Characterisation and functional analysis of canine TLR5

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Abstract

In this study, we characterised the single exon TLR5 gene of the Chinese rural dog. Sequence analysis revealed a 2577 nucleotide-long open reading frame of canine TLR5, encoding an 858 amino acid-long protein. The putative amino acid sequence of canine TLR5 consisted of a signal peptide sequence, 15 LRR domains, a LRR C-terminal domain, a transmembrane domain and an intracellular Toll-IL-1 receptor domain. The amino acid sequence of the canine TLR5 protein shared 95.4% identity with vulpine, 72.2% with feline and 64.7% with human TLR5. Plasmids expressing canine TLR5 and NF-κB-luciferase were constructed and transfected into HEK293T cells. Expression was confirmed by indirect immunofluorescence assay. These HEK293T cells transfected with the canine TLR5- and NF-κB-luciferase plasmids significantly responded to flagellin from *Salmonella enteritidis* serovar Typhimurium, indicating that it is a functional TLR5 homolog. In response to stimulation with *Salmonella enteritidis*, the level of TLR5 mRNA significantly increased over the control in PBMCs at 4 h. The levels of IL-8, IL-6 and IL-1β also increased after exposure. The highest levels of TLR5, IL-8 and IL-1β expression were detected at 8, 4 and 12 h after stimulation, respectively. These results imply that the expression of canine TLR5 may participate in the immune response against bacterial pathogens.

Keywords

Toll-like receptor 5, dogs, Salmonella enteritidis, cytokines, flagellin, sequence analysis

Introduction

The innate immune system in most multicellular organisms acts as the frontline of defence against invading micro-organisms by recognising PAMPs through PRRs. TLRs have emerged as key components of the innate immune system that sense microbial infections and elicit antimicrobial host defence responses. TLRs are a family of type I transmembrane receptors classified as PRRs that play an important role in the recognition of PAMPs from invading pathogens.[1] This recognition leads to the activation of the subsequent signalling pathway, which results in the development of host immune responses.[2]

To date, 10 mammalian TLRs have been identified.[3] TLR family members consist of an extracellular LRR domain for ligand recognition, a transmembrane domain and an intracellular Toll/Il-1 receptor (TIR) signalling domain.[4] The different structures of the LRR domain in variable TLRs correspond to distinct components of pathogens.[5] Among the identified

TLRs, TLR5 is important in the host defence against bacterial pathogens in several species. TLR5 recognises flagellin,[6] which contributes to the motility of bacterial pathogens, triggers the MyD88-dependent signalling pathway and activates NF-κB to stimulate transcription of several pro-inflammatory genes.[6–8] TLR5-deficient

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mice lack the flagellin-induced pulmonary inflammatory response[9] and are more susceptible to *Escherichia coli* infection of the urinary tract,[10] which indicates that TLR5 plays a role in restricting the entry of flagellated pathogens in mammals.

Flagellins from Gram-negative bacteria are involved in a direct interaction with the leucine-rich regions in TLR5[11] and activate a range of inflammatory cells via a TLR5-dependent signalling pathway.[6,11] The extracellular pattern recognition domain of TLR5 has been found to be conserved among mammals, which shows evidence of adaptive positive selection.[12] Polymorphism in the coding region of TLR5 has been found to be associated with altered signalling and cytokine production.[13] Missense mutations of human TLR5 have been shown to result in the abolition of bacterial-induced signal transduction.[14]

In this study, we cloned and characterised the canine TLR5 gene, analysed its functional response to flagellin stimulation by luciferase assay and evaluated its function in PBMCs following exposure to *Salmonella enteritidi*s by detection of specific cytokines. These data expand our knowledge of the relationship between TLR5 and innate immunity in dogs.

Materials and methods

Molecular cloning of canine TLR5

The procedures described in this study were approved by the Institutional Animal Care and Use Committee of Jiangsu Normal University. Blood samples were collected from a healthy Chinese rural dog from the Xuzhou Chengxi Companion Animal Hospital (Xuzhou, PR China), and PBMCs were isolated using lymphocyte separation medium (Shanghai Solarbio Bioscience and Technology Company, Shanghai, PR China). Genomic DNA was extracted from the PBMCs using a Universal Genomic DNA Extraction Kit v3.0 (Takara Bio, Dalian, PR China) according to the manufacturer's instructions. To clone the canine TLR5 gene, primer pairs covering the entire open reading frame (ORF) were designed based on the well-conserved upstream and downstream sequences of TLR5 from dogs (GenBank ID: NM_001197176) and foxes (GenBank ID: XP 025844601.1). PCR was performed using the designed primers (Table 1) and canine genomic DNA as a template. The amplified PCR product was purified, cloned into pCR2.1-T cloning vector with a TA Cloning Kit (Invitrogen, Carlsbad, CA) and sequenced by Genscript (Nanjing, PR China).

Sequence analyses

The nucleotide and deduced amino acid sequences of canine TLR5 were analysed using DNAstar software and the Expasy search programme (http://au.expasy.org/tools/). The Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de) was used to predict the protein domain structure of canine TLR5. TLR5 sequences from different species were compared using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A multiple sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/) and edited with the Genedoc software (Free Software Foundation, Boston, MA). Phylogenetic analysis was conducted

Table 1. PCR primers used in this study.

Primers name	Primer sequence (5′ to 3′)	Usage	GenBank No.
CanineTLR5			
CanineTLR5 F	AAAGAATTCGCCACCATGGGCCGCCAGCTGGGC	Clone of canine TLR5	This study
CanineTLR5 R	AAATCTAGACTAGGCGACGGCCGCCACG	Clone of canine TLR5	
TLR5			
TLR5 F	TCAACCTGTCGCACAATCTC	Detection of mRNA	NM_001197176
TLR5 R	CTGAACGTCTGGTCCTGGAT	Detection of mRNA	
IL-6			
IL-6 F	CCACTCACCTCTGCAAACAA	Detection of mRNA	U12234.1
IL-6 R	GTGCCTCTTTGCTGTCTTCA	Detection of mRNA	
IL-1β			
IL-1β F	CCCTGGAAATGTGAAGTGCT	Detection of mRNA	NM_001037971
IL-1β R	CTGACACGAAATGCCTCAGA	Detection of mRNA	
IL-8			
IL-8 F	GGCAGCTTTTGTCCTTTCTG	Detection of mRNA	NM_001003200
IL-8 R	ATTTGGGATGGAAAGGTGTG	Detection of mRNA	
β-Actin			
β-Actin F	GGACCTGACCGACTACCTCA	Detection of mRNA	AF327898
β-Actin R	AGTCCAGGGCCACATAACAC	Detection of mRNA	

on amino acid sequences using MEGA v7.0, and a phylogenetic tree was constructed with the neighbour-joining (NJ) method using a Poisson correction model with 1000 bootstrap replicates.

Construction of canine TLR5 expression plasmid

An expression recombinant plasmid pcDNA3.1-dogTLR5 was produced by cloning the full length of canine TLR5 into the restriction endonuclease *Eco*R I and *Xba* I sites of pcDNA3.1(+) expression vector (Invitrogen). The plasmid DNA pCR2.1-T containing the full length canine TLR5 ORF was digested with *Eco*R I and *Xba* I (Takara Bio) and subcloned into the same restriction enzymes site of the pcDNA3.1(+) expression vector.

Transfection and luciferase assay

To determine the functional response of canine TLR5 to flagellin, HEK293 cells were grown on 24-well tissue culture plates in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) until 70% confluence was reached. Cells were washed with PBS before transfection and then replaced with Opti-MEM (Invitrogen) medium. Transient transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Equal amounts of pcDNA3.1-canine TLR5 or pcDNA3.1-empty and reporter pGL4.32[luc2P/NF-B-RE/Hygro] plasmid DNA (Promega, Madison, WI) were transfected into the HEK293 cells. Twenty-four h after transfection, purified flagellin from *Salmonella enterica* serovar Typhimurium (Enzo Life Sciences, Farmingdale, NY) was added to the medium. After stimulation for 5 h, cells were harvested, and NF-κB-induced luciferase activity was measured using the Bright-Glo Luciferase Assay system (Promega) according to the manufacturer's instructions. Data were expressed as fold induction relative to the unstimulated cells and represent the mean $\pm$ SD of three independent experiments.

Gene expression profiling of canine TLR5 and cytokines in PMBCs after Salmonella stimulation

PBMCs from three dogs (M_{age}=3.0 yr) were isolated from whole blood using lymphocyte separation medium and washed three times with RPMI 1640 (Hyclone Laboratories, Logan, UT). Cell viability and number were determined by trypan blue exclusion. Cells were plated onto 24-well plates at 5×10^7 cells/l in 1 ml RPMI 1640 with 10% FBS (Hyclone) and cultured overnight (about 16 h, 37°C, 5% CO₂). *Salmonella enteritidis* strain 50041, in log growth phase, was suspended in RPMI 1640 at 1×10^8 cfu/ml. Either 100 μl *Salmonella enteritidis* or culture medium

was added into each well of PBMCs after overnight culture. PBMCs were harvested at 0, 2, 4, 8 and 12 h post exposure. There were three wells per treatment per time point. Mononuclear cells were collected from the plates interface, washed with PBS and centrifuged for 10 min at 200 *g*. The pellets were finally re-suspended in RPMI 1640. Total RNA was extracted from PBMCs using RNAiso Plus, treated with RNase-Free DNaseI to remove contamination with genomic DNA and reverse transcribed into cDNA using the PrimeScript RT Reagent Kit (Perfect Real Time; Takara Bio) according to the manufacturer's instructions.

The mRNA expression levels of canine TLR5 (NM 001197176), IL-6 (U12234.1), IL-1β (NM 001037971) and IL-18 (NM 001003200) in the exposed and control cells were determined by quantitative real-time PCR using SYBR Premix Ex TaqTM II (Perfect Real Time; Takara Bio). The primer sequences are listed in Table 1. Quantitative real-time PCR was carried out on an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA).

Each PCR reaction was performed in triplicate, and a non-template control was included on every plate to ensure that there was no contamination in the master mix. Standard curves were produced for each primer set of TLR5, IL-6, IL-1β and IL-8 (Table 1). The expression levels of each gene were normalised to the expression level of the endogenous control (β-Actin)[15] and were expressed as fold changes relative to the control group at each time point using the $2^{-\Delta\Delta Ct}$ method.[16]

Statistical analysis

The significance of the difference in the experimental data between treated and control groups was determined by Student's *t*-test. Statistical significance was determined at $P<0.05$.

Results

Characterisation of canine TLR5 gene

The entire coding region of the TLR5 gene from the Chinese rural dog was successfully amplified from genomic DNA. Sequencing results showed the canine TLR5 gene contained an ORF of 2577 bp encoding a protein of 858 amino acids. Prediction of protein domains by the SMART programme revealed that the putative amino acid sequence consisted of a signal peptide sequence encompassing the first 21 amino acid residues of the N-terminal region, 15 LRR domains, an LRR-CT domain, a transmembrane domain and a 148-amino acid TIR domain at positions 693–840 of

the carboxy-terminus (Figure 1). The deduced amino acid sequence of the canine TLR5 was aligned with reported sequences from human (GenBank ID: NM 003268), mouse (GenBank ID: AF186107), cat (GenBank ID: XP 023103200.1) and fox (GenBank ID: XP 025844601.1) using the DNAMAN programme (Figure 1). Comparison of the amino acid sequences of the TIR domains revealed that the canine TLR5 shared 64.7%, 64.9%, 72.2% and 95.4% identity with human, murine, feline and vulpine TLR5, respectively.

Figure 1. Alignment of canine (DOG), human (HUM), murine (MOU), feline (CAT) and vulpine (FOX) TLR5 amino acid sequences performed using the Clustal W programme and edited using the DNAMAN program. In the canine sequence, the predicted domains are marked by arrows with different colours. The signal peptide is marked in green, and the LRR are in black. The arrow in yellow denotes LRR C-terminal (LRR-CT) domain, the blue one indicates a transmembrane domain and the red one represents TIR domain. Shading was performed using the conserved mode (blue-black shading for conserved residues, and pink or light blue shading for similar residues). GenBank numbers for the aligned sequence: human NM_003268, mouse AF186107, cat XP_023103200.1, fox XP_025844601.1.

Phylogenetic analysis of TLR5

Phylogenetic analyses were performed on the amino acid sequences of the full coding region of TLR5 using the NJ method. The resulting phylogenetic tree, consisting of 12 protein sequences, was composed of two major branches (Figure 2). TLR5 protein sequences from the avian species, that is, chicken (GenBank ID: HM747028) and mallard (GenBank ID: KF255555.1), were in one subgroup. The sequences from the mammals, including polar bear (GenBank ID: XP 008693736.1), panda (GenBank ID: XP 011227214.1), fox (GenBank ID: XP_025844601.1), leopard (GenBank ID: XM 019436125), cat (GenBank ID: XP 023103200.1), pig (GenBank ID: AB208697.2), monkey (GenBank ID: NM 001130429), mouse (GenBank ID: AF186107) and human (GenBank ID: NM 003268), were in another subgroup. The canine and vulpine TLR5 sequences were the most closely related, as expected. The observed relationships within this cluster reflected the taxonomic positions of these species.

Response of canine TLR5 to flagellin

To evaluate the response of canine TLR5 to flagellin, the effect of flagellin stimulation on NF-κB activity was determined. The canine TLR5 gene was cloned into the pcDNA3.1(+) expression vector and transfected into HEK293 cells, and NF-κB-induced luciferase activity was assayed after flagellin stimulation. The NF-κB-induced luciferase activity in canine TLR5-transfected HEK293T cells was significantly higher than in cells transfected with an empty vector control at 5 h after stimulation. The result suggests that canine TLR5 is a pathogen receptor that plays a role in the recognition of flagellin (Figure 3).

Effect of S. enteritidis on the expression of canine TLR5 and inflammatory cytokines

To characterise the effect of *Salmonella enteritidis* on the expression of TLR5 and inflammatory cytokines in PBMCs, the mRNA expression levels of canine TLR5 and inflammatory cytokines, including IL-1β, IL-6 and IL-8, in the PBMCs were detected by real-time PCR. Canine TLR5 gene expression in PBMCs was significantly increased compared to the control after PMBCs exposure to *S. enteritidis*. The mRNA expression levels of IL-8 and IL-1β were also found significantly increased. However, there was no notable difference in the mRNA expression levels of IL-6 (Figure 4).

Discussion

The TLR family represents a major component of the vertebrate PRR system that affords the ability to detect invading micro-organisms and has a fundamental role
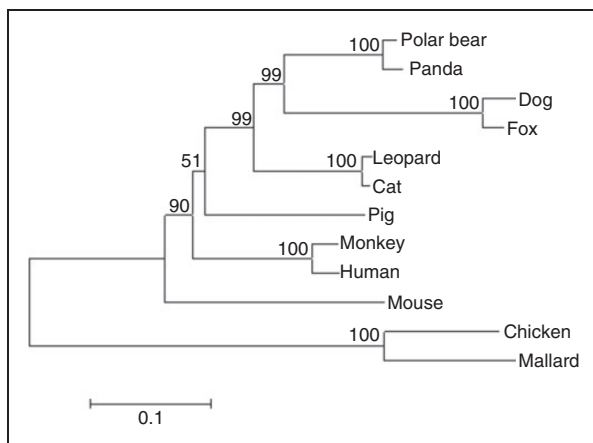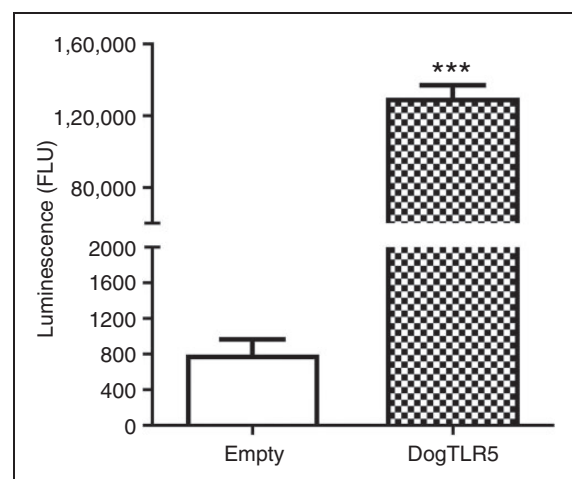

Figure 2. Phylogenetic tree of the TLR5 amino acid sequences from different species. The tree was constructed by the neighbour-joining method based on the Poisson correction model with 1000 bootstrap replicates using MEGA v7.0. The numbers at the nodes indicate bootstrap values. The bar (0.1) indicates the genetic distance. GenBank numbers: human NM_003268, cat XP_023103200.1, chicken HM747028, panda XP_011227214.1, fox XP_025844601.1, mouse AF186107, polar bear XP_008693736.1, monkey NM_001130429, leopard XM_019436125, mallard KF255555.1, pig AB208697.2.

Figure 3. Effect of flagellin stimulation on NF-κB activity. HEK293 cells were transfected with expression vector (pcDNA3.1-dogTLR5 or pcDNA3.1-empty) and reporter vector (pGL4.32luc2P/NF-B-RE/Hygro). Twenty-four h post transfection, 100 ng/ml flagellin was added to the transfected cells, and NF-κB luciferase activity was measured after stimulation for 5 h. Data are depicted as fold induction relative to the unstimulated cells. All data are the mean ± *SD* from three independent experiments. Asterisks indicate significant differences by a *t*-test between canine TLR5 and empty transfected into HEK293 cells ($P<0.05$). (a) Empty: pcDNA3.1-transfected cells; (b) canine TLR5: pcDNA3.1-TLR5-transfected cells.
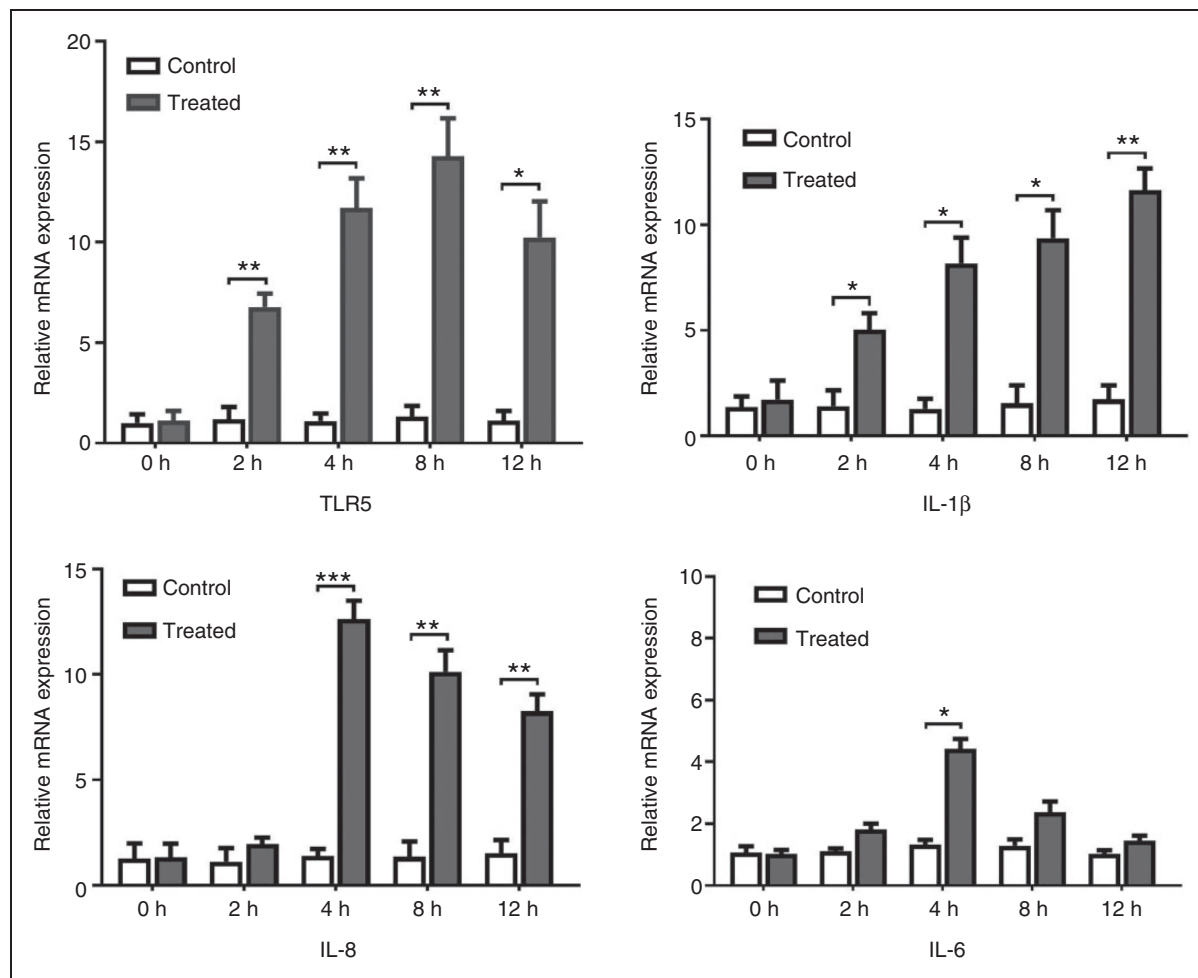
Figure 4. Induction of canine TLR5 and inflammatory cytokines expression in PBMCs following *S. enteritidis* exposure. Total RNA was extracted from the PBMCs of three dogs at 2, 4, 8 and 12 h after *S. enteritidis* exposure. The mRNA levels of TLR5 (a), IL-1β (b), IL-6 (c) and IL-8 (d) were measured by real-time PCR. Fold induction values are given as the mean ± SD. Asterisks represent a significant difference between the gene expression levels of the infected and control samples by *t*-test ($P < 0.05$).

in triggering immune responses. The demonstrated response of TLR5 to flagellin[9] implicates a similar role of canine TLR5 homolog in the immune response of mammals. Canine and vulpine TLR5 genes, which are closely related species, shared 95% amino acid identity in this study. In general, the canine TLR5 gene has a typical TLR structure that consists of extracellular LRRs, a transmembrane domain and an intracellular TIR domain.[3] However, canine TLR5 exhibits some structural features that are different from other TLR5. The repeat numbers of LRRs and their 'phasing' in TLRs differ with isoforms and species. For example, some mammalian TLR5 have 22 LRRs,[17] while canine TLR5 possesses 15 LRRs. Additionally, there are also some amino acid differences in the LRR domains of TLR5 among different species (Figure 1). The structures of LRRs in TLRs are speculated to mediate specific recognition of PAMPs.[18] Thus, the

differences in the LRRs of TLR5 among different species may influence its functional role as a pathogen receptor. As known, TLR5 recognises bacterial flagellin. To evaluate the response of canine TLR5 to flagellin, we determined the effect of flagellin stimulation on NF-κB activity. After stimulation with flagellin, NF-κB-induced luciferase activity in canine TLR5-transfected HEK293 cells was significantly higher than the induced level in cells transfected with the empty plasmid vector. This result suggests that canine TLR5 as a pathogen receptor plays a role in the recognition of flagellin.

Cytokines are an integral part of the immune response to *Salmonella*. Specifically, increased mRNA expression and secretion of chemokines, as well as proinflammatory and Th1 cytokines, are observed following infection with some *Salmonella* serotypes.[19] The most important inflammatory cytokine is IL-1β,

which is a molecular form of the potent pro-inflammatory cytokine IL-1 and is produced by monocytes, macrophages and other cell types.[20] IL-1β is produced as a precursor, and its maturation is preceded by an inflammasome activated caspase cascade.[21] In this study, *S. enterica* could induce IL-1β secretion in canine PBMCs, which was consistent with data in other species.[22,23]

IL-6 is a prototypical cytokine featuring pleiotropic and redundant activity. It is a pro-inflammatory cytokine involved in the transition from innate to acquired immunity and plays a key role in the recruitment of immune cells to infection sites.[24] IL-8, another pro-inflammatory cytokine, serves as a chemoattractant involved in the activation of neutrophils, basophils and some subpopulation of lymphocytes.[25]

Flagellin has been shown to stimulate the production of cytokines, including IL-6, from various human cell lines.[26,27] *Salmonella* expressing flagellin stimulated up-regulation of IL-6 and IL-8 in naive porcine PBMCs.[28] Exposure of chicken PBMCs to *Salmonella enteritidis* can induce a rapid change in both pro-inflammatory (IL-6 and IL-8) cytokine gene expression. The down-regulation of mRNA for both pro-inflammatory cytokines may be a consequence of *in vitro* invasion of PBMCs by *S. enteritidis*.[29] Cheeseman et al. found that mRNA expression of IL-6 and IL-8 was not significantly different in the spleen of chickens after oral inoculation with *S. enteritidis* compared to unexposed birds.[30] However, other groups have indicated that stimulation of *Salmonella* or flagellin significantly increased IL-8 and IL-6 expression in PBMCs.[28,31,32] In this study, IL-6 and IL-8 showed a significant increase in the expression levels in PBMCs exposed to *S. enterica*. Our results are in accordance with these studies, and we can speculate that the increased expression of IL-8 or IL-6 mRNA would promote the canine immune response against intracellular pathogens such as *Salmonella*. Several components of the experimental design may have contributed to these differences in IL-6 and IL-8 mRNA expression, including different hosts.

In conclusion, we cloned and sequenced the canine TLR5 gene. We then characterised its predicted protein domains and determined the mRNA expression of the canine TLR5 and cytokines in response to infection with *S. enteritidis* in PMBCs. Further research is needed to understand the structural characteristics better of the ligand-binding sites of canine TLR5 and its exact roles in response to different bacterial infections. The results will significantly improve our understanding of the innate immune response of dogs.

Declaration of conflicting interests

Funding

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References

1. Akira S, Uematsu S and Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; 124: 783–801.
2. Takeda K and Akira S. TLR signaling pathways. *Semin Immunol* 2004; 16: 3–9.
3. Akira S. Mammalian Toll-like receptors. *Curr Opin Immunol* 2003; 15: 5–11.
4. Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol* 2001; 1: 135–145.
5. Ng A and Xavier RJ. Leucine-rich repeat (LRR) proteins: integrators of pattern recognition and signaling in immunity. *Autophagy* 2011; 7: 1082–1084.
6. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 2001; 410: 1099–1103.
7. Didierlaurent A, Ferrero I, Otten LA, et al. Flagellin promotes myeloid differentiation factor 88-dependent development of Th2-type response. *J Immunol* 2004; 172: 6922–6930.
8. Gewirtz AT, Navas TA, Lyons S, et al. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* 2001; 167: 1882–1885.
9. Feuillet V, Medjane S, Mondor I, et al. Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria. *Proc Natl Acad Sci U S A* 2006; 103: 12487–12492.
10. Andersen-Nissen E, Hawn TR, Smith KD, et al. Cutting edge: Tlr5–/– mice are more susceptible to *Escherichia coli* urinary tract infection. *J Immunol* 2007; 178: 4717–4720.
11. Mizel SB, West AP and Hantgan RR. Identification of a sequence in human Toll-like receptor 5 required for the binding of Gram-negative flagellin. *J Biol Chem* 2003; 278: 23624–23629.

12. Smith SA, Jann OC, Haig D, et al. Adaptive evolution of Toll-like receptor 5 in domesticated mammals. *BMC Evol Biol* 2012; 12: 122.

13. Ruan W, Wu Y and Zheng SJ. Different genetic patterns in avian Toll-like receptor (TLR)5 genes. *Mol Biol Rep* 2012; 39: 3419–3426.

14. Merx S, Zimmer W, Neumaier M, et al. Characterization and functional investigation of single nucleotide polymorphisms (SNPs) in the human TLR5 gene. *Hum Mutat* 2006; 27: 293.

15. Fang Q, Pan Z, Geng S, et al. Molecular cloning, characterization and expression of goose Toll-like receptor 5. *Mol Immunol* 2012; 52: 117–124.

16. Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402–408.

17. Matsushima N, Miyashita H, Enkhbayar P, et al. Comparative geometrical analysis of leucine-rich repeat structures in the Nod-Like and Toll-Like receptors in vertebrate innate immunity. *Biomolecules* 2015; 5: 1955–1978.

18. Werling D, Jann OC, Offord V, et al. Variation matters: TLR structure and species-specific pathogen recognition. *Trends Immunol* 2009; 30: 124–130.

19. Eckmann L and Kagnoff MF. Cytokines in host defense against Salmonella. *Microbes Infect* 2001; 3: 1191–1200.

20. Ferrero-Miliani L, Nielsen OH, Andersen PS, et al. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. *Clin Exp Immunol* 2007; 147: 227–235.

21. Martinon F, Burns K and Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 2002; 10: 417–426.

22. Kim J, Ahn H, Woo HM, et al. Characterization of porcine NLRP3 inflammasome activation and its upstream mechanism. *Vet Res Commun* 2014; 38: 193–200.

23. Ahn H, Kim J, Kwon S, et al. Triggers of NLRC4 and AIM2 inflammasomes induce porcine IL-1beta secretion. *Vet Res Commun* 2018; 42: 265–273.

24. Kaplanski G, Marin V, Montero-Julian F, et al. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. *Trends Immunol* 2003; 24: 25–29.

25. Baggiolini M and Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett* 1992; 307: 97–101.

26. Eaves-Pyles T, Murthy K, Liaudet L, et al. Flagellin, a novel mediator of Salmonella-induced epithelial activation and systemic inflammation: I kappa B alpha degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. *J Immunol* 2001; 166: 1248–1260.

27. Rossi O, Caboni M, Negrea A, et al. Toll-like receptor activation by generalized modules for membrane antigens from lipid A mutants of Salmonella enterica serovars Typhimurium and Enteritidis. *Clin Vaccine Immunol* 2016; 23: 304–314.

28. Won G and Lee JH. Multifaceted immune responses and protective efficacy elicited by a recombinant autolyzed Salmonella expressing FliC flagellar antigen of F18(+) Escherichia coli. *Vaccine* 2016; 34: 6335–6342.

29. Kaiser MG, Cheeseman JH, Kaiser P, et al. Cytokine expression in chicken peripheral blood mononuclear cells after in vitro exposure to Salmonella enterica serovar Enteritidis. *Poultry Sci* 2006; 85: 1907–1911.

30. Cheeseman JH, Kaiser MG, Ciraci C, et al. Breed effect on early cytokine mRNA expression in spleen and cecum of chickens with and without Salmonella enteritidis infection. *Dev Comp Immunol* 2007; 31: 52–60.

31. Khani MH, Bagheri M, Zahmatkesh A, et al. Immunostimulatory effects of truncated and full-length flagellin recombinant proteins. *Microb Pathog* 2019; 127: 190–197.

32. Ma YU, Zhang LI and Li Q. Expression levels of cytokines and chemokines increase in human peripheral blood mononuclear cells stimulated by activation of the Toll-like receptor 5 pathway. *Exp Ther Med* 2016; 11: 588–592.