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Short communication

Mutational analysis identifies leucine-rich repeat insertions crucial for pigeon toll-like receptor 7 recognition and signaling

Dan Xiong[a,b,1], Li Song[a,b,1], Yang Jiao[a,b], Xilong Kang[a,b], Xiang Chen[a,b], Shizhong Geng[a,b], Zhiming Pan[a,b,*], Xinan Jiao[a,b,*]

[a] Jiangsu Key Laboratory of Zoonosis, Yangzhou University, Yangzhou, Jiangsu 225009, China
[b] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, Jiangsu 225009, China

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ABSTRACT

Toll-like receptor 7 (TLR7) is responsible for recognizing viral single-stranded RNA and antiviral imidazoquinoline compounds, leading to the activation of the innate immune response. In this study, mutated pigeon TLR7 fragments, in which the insertion at position 10 of leucine-rich repeat 10 (LRR10) or at position 15 of LRR2/11/13/14 was deleted, were amplified with an overlap-PCR method, and inserted into the expression vector pCMV. The immune functions of the TLR7 mutants were determined with an NF-κB luciferase assay of transfected cells. The deletion of the insertions absolutely abolished TLR7–NF-κB signaling. With quantitative real-time PCR and sandwich enzyme-linked immunosorbent assay, we observed that stimulation with R848 failed to induce the expression of interleukin 8 (IL-8) in any of the mutant-TLR7-transfected cells, consistent with their lack of NF-κB activity. However, the expression of interferon α (IFN-α) and tumor necrosis factor α (TNF-α) was significantly upregulated in the Del10IN10 and Del14IN15 groups. Remarkably, the levels of pigeon TLR7 expression were significantly increased in all the TLR7-mutated groups. Therefore, we speculate that another part of the deficient TLR7 mediates the induction of IFN-α and TNF-α by increasing the expression of TLR7 as compensation. However, the increased expression of TLR7 in the Del11IN15 group failed to induce the production of IFN-α, IL-8, or TNF-α, indicating that a false compensation occurred when the crucial LRR insertion was deleted.

1. Introduction

The innate immune system is activated through pattern recognition receptors (PRRs) to defend against invading pathogens by recognizing pathogen-associated molecular patterns (PAMPs) (Takeda et al., 2003). Toll-like receptor 7 (TLR7) recognizes viral single-stranded RNAs and antiviral imidazoquinoline compounds (Akira et al., 2006). Upon activation, TLR7 initiates the activation of two major signaling pathways through myeloid differentiation primary response protein 88 (MyD88) and/or interferon regulatory factor 7 (IRF7), resulting in the expression of proinflammatory cytokines and/or type I interferons (IFNs) (Negishi et al., 2006). A study has shown that an amino acid insertion in leucine-rich repeat 8 (LRR8) of the human TLR8 ectodomain is essential for TLR signaling (Gibbard et al., 2006). Current evidence showed that the residues inserted at position 15 in LRR14 of human TLR5 contributed to flagellin binding, indicating that these irregular LRRs are crucial for PAMP recognition (Mizel et al., 2003).

Recently, we cloned the pigeon TLR7 (PiTLR7) gene and identified its immune function (Xiong et al., 2015). The prevailing LRR consensus sequence of the TLRs is the 24-residue motif XLXXLXLXXNXφXXφXXXXFXXLX (Medzhitov, 2001). A sequence analysis of PiTLR7 showed that it contains 15 instances of this motif. However, several LRRs have been modified with amino acid insertions relative to the general motif. In particular, various amino acids are inserted at position 15 of LRR2, LRR11, LRR13, and LRR14. LRR10 is markedly different from the other repeats, containing long insertions at position 10 after the consensus Asn residue (Xiong et al., 2015). In the present study, PiTLR7 mutants

* Corresponding authors at: Jiangsu Key Laboratory of Zoonosis, Yangzhou University, 48 East Wenhui Road, Yangzhou, Jiangsu 225009, China.
E-mail addresses: xiongdan_yzu@163.com (D. Xiong), songli_yzu@163.com (L. Song), 983899312@qq.com (Y. Jiao), 455157409@qq.com (X. Kang), chenxiang@yzu.edu.cn (X. Chen), gszhong@yzu.edu.cn (S. Geng), zmpan@yzu.edu.cn (Z. Pan), jiao@yzu.edu.cn (X. Jiao).
[1] These authors contributed equally to this work.

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Table 1
PCR primers used in this study.

Primer name	Primer sequence (5′ → 3′)	Size (bp)	GenBank	Application
PiTLR7a F	ACGCGTCGACCATGGTACTTAGTGCAGAAGAGCCAAATAC	3144	KM086724	Amplification of Pigeon TLR7
PiTLR7b R	CCCCGGTACCCTAAACAGTTTCTTGGAGAAGCTTGTTG			
Del2IN15a R	TCCTTTAAAGTAGATGACAGAATCTGAGTTAAGTTGTTAG	3135		Amplification of Pigeon TLR7 – DelLRR2IN15
Del2IN15b F	CTAACAACTTAACTCAGATTCTGTCATCTACTTTAAAGGA			
Del10IN10a R	CCTCTGAATTCTAAAATCTCATTCATCATCAGCTTCTTCA	3093		Amplification of Pigeon TLR7 – DelLRR10IN10
Del10IN10b F	TGAAGAAGCTGATGATGAATGAGATTTTAGAATTCAGAGG			
Del11IN15a R	ACCTTGAGTCCTGGAGGCATCAAAAAACTGAGTGAGTTGA	3123		Amplification of Pigeon TLR7 – DelLRR11IN15
Del11IN15b F	TCAACTCACTCAGTTTTTTGATGCCTCCAGGACTCAAGGT			
Del13IN15a R	TCTTGCAGCACTGAAGAGCAAACAGTAGTCAGAAGGTTAT	3126		Amplification of Pigeon TLR7 – DelLRR13IN15
Del13IN15b F	ATAACCTTCTGACTACTGTTTGCTCTTCAGTGCTGCAAGA			
Del14IN15a R	TATTTCAATTGAAAAGCACCTAGCCGTTGAATGCGATTGT	3126		Amplification of Pigeon TLR7 – DelLRR14IN15
Del14IN15b F	ACAATCGCATTCAACGGCTAGGTGCTTTTCAATTGAAATA			
Huβ-actin F	GGACTTCGAGCAAGAGATGG	234	X00351	Quantitative real-time PCR
Huβ-actin R	AGCACTGTGTTGGCGTACAG			
PiTLR7 F	ACCAGCGGCTTCTAGATGAA	158	KM086724	Quantitative real-time PCR
PiTLR7 R	CTGCCAAAAGTAGGGCTGAG			
HuIFN-α F	GCAAGCCCAGAAGTATCTGC	241	NM_024013	Quantitative real-time PCR
HuIFN-α R	ACTGGTTGCCATCAAACTCC			
HuIL-8 F	TAGCAAAATTGAGGCCAAGG	227	NM_000584	Quantitative real-time PCR
HuIL-8 R	AAACCAAGGCACAGTGGAAC			
HuTNF-α F	TCCTTCAGACACCCTCAACC	173	NM_000594	Quantitative real-time PCR
HuTNF-α R	AGGCCCCAGTTTGAATTCTT			

were constructed in which the insertions in the LRRs were deleted. Using transfected cell lines as the model system, we analyzed the potential importance of the insertions in these LRRs in receptor signaling.

2. Materials and methods

2.1. DNA constructs

Vectors expressing each individual mutated PiTLR7 gene were constructed as described previously (Xiong et al., 2015). The mutated TLR7 fragments, in which the insertions at position 10 of LRR10 or at position 15 of LRR2, LRR11, LRR13, or LRR14 were deleted, were amplified with an overlap-PCR method using two pairs of primers: PiTLR7a-F/DelxINya-R and DelxINyb-F/PiTLR7b-R (x refers to LRR2, 10, 11, 13, or 14, and y refers to position 10 or 15) (Table 1). *Sal*I and *Kpn*I restriction enzyme sites were introduced into PiTLR7a-F and PiTLR7b-R, respectively (underlined in Table 1). The mutated TLR7 fragments were inserted into the eukaryotic expression vector pCMV-HA (Invitrogen, Carlsbad, CA, USA) digested with *Sal*I and *Kpn*I (Takara Biotechnology Co., Dalian, China) to create pCMV-PiTLR7DelxINy. The sequences of the resulting constructs were confirmed with DNA sequencing (Genscript, Nanjing, China).

2.2. Cell culture, transfection, and stimulation

The human embryonic kidney HEK293T cells from American Type Culture Collection (ATCC, Manassas, VA, USA) were grown in 24-well tissue culture plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) until they reached 70% confluence. The cells were washed with phosphate-buffered saline before transfection, and the medium was then replaced with Opti-MEM™ medium (Invitrogen). The cells were transiently transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. HEK293T cells were transfected with equal amounts of the DNA constructs, including pCMV-PiTLR7, the pCMV-PiTLR7 mutants, or pCMV-empty, and 200 ng of the reporter plasmid pGL4.32 (luc2P/NF-κB-RE/Hygro) (Promega, Madison, WI, USA). The equal amount of complete DMEM was added five hours after transfection. Twenty-four hours after transfection, endotoxin-free R848 (Enzo Life Sciences, Farmingdale, NY, USA), a TLR7 agonist, was added to a final concentration of 2.5 μg/mL. After stimulation for 5 h, the cells were harvested for analysis with a luciferase assay or quantitative real-time PCR, and the supernatants were collected for analysis with sandwich enzyme-linked immunosorbent assay (ELISA).

2.3. Luciferase assay

To determine the immune function of PiTLR7 and the mutants after R848 stimulation, NF-κB-induced luciferase activity was measured using the Bright-Glo Luciferase Assay System (Promega), according to the manufacturer's instructions.

2.4. RNA isolation, RT-PCR, and quantitative real-time PCR

The harvested cells were homogenized in TRIzol Reagent (Invitrogen), and total RNA was prepared as described by the manufacturer, and reverse transcribed into cDNA using the PrimeScript RT Reagent Kit (Perfect Real Time; Takara Biotechnology Co.), according to the manufacturer's instructions. The cDNA was quantified with a Nanodrop™ spectrophotometer, and then diluted to 40 ng/μL. Quantitative real-time PCR (qRT-PCR) was used to measure the mRNA expression levels of pigeon TLR7, human interferon α (IFNA), human interleukin 8 (IL8), and human tumor necrosis factor α (TNFA) using SYBR Premix Ex Taq II (Perfect Real Time; Takara Biotechnology Co.) with the ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and specifically designed primers (Table 1). The amplification reaction and the real-time PCR program were conducted as described previously (Xiong et al., 2015). A dissociation analysis of the amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The data were analyzed with the ABI 7500 SDS software (Applied Biosystems, Foster, CA, USA) with the baseline set automatically by the software. The threshold method was used to quantify the mRNA levels (Livak and Schmittgen, 2001). The ΔC_T values were calculated based on the internal standard signal (*ACTB*, β-actin) and the results were expressed as $2^{-\Delta\Delta C(T)}$.

2.5. Enzyme-linked immunosorbent assay

To determine the secreted cytokines of HEK293T cells transfected with the PiTLR7 or mutants after R848 stimulation, supernatants were collected and expression levels of cytokines