Immunology Letters 178 (2016) 50–60

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Identification and characterization of the toll-like receptor 8 gene in the Chinese raccoon dog (*Nyctereutes procyonoides*)

Yong Yang[a,b,1], Mingwei Tong[b,1], Li Yi[b,1], Yuening Cheng[b], Miao Zhang[b], Zhigang Cao[b], Jianke Wang[b], Peng Lin[b], Shipeng Cheng[b,*]

[a] *Wu Xi Medical School, Jiangnan University, China*
[b] *Institute of Special Economic Animal and Plant Science, Chinese Academy of Agricultural Sciences, China*

ARTICLE INFO

Article history:
Received 17 March 2016
Received in revised form 11 July 2016
Accepted 25 July 2016
Available online 29 July 2016

Keywords:
TLR8
Nyctereutes procyonoides
Sequence analysis
Gene expression

ABSTRACT

TLR8 is an important sensor of single-stranded RNA (ssRNA) from the viral genome and plays an essential role in innate antiviral responses via the recognition of conserved viral molecular patterns. In this report, TLR8 in the Chinese raccoon dog was characterized and analyzed for the first time. The full-length sequence of raccoon dog TLR8 (RdTLR8) cDNA was cloned by rapid amplification of cDNA ends (RACE) and is 3191bp with a 3117-bp open reading frame (ORF) encoding 1038 amino acids. The putative protein exhibits typical features of the TLR families, with 19 leucine-rich repeats (LRRs) in the extracellular domain and a cytoplasmic TIR domain. Comparative analyses of the RdTLR8 amino acid sequence indicated a 73.6–99.4% sequence identity with dog, horse, pig, sheep, cattle, human and mouse TLR8. Phylogenetic analysis grouped 71 mammalian TLR proteins into five sub-families, wherein RdTLR8 was clustered into a monophyletic TLR8 clade in the TLR9 family, which was completely coincident with the evolutionary relationship among mammals. Quantitative real-time PCR analysis revealed extensive expression of RdTLR8 in tissues from healthy Chinese raccoon dogs with the highest expression in the peripheral blood mononuclear cells (PBMCs) and the lowest expression in the skeletal muscle. HEK293 cells cotransfected with a RdTLR8 expression plasmid and an NF-κB-luciferase reporter plasmid significantly responded to the agonist 3M-002, indicating a functional TLR8 homolog. In addition, raccoon dog PBMCs exposed to the canine distemper virus (CDV) wild strain CDV-PS and the TLR8 agonist 3M-002 showed significant upregulation of RdTLR8 mRNA and proinflammatory cytokines TNF-α and IFN-α, suggesting that RdTLR8 might play an important role in the immune response to viral infections in the Chinese raccoon dog.

1. Introduction

The innate defense system provides an essential first line of protection against invading microorganisms and depends on germline-encoded pattern recognition receptors (PRRs) that have evolved to detect relatively conserved components of pathogens referred to as pathogen associated molecular patterns (PAMPs) [1,2]. One of the first discovered and the most extensively studied PRR is the family of Toll-like receptors (TLRs) [3,4]. TLRs are type I transmembrane proteins that contain an N-terminal ectodomain (ECD) with a leucine-rich repeat region (LRR) and an intracellular C-terminus with a Toll–interleukin (IL)-1 receptor (TIR) domain. The LRR is involved in the recognition of PAMPs, while the cytoplasmic TIR domain is required for downstream signal transduction [5,6]. To date, at least ten mammalian TLRs have been well characterized. Based on the phylogenetic, structural, and functional similarities of those TLRs, TLR7, TLR8 and TLR9 form a subfamily of TLRs that are mainly expressed on endosomal membranes, with the exception of a small population of TLR8 that is also expressed on the cell surface [7].

TLR8 can recognize microbial ssRNA and is involved in the protection against viruses. Activation of TLR8 induces production of the proinflammatory cytokines TNF-α and type I IFNs [8], verifying its essential role in the host immune system. In humans, variations in the TLR8 gene have been linked to a higher susceptibility to asthma, TB and HIV infections [9]. Polymorphism within TLR8 has recently been reported to be associated with the modulation of TLR8-dependent microbicidal response of infected macrophages [10]. Furthermore, a novel role for TLR8 has been studied. These

* Corresponding author.
E-mail address: tcs_csp@163.com (S. Cheng).
[1] Yong Yang, Ming wei Tong and Li Yi had contributed equally to this article.

http://dx.doi.org/10.1016/j.imlet.2016.07.012
0165-2478/

Table 1
Primers used for this study.

Primer names	Primer sequences	Products	Purpose	Annealing temperature
F1	CTTGGGTGTCAACTTTATT	P1	Partial cloning	47
R1	CTAACTCGGGCAGATCACT			
F2	AAACCAGTTACTCCTTGAA	P2	Partial cloning	52
R2	TTGAGAAGAAGAGCCATTT			
F3	TGAGCCACAACAGCATCTA	P3	Partial cloning	53
R3	AAATCTCCAATGTCGCAAG			
F4	GAAAACATGAGCCCTCGGTC	P4	Partial cloning	53
R4	CTTCAGGGAGCTTGGCAGT			
F5	TACTTGCGACATTGGAGATT	P5	Partial cloning	52
R5	TTATTATGTTGGCGGGTCAT			
F6	ATGAGCCCTCGGTCTTTGGTTCTG		Sequence verification	54
R6	GTTGGCGGGTCATGGCTTAA			
GAPDH F	GGAGAAAGCTGCCAAATATG		Tissue distribution	58
GAPDH R	ACCAGGAAATGAGCTTGACA			
TLR8 F	TCAGCGGATATGGAGTTTG		Tissue distribution	50
TLR8 R	CCTGCTATTCGGAAGTAGTG			
TNF-α F	ACGTGCCAATGCCCTCCTGG		qRT-PCR	58
TNF-α R	AGACGGCGAAGCGGCTGATG			
IFN-α F	CTTTAGTCATCCGCAAACCTG		qRT-PCR	50
IFN-α R	CTTGCCCTGAGCCAAAGATAG			

studies suggest that it can act as a suppressor of neurite outgrowth and an inducer of neuronal apoptosis, indicating that it may be involved in a variety of physiological processes in mammals [11]. Until now, TLR8 has been identified in humans, sheep, cows, pigs and goats [12–15]. Although found in many mammals, TLR8 has not been reported in the raccoon dog (*Nyctereutes procyonoides*), which is an important commercial fur animal species and is widely cultured in North-Eastern China and other parts of the world. In recent years, the health of the raccoon dog has constantly been threatened by outbreaks of diseases caused by various pathogens including canine distemper virus (CDV) resulting in huge economic losses [16].

Therefore, this work was undertaken to describe the cloning and characterization of the RdTLR8 sequence and analysis of its expression in various tissues. Additionally, raccoon dog PBMCs were stimulated with the mammalian TLR8 agonist 3M-002 and infected with CDV *in vitro*. We used a qRT-PCR assay to detect RdTLR8 and the relative mRNA expression levels of the proinflammatory cytokines TNF-α and IFN-α. Overall, these data yield insights into the immunological characteristics of RdTLR8, and these results will provide a molecular foundation for further investigation into the potential role of RdTLR8 in anti-viral therapeutics and vaccine design.

2. Materials and methods

2.1. Animal virus strains and tissue samples

Biological samples were collected from raccoon dogs (approximately 12 months old) that were provisionally kept under the same nutrient and management conditions at the animal house of the Central Laboratory for Animal Diseases of the Institute of Special Animal and Plant Sciences, Jilin, China. These raccoon dogs had not been vaccinated against any diseases and had no prior record of any disease. All experiments were performed in accordance with the Animal Experimentation Guidelines of the Institute of Special Animal and Plant Sciences and approved by the Institution Animal Care and Use Committee of the Institute of Special Animal and Plant Sciences. The canine distemper virus, CDV-PS strain (a high virulence field isolate), which has a $10^{-3.13}$/mL tissue culture infective dose ($TCID_{50}$), was provided by the Central Laboratory for Animal Diseases of the Institute of Special Animal and Plant Sciences.

Peripheral blood and tissue samples were collected following humane sacrifice of raccoon dogs. PBMCs were separated using Histopaque 1077 (Sigma-Aldrich, MO, USA) according to the manufacturer's instructions. Cells were subsequently washed three times with phosphate buffered saline (PBS), and 5×10^6 live cells were used for RNA isolation. Tissue samples containing lymph node, spleen, skin, liver, duodenum, lung, kidney, heart, uterus and skeletal muscle were collected in sterile tubes, frozen on dry ice and stored at −80 °C for the extraction of total RNA.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from PBMCs and collected tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's protocol. RNA samples were incubated with gDNA Removal (TransGen Biotech, Beijing, China) to avoid contamination with genomic DNA. The concentration of RNA was determined using BioPhotometer plus by measuring absorbance at 260 nm. The ratios of absorption (260/280 nm) of all samples were between 1.8 and 2.0 Aliquots of RNA were subjected to electrophoresis through a 1.4% agarose formaldehyde gel to verify their identities. The cDNAs were synthesized from one microgram of total RNA extracted from each tissue using a cDNA Synthesis Kit (TransGen Biotech, Beijing, China). The cDNA samples were stored at −80 °C until used.

2.3. Cloning and characterization of RdTLR8

Primers were designed with sequences that were deduced from multiple alignments of the conserved regions of canine, human and mouse TLR8 (GenBank accession numbers JF681168, NM_016610 and NM_133212, respectively). The partial sequence of RdTLR8 was amplified using primers listed in Table 1. PCR amplification was carried out with Ex Taq DNA polymerase (TaKaRa, Dalian, Liaoning, China) under the following conditions: 1 cycle at 94 °C for 5 min; 35 cycles at 94 °C for 30 s, at each primer annealing temperature 47–53 °C (Table 1) for 1 min; and at 72 °C for 2 min; and 1 cycle at 72 °C for 5 min. The amplified PCR products were purified and cloned into pMD18-T with a TA Cloning Kit (TaKaRa, Liaoning, China). Positive colonies were sequenced by the Beijing Genomics Institute (Beijing, China).

After sequencing and BLAST analysis, 5′- and 3′-rapid amplification of cDNA ends (RACE) using the SMART™ RACE Amplification Kit (BD Biosciences Clontech, CA, USA) was performed to obtain the full-length cDNA sequences of RdTLR8. The sequences of gene-specific primers for RACE methods (Table 1) were designed according to the obtained RdTLR8 partial nucleotide sequence. The