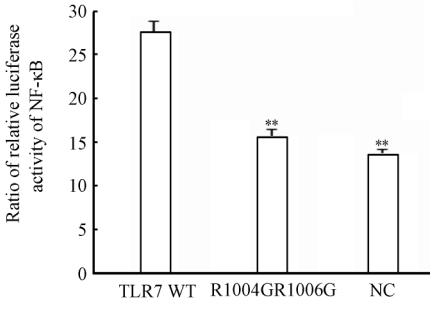

Fig 4　Activation of NF-κB by TLR7 RXR motif mutant in HEK293T cells

HEK293T cells were transfected with wild-type TLR7(TLR7 WT), the mutant TLR7 R1004GR1006G or empty vector as in Fig 1. After R848 stimulation for 6 h, relative luciferase activity of NF-κB was measured. NC: Negative control. ** $P<0.01$ vs TLR7 WT. $n=3$, $\bar{x}\pm s$

2.3　Arginines at 1004 and 1006 have different effects on TLR7 signal transduction　In order to validate whether there are interactions between these two arginines and their adjacent amino acids, five single amino acid mutants, TLR7 L1003P, TLR7 R1004G, TLR7 K1005E, TLR7 R1006G and TLR7 L1007P, were constructed by site-direct mutagenesis (Fig. 5). DNA sequences were confirmed by sequencing.

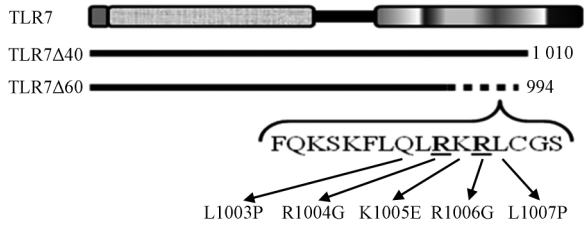

Fig 5　Schematic diagram of TLR7 truncation mutants used in this study

TLR7 L1003P signifies a mutant with leucine replaced with proline at position 1003. Others are named in the same manner

Reporter gene assay revealed that, after stimulated by R848, only TLR7-R1004G and TLR7-R1006G depressed the relative luciferase activity of NF-κB, while other mutants remained the level compared with wild-type TLR7 (Fig. 6). However, it is worth noting that the impact to NF-κB activation by R1004G is higher than by R1006G. These results suggested that although both arginines at 1004 and 1006 could impact TLR7 signal transduction, R1004G is more effective than R1006G.

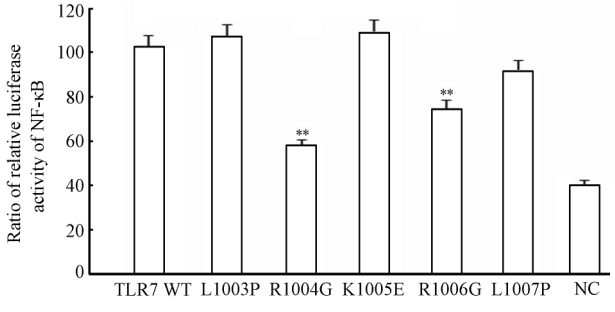

Fig 6　Activation of NF-κB by TLR7 site-direct mutants in HEK293T cells

HEK293T cells were transfected with wild-type TLR7(TLR7 WT), TLR7 site-direct mutants or empty vector as in Fig 1. After R848 stimulation for 6 h, relative luciferase activity of NF-κB was measured. NC: Negative control. ** $P<0.01$ vs TLR7 WT. $n=3$, $\bar{x}\pm s$

2.4　Arginine at 1004 is the only amino acid essential for binding with MyD88　MyD88, as an adapter protein, is reported to be essential for TLR7 signal pathway. However, the region of TLR7 TIR domain binding to MyD88 is not clear. To examine the relationship between TLR7 mutants and MyD88, co-immunoprecipitation studies were performed.

Firstly, we detected the combination between TLR7 truncating mutants and MyD88. HEK293T cells were co-transfected with MyD88-Myc and wild-type TLR7 or TLR7 truncation mutants. By using immunoprecipitation and immunoblotting assay, we found three truncation mutants, TLR7Δ60, TLR7Δ80 and TLR7ΔTIR, did not co-immuoprecipitate with MyD88-Myc after R848 stimuli (Fig. 7A). The results coincided with reporter gene assay, suggesting that the crucial region between TLR7Δ40 and TLR7Δ60 would impact signal transduction via changing the ability of binding MyD88.

Then we tested TLR7 RXR signal motif mutant by using of immunoprecipitation and immunoblotting assay (Fig. 7B). Compared with wild-type TLR7, the mutants' ability of binding to MyD88 was greatly decreased. The result indicated that RXR motif between TLR7Δ40 and TLR7Δ60 is significant for TLR7 signal transduction by impacting the binding with MyD88.

We also performed immunoprecipitation and immunoblotting to further investigate the binding site to MyD88 in TLR7 TIR region (Fig. 7C). To our surprise, TLR7 R1004G was the only mutant which could not co-precipitate with MyD88. These results suggested that arginine at 1004 is the only amino acid critical for activation of TLR7 signal pathway by impacting the binding with MyD88.

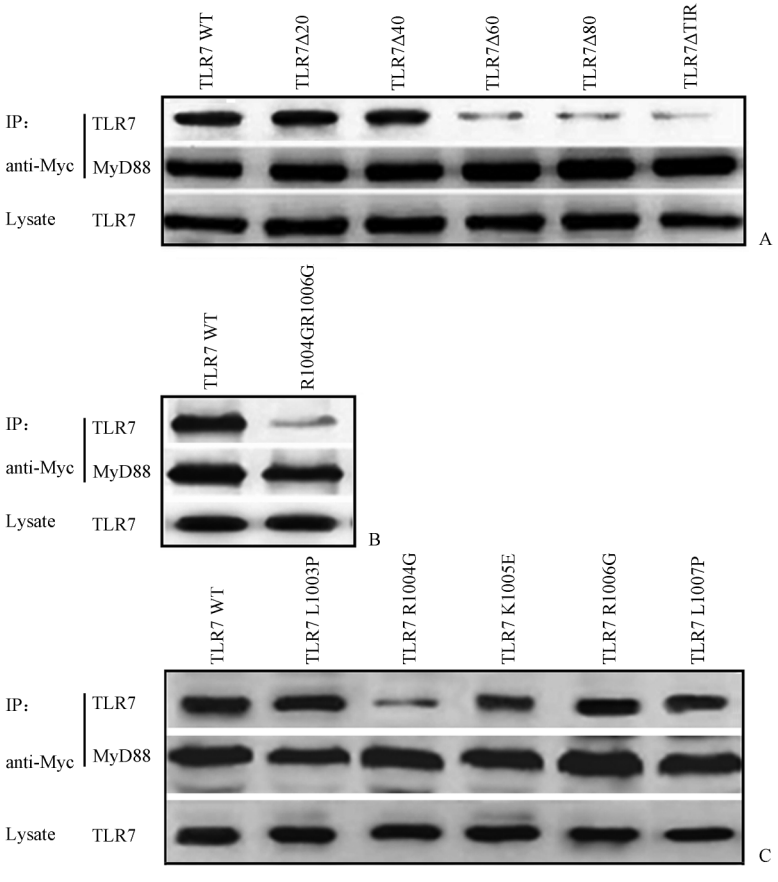

Fig 7　Binding between MyD88 and TLR7 mutants in HEK293T cells

HEK293T cells were co-transfected with plasmids expressing MyD88-Myc and TLR7 truncating mutants (A), TLR7 RXR motif mutant (B), TLR7 site-direct mutants(C), or wild-type TLR7 (TLR7 WT). After 24 h, cells were stimulated with R848 for 6 h, then cells were collected and lysed by using of the lysis buffer. MyD88-myc was immunopecipitated from lysates of the cells using anti-c-Myc affinity agarose (Thermo). The immunoprecipitates were then subjected to immunoblotting with anti-TLR7 antibodies to detect the co-immunoprecipitation of MyD88-TLR7 mutants. The immunoprecipitates were also subjected to immunoblotting with anti-MyD88 as control groups. Immunoblottings with anti-TLR7 were also performed in whole cell lysates to confirm comparable levels of TLR7 mutants and wild-type expression

3 Discussion

TLR7 signal pathway results in activation of transcription factor NF-κB, which then leads to production of inflammatory cytokines and type Ⅰ IFN[2]. In this study, we performed truncating mutagenesis and site-direct mutagenesis analysis to illustrate the mechanisms of TLR7 intracellular signal transduction. We identified a region, locating between TLR7Δ40 and TLR7Δ60, consisting of 16 amino acids, could be a crucial part for TLR7 signal transduction. This region was proven to be essential for the binding between TLR7 and MyD88. Further study indicated that the two arginines at 1004 and 1006 in this region could impact the activation of NF-κB, and only arginine at 1004 is essential for conjunction of MyD88.

Studies by population genetics have indicated that there are many single-nucleotide polymorphisms in TLR7 that could be associated with the risk of many diseases[10]. For example, rs179008, the glutamine at 32, changes into leucine or proline, increases the risk of age-related macular degeneration (AMD), asthma and related disorders[10-11]. Another SNP in TLR7, c. 1-120T＞G, is reported to protect male patients with chronic HCV-infection from inflammation and fibrosis[12]. As the mutants at 1004 or 1006 could impair TLR7 signal transduction dramatically, those arginines could be a potential target for therapy of diseases which depend on TLR7 signal pathway.

As TLR7 recognizes ssRNAs specifically, TLR7 signal pathway is indispensable for organism to resist virus infection. Our previous study has shown that TLR7 is a essential receptor for IFN induction during HCV infection[13]. In this study, we found the crucial site for TLR7 signal activation. So our findings provide important information for understanding TLR7 activation during natural immune response.

TLR signal transduction depends on the interaction between TIR domain and TIR-containing adaptor molecules such as MyD88. BB loop is reported to enable interactions between certain TLRs and certain adaptor molecules[14]. For example, a mutation within the TLR4 BB loop, Pro712His, in the C3H/HeJ mouse has been identified because it could abolish LPS signaling[15-16]. Importantly, TLR4 Pro712His does not change the overall structure of the TIR domain, and the association of TLR4 Pro712His and its adaptor molecules is reported to be intact[17-18]. In contrast with TLR4, it is reported that Pro434 mutation in BB loop could arrest signal transduction by impacting homo-oligomerization in TLR3 pathway[19]. In our study, interestingly, TLR7 transmits its signal in a BB loop-independent manner. We found two arginines in the same signal motif that could abolish TLR7 signal transduction, but only Arg1004 had the ability to impact the binding between TLR7 and MyD88. To conclude, our findings offer new evidences for understanding TLR7 signal transduction, and provide new potential targets for immunotherapy through TLR7 pathway.

4 Conflict of interest

The authors declare that there is no conflict of interest.

[References]

[1] Botos I, Liu L, Wang Y, Segal D M, Davies D R. The Toll-like receptor 3:dsRNA signaling complex[J]. Biochim Biophys Acta, 2009, 1789(9-10):667-674.

[2] Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors[J]. Nat Immunol, 2010, 11:373-384.

[3] Jin M S, Lee J O. Structures of the Toll-like receptor family and its ligand complexes[J]. Immunity, 2008, 29: 182-191.

[4] Lysakova-Devine T, Keogh B, Harrington B, Nagpal K, Halle A, Golenbock D T, et al. Viral inhibitory peptide of TLR4, a peptide derived from vaccinia protein A46, specifically inhibits TLR4 by directly targeting MyD88 adaptor-like and TRIF-related adaptor molecule[J]. J Immunol, 2010, 185:4261-4271.

[5] Takada E, Okahira S, Sasai M, Funami K, Seya T, Matsumoto

M. C-terminal LRRs of human Toll-like receptor 3 control receptor dimerization and signal transmission[J]. Mol Immunol, 2007,44:3633-3640.

[6] Bell J K,Mullen G E,Leifer C A,Mazzoni A,Davies D R,Segal D M. Leucine-rich repeats and pathogen recognition in Toll-like receptors[J]. Trends Immunol,2003,24:528-5233.

[7] Smits E L,Ponsaerts P,Berneman Z N,Van Tendeloo V F. The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy[J]. Oncologist,2008,13:859-875.

[8] Kawai T,Akira S. Innate immune recognition of viral infection [J]. Nat Immunol,2006,7:131-137.

[9] Christensen S R,Shupe J,Nickerson K,Kashgarian M,Flavell R A,Shlomchik M J. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus[J]. Immunity,2006, 25:417-428.

[10] Edwards A O,Chen D,Fridley B L,James K M,Wu Y,Abecasis G,et al. Toll-like receptor polymorphisms and age-related macular degeneration[J]. Invest Ophthalmol Vis Sci,2008,49: 1652-1659.

[11] Møller-Larsen S,Nyegaard M,Haagerup A,Vestbo J,Kruse TA,Børglum A D. Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders[J]. Thorax,2008,63:1064-1069.

[12] Schott E,Witt H,Neumann K,Taube S,Oh D Y,Schreier E,et al. A Toll-like receptor 7 single nucleotide polymorphism protects from advanced inflammation and fibrosis in male patients with chronic HCV-infection[J]. J Hepatol,2007,47:203-211.

[13] Zhang Y L,Guo Y J,Bin Li,Sun S H. Hepatitis C virus single-stranded RNA induces innate immunity via Toll-like receptor 7 [J]. J Hepatol,2009,51:29-38.

[14] Toshchakov V Y,Vogel S N. Cell-penetrating TIR BB loop decoy peptides a novel class of TLR signaling inhibitors and a tool to study topology of TIR-TIR interactions[J]. Expert Opin Biol Ther,2007,7:1035-1050.

[15] Poltorak A,He X,Smirnova I,Liu M Y,Van Huffel C,Du X,et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene[J]. Science, 1998, 282: 2085-2088.

[16] Toshchakov V Y,Fenton M J,Vogel S N. Cutting edge: differential inhibition of TLR signaling pathways by cell-permeable peptides representing BB loops of TLRs[J]. J Immunol,2007, 178:2655-2660.

[17] Fitzgerald K A,Palsson-McDermott E M,Bowie A G,Jefferies C A,Mansell A S,Brady G,et al. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction[J]. Nature,2001,413:78-83.

[18] Dunne A,Ejdeback M,Ludidi P L,O'Neill L A,Gay N J. Structural complementarity of Toll/interleukin-1 receptor domains in Toll-like receptors and the adaptors Mal and MyD88 [J]. J Biol Chem,2003,278: 41443-41451.

[19] Funami K,Sasai M,Oshiumi H,Seya T,Matsumoto M. Homooligomerization is essential for Toll/interleukin-1 receptor domain-containing adaptor molecule-1-mediated NF-kappaB and interferon regulatory factor-3 activation[J]. J Biol Chem,2008, 283:18283-18291.

[**Editor**] JIA Ze-jun

Toll 样受体 7 胞内区功能关键位点筛选及其机制研究

魏　伟,章意亮,郭瀛军,孙树汉*

第二军医大学基础部医学遗传学教研室,上海 200433

[**摘要**] **目的**　筛选出 TLR7 TIR 区对其信号传递起关键作用的位点,并解释该位点的作用机制。**方法**　针对 TLR7 野生型序列,设计构建一系列 TLR7 缺失突变与点突变质粒。将这些质粒转染入 HEK293T 细胞,利用双荧光素酶报告基因检测这些质粒对 NF-κB 信号通路的影响。利用免疫共沉淀与蛋白质印迹实验验证 TLR7 突变体与其下游接头蛋白 MyD88 结合能力的变化。**结果**　通过对 TLR7 胞内区截短突变分析,发现在其 TIR 区存在一个由 16 个氨基酸残基组成的区域对其信号传递至关重要。进一步的研究发现,该区域存在一个 RXR 信号基序。该基序位于 1004 位与 1006 位的两个保守精氨酸残基,为 TLR7 正常信号传递所必需。通过免疫共沉淀与蛋白质印迹实验,可以发现 TLR7 位于 1004 位的精氨酸对其与下游蛋白 MyD88 的结合至关重要。**结论**　成功鉴定出 TLR7 TIR 区 1004 位的精氨酸对其信号传递起重要作用,该位点是通过影响 TLR7 与 MyD88 结合的方式发挥功能。

[**关键词**] Toll 样受体 7;天然免疫;信号转导

[**中图分类号**] R 392.111　　[**文献标志码**] A　　[**文章编号**] 0258-879X(2012)03-0233-07