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CpG oligodeoxynucleotide-specific duck TLR21 mediates activation of NF-κB signaling pathway and plays an important role in the host defence of DPV infection

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ABSTRACT

TLR21 can recognize unmethylated cytosine-phosphate-guanine oligodeoxynucleotides (CpG-ODN) and activates NF-κB immune signaling pathway. However, the function of TLR21 in duck remains largely unclear. Here, the complete duck TLR21 (duTLR21) cDNA was cloned from Cherry Valley duck for the first time, and its immune response was preliminarily studied. Tissue specificity analysis showed duTLR21 was higher expressed in the peripheral blood, spleen, bursa of Fabricius and cecum. The expression of duTLR21 was significantly upregulated after stimulation with CpG-ODN or duck plague virus (DPV), but not Tembusu virus (TMUV), LPS or Poly (I:C). In addition, the transfection of DEF with duTLR21 stimulated by CpG-ODN activated NF-κB, through this signal pathway, the transcription of IL-1β, IL-6 and IFN-α were promoted, whereas knockdown of duTLR21 impaired the transcription of these genes. Furthermore, the overexpression of duTLR21 inhibited the replication of the DPV and the knockdown of duTLR21 by shRNA significantly promoted DPV replication in vitro. Altogether, these results indicate that duTLR21 can be activated by CpG-ODN, which mediates activation of NF-κB signaling pathway, and plays an important role in the host defence of DPV infection.

1. Introduction

Toll-like receptors (TLRs) are important pattern recognition receptors (PRRs) that recognize pathogens' highly conserved microbial structures known as pathogen associated molecular patterns (PAMPs) (Ausubel, 2005), playing a vital role in early responses to infections. Those receptors are composed of an extracellular LRR domain that is responsible for detecting and binding PAMPs, a TM domains, and an intracellular TIR domain that can mediate the signal transduction, resulting in an inflammatory response and the release of inflammatory cytokines (Gay and Keith, 1991; Schooley and Qwarnstrom, 2000). To date, 10 human TLRs, 13 murine TLRs, and more than 20 non-mammalian TLRs have been characterized. Those TLRs can be divided into six families (TLR1, TLR3, TLR4, TLR5, TLR7, and TLR11) (Roach et al., 2005). They can recognize various PAMPs like lipopolysaccharide (LPS), lipopeptide, flagellin, imidazoquinoline, CpG DNA, single and double-stranded RNA (Akira et al., 2006; Roach et al., 2005).

Although TLRs are widely present in animals and evolutionarily conserved, the avian TLRs have something in common as well as something different compared to that of mammals. Avian TLR3, TLR4, TLR5, TLR7 and duplicated genes TLR2A and TLR2B are clear orthologous to those found in other vertebrates, avian TLR15, TLR1La and TLR1Lb appear to be unique to avian species, and avian TLR21 is an ortholog of TLR21 in fishes and amphibians (Brownlie and Allan, 2011; Chen et al., 2013; Temperley et al., 2008). It was confirmed that chTLR21 has an immune function similar to mammalian TLR9, it can recognize CpG DNA (Brownlie et al., 2009; Keestra et al., 2010). Just like human TLR9 (huTLR9), chTLR21 is located in the endoplasmic reticulum, and bafilomycin A can block its activation. The later studies demonstrated that chTLR21 can be activated by chromosomal DNA

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isolated from five strains of Campylobacter, while huTLR9 is poorly activated by low GC DNA but highly activated by bacterial DNA with high GC chromosomal DNA (Dalpke et al., 2006; de Zoete et al., 2010), this indicated that avian TLR21 may have broader recognition profiles than mammalian TLR9. Signals via the MyD88 to induce a Interferon (IFN) typeI, III, and inflammatory cytokine response When TLR9 is activated by DNA binding (Luecke and Paludan, 2015). Expression of chTLR21 in Human embryonic kidney (HEK293) cells resulted in activation of NF-κB in response to synthetic CpG-ODN, typically recognized by mammalian TLR9 (Brownlie et al., 2009). However, the release of the downstream cytokines is distinguishing in different types of avian cells. For example, after stimulation with class B CpG-ODN, mRNA expression level of IL-1β was significantly increased in chicken spleen, but decreased in bursal cells, and did not significantly change in harderian gland (HG) cells (Patel et al., 2008). In addition, class B CpG-ODN could increase IFN-γ mRNA expression level in HG cells (Chrzastek et al., 2014). Low concentration of CpG-ODN could significantly upregulated IL-10 mRNA expression level in bursal B cells (St Paul et al., 2012). Complete TLR21 gene sequence has been identified in chickens, gooses, fishes, frogs and many other animals, but not in ducks yet.

Duck plague virus (DPV), also known as duck enteritis virus, belongs to the subfamily *Alpha-herpesvirinae* of the family *Herpesviridae*. Having a globular shape of diameter of about 120–130 nm, DPV is an enveloped virus and has an icosahedral capsid and a linear double-stranded DNA (Dhama et al., 2017; Yuan et al., 2005). Considering that DPV is a member of *Herpesviridae*, the previous reports suggested that mammalian TLR9 is able to recognize herpes simplex virus (HSV) (Luecke and Paludan, 2015; Lund et al., 2003; Paludan et al., 2011), so we wonder whether duTLR21 can recognize DPV, which belongs to the same genus as HSV.

In this study, we identified and characterized the CDS of duTLR21 gene, and its gene expression was measured in various tissues from uninfected ducks. We demonstrated that duTLR21 gene plays an important role in recognition of CpG-ODN and host anti-duck plague virus infection.

2. Materials and methods

2.1. Ethics statement

All animal experiments were approved by the committee of experiment operational guidelines and animal welfare of Sichuan Agriculture University, China (approved permit number is XF2014-18).This study was performed in strict accordance with the recommendations of the ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines).

2.2. Animals, cells, virus and ligands

Cherry Valley ducks were purchased from a farm located in Yaan, China. Duck embryo fibroblasts (DEFs) were cultured in Dulbecco's modified Eagle's Medium (DMEM, Sigma, St Louis, MO, USA) complemented with 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA) The DPV (CHv strain, GenBank ID: JQ647509.1) and DTMUV (CQW1 strain, GenBank ID: KM233707.1) were provided by our lab. The DPV and DTMUV titers were determined by the median tissue culture infective dose (TCID50)(Reed and Muench, 1938), and were $10^{-6.7}$/0.1 ml and $10^{-6.0}$ /0.1 ml respectively. CpG-ODNs were synthesized by Sangon Biotech (Shanghai, China), LPS and poly (I:C) was purchased from Sigma (St Louis, MO, USA).

2.3. Molecular cloning of duTLR21

To clone the duTLR21, primers p5′ TLR21F/R and p3′ TLR21F/R (Table 1) were designed based on the partial mRNA sequences of *Anas platyrhynchos* TLR21 (GenBank ID: JN573269) and Anser cygnoides TLR21 (GenBank ID: KT735043.1). The Total RNA was extracted from duck spleen by using RNAiso plus (Takara, Dalian, China) and the first-strand cDNA was synthesized with reverse transcription kit (Takara, Dalian, China) according to the manufacturer's protocol.

The 5′ and 3′ ends of the duTLR21 cDNA were acquired by rapid-amplification of cDNA ends (RACE) assay. Gene-specific primers (Table 1) were designed according to the partial sequence of duTLR21 we have obtained above. One adaptor primer (Table 1) binds the 5′ or 3′ ends of the target sequence and another gene-specific primer binds to the middle of the 5′ or 3′ partial sequence, Nested PCR was performed. Finally, primers TLR21 F/R (Table 1) were designed and used to amplify the duTLR21 open reading frame (ORF) fragment. The PCR products were cloned into pGEM-T (Promega, Beijing, China) vector and transformed into *E. coli* DH5α competent cells.

2.4. duTLR21 bioinformatics analysis

BLAST from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for sequence analysis. The ORF of duTLR21 was predicted using ORF Finder (Open Reading Frame Finder, http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi). Multiple alignment of the amino acid sequence was performed by using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2) and then edited with BOXSHADE (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html). The Simple Modular Architecture Research Tool (SMART, http://smart.emblheidelberg.de/) was used to identify the protein motifs in duTLR21. The phylogenetic tree was constructed by MEGA7. The SWISS MODEL (http://www.swissmodel.expasy.org/) was used to predict the tomograph of duTLR21 protein.

2.5. duTLR21 tissue distribution analysis

To study the tissue distribution and mRNA expression levels of TLR21 in duck, the brain, heart, liver, spleen, lung, kidney, thymus, bursa of Fabricius, Harderian gland, duodenum, jejunum, ileum, cecum, rectum and blood were collected from 7 days old and 35 days old Cherry Valley ducks. Total RNA was prepared by using the RNAiso plus (Takara, Dalian, China) and was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). The quantitative real-time PCR (qPCR) analysis was carried out to assess the abundance of the duTLR21 mRNA transcripts.

2.6. Stimulation of DEF cells

CpG motifs are oligodeoxynucleotides which have strong immunostimulating functions in animals. A CpG-ODN's immunostimulatory activities are dependent on its length, the number of CpG motifs, and the position, spacing, and surrounding bases of these CpG motifs (Yeh et al., 2013). Researches show that chTLR21, goTLR21, huTLR9, zebTLR9 and zebTLR21 could recognize class B CpG-ODN like CpG-2007, and zebTLR21 also could recognize CpG-2395 which belongs to class C CpG-ODN (Chrzastek et al., 2014; Pohar et al., 2017, 2015b; Qi et al., 2016; Yeh et al., 2013). So in this study, class B CpG-ODN (CpG-2007, CpG-HC4040), and class C CpG-ODN (CpG-2395) were used to stimulate DEFs. Control groups received either non-CpG-ODN or DMEM. DEFs were treated with CpG-ODNs (Table 2) at 3 μM, LPS at 0.2 μg/ml, Poly (I:C) at 5 μg/ml when 80% cell confluence was reached. The cells were harvested at 6 h post stimulation and the relative expression level of duTLR21 was determined by qPCR.

2.7. Plasmid construction, transfection and dual-luciferase reporter assay

The DNA fragment containing the entire ORF of duTLR21 that added *Bam*H I and *Eco*R I restriction sites was subcloned into the pcDNA3.1(+) expression vector, named pcDNA3.1(+)- duTLR21-Flag.

Table 1
Primer names, sequences and their applications in molecular cloning of duTLR21.

Primer name	Sequence(5'→3')	Note
p5'TLR21 F	CCCTACGGCTTCCGCAACT	Partial cloning
p5'TLR21 R	CACACCTTCTCCACCCCG	
p3'TLR21 F	CCTGGGCTTCTACCTCTTC	
p3'TLR21 R	CCACGATGTTGTCGATGAT	
3'RACE-AP	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC (T)18	3' RACE
3'AP outer	CCAGTGAGCAGAGTGACGAG	
3'AP inner	TGAGCAGAGTGACGAGGAC	
3'GSP outer	TGGGCTTCTACCTCTTCACCG	
3'GSP inner	AGTGGCGGCGGGAGGAGG	
5'GSRP	CGAGATGCCGTTGT	5' RACE
5'AP outer	AAGCAGTGGTATCAACGCAGAGTACGCGGGGGGGGGG	
5'AP inner	AAGCAGTGGTATCAACGCAGAGT	
5'GSP outer	GCAGCCACAGCGAKGACAGGT	
5'GSP inner	TGTGGGAGAGGTTGAGSGCGTAG	
TLR21 F	CGGGATCCAGCCGAGGATGACCGAACCA	CDS cloning
TLR21 R	GAATTCTGCCTTCTCCTCTTTCTCCCC	

Table 2
Sequences of phosphorothioate-modified CpG-ODN used in this study.

CpG-ODN	Sequence(5'→3')
non-CpG-ODN	GCTTGATGACTCAGCCGGAA
CpG-2007	TCGTCGTTGTCGTTTTGTCGTT
CpG-HC4040	TGACTGTGAACGTTCGAGATGA
CpG -2395	TCGTCGTTTTCGGCGCGCGCCG

Table 3
Primer names, sequences and their applications used in qPCR.

Primer name	Sequence(5'→3')	Tm(℃)	Note
qTLR21 F	GGGGCTTGGCAACCTGACGGA	56	qPCR
qTLR21 R	TGAAGATGCCGGGGAAGAGGA		
qGAPDH F	CAAGGCTGAGAATGGGAAAC	54	
qGAPDH R	CTGCCCACTTGATGTTGC		
qIL-1β F	TCATCTTCTACCGCCTGGAC	64	
qIL-1β R	GTAGGTGGCGATGTTGACCT		
qIL-6 F	AAGTTGAGTCGCTGTGCT	61	
qIL-6 R	GCTTTGTGAGGAGGGATT		
qIFN-α F	TCCTCCAACACCTCTTCGAC	64	
qIFN-α R	GGGCTGTAGGTGTGGTTCTG		
qDPV-UL30-F	TTTCCTCCTCCTCGCTGAGTG	61	
qDPV-UL30-R	CCAGAAACATACTGTGAGAGTG		

NF-κB-Luc and pRL-TK were kindly provided by Wu (S Chen et al., 2017). Transient transfection was performed using lipofectamine 3000 (invitrogen, Shanghai, China). The DEFs were seeded in 24-well plates and incubated until 60–80% confluent. Then the cells were co-transfected with 470 ng/well pcDNA3.1(+)-duTLR21-Flag or empty vector, together with 500 ng/well reporter plasmid NF-κB-Luc, as well as 30 ng/well pRL-TK (Promega, Beijing, China) as an internal control vector. After 48 h, the cells were stimulated by different sequences of phosphorothioate-modified CpG-ODNs (Table 3), Non-CpG DNA, LPS, Poly (I:C). The cells were harvested at 6 h after stimulation, and firefly luciferase activity was measured by the dual-luciferase assay system (Promega, Beijing, China) according to the manufacturer's directions.

2.8. Western blotting analysis

The DEFs were cultured in 6-well plates and transfected with the expression plasmids or empty control plasmids. The cells were lysed in RIPA Buffer (Solarbio, Beijing, China) and harvested at 48 h post-transfection. The protein samples (about 30 μg) were electrophoresed by 10% SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% nonfat dry milk at 37℃ for 1 h, and incubated with mouse anti-Flag antibodies (ProteinTech, Shenzhen, China) at a 1:1000 dilution or mouse anti-β-actin monoclonal antibodies (Santa Cruz Biotechnology, Shanghai, China) at a 1:200 dilution over night at 4℃. Goat anti-mouse IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Shanghai, China) was used as the secondary antibody at a 1:5000 dilution. The chemiluminescence using an ECL kit (Bio-Rad, California, USA) was used to visualize proteins.

2.9. Antiviral activity of duTLR21

DEFs were seeded in 6-well plates and incubated overnight. The DEFs were infected with DPV (10^3 $TCID_{50}$), DTMUV (10^3 $TCID_{50}$) or PBS. Cells were collected at 24 and 48 h post infection (hpi) and the relative expression levels of duTLR21, IL-1β, IL-6 and IFN-α were determined.

The newborn ducks were fed over 1 week before use. Then forty DPV-free 7-day-old ducklings were divided into the same amount of two groups randomly. One group was infected with 0.5 ml DPV (5×10^6 $TCID_{50}$) suspension by injection into muscle, the other group was inoculated with 0.5 ml sterile PBS as the control. The spleen and bursa of Fabricius from each group were collected for RNA extraction at 2/3/4 days post-infection (d.p.i) (Spleen and bursa of Fabricius of 6 ducklings were collected in each group at 2 day post-infection, spleen and bursa of Fabricius of 7 ducklings were collected in each group at 3/4 days post-infection.) and the relative expression levels of duTLR21, IL-1β, IL-6 and IFN-α were determined.

DEFs were seeded in 6-well plates and were transfected with the duTLR21 expression plasmids as described above. The DEFs were infected with DPV (10^3 $TCID_{50}$) at 48 h post-transfection. Then the cells were collected for DNA extraction at 24/48 hpi. The extraction of DNA was performed with DNA extraction kit (TIANGEN, Beijing, China) according to the manufacturer's protocol. The DPV absolute quantitative curve was created as previously described (Zhou et al., 2016).

2.10. Quantitative real-time PCR

SYBR Green real-time PCR assay (CFX96 Bio-Rad, Hercules, CA, USA) was used to perform the qPCR. Total RNA was extracted from DEF using RNAiso plus and the cDNA fragments were amplified by RT-PCR.

Table 4
The sequence of shRNAs used in this research.

Target Gene	Name	Sequence(5'→3')
duTLR21	shRNA-1057	GCACAACGGCTTCCAGAAGAT
	shRNA-1344	GGGTCTTGAAGCTCAACATCA
	shRNA-2122	GCTGAAGACCTTCAACTACAG
Negative control	shRNA-NC	GTTCTCCGAACGTGTCACGT

The relative expression levels of duTLR21, IL-1β, IL-6 and IFN-α were determined. Duck glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank ID: GU564233.1) was used as an internal control. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method and represented (Livak and Schmittgen, 2001) as the mean ± SD (n = 3). The primer sequences are listed in Table 3.

2.11. RNA interference

Three small interfering RNAs (siRNAs) that targeted duTLR21 gene were inserted into a pGPU6-GFP-Neo (GenePharma, shanghai, China), resulting in shRNA-1057, shRNA-1344 and shRNA-2122 (Table 4). A non-targeting shRNA (shRNA-NC) was synthesized as a negative control. The pcDNA3.1(+)-duTLR21-Flag was cotransfected with shRNA at 500 ng/well into the DEFs to identify the effect of a specific target

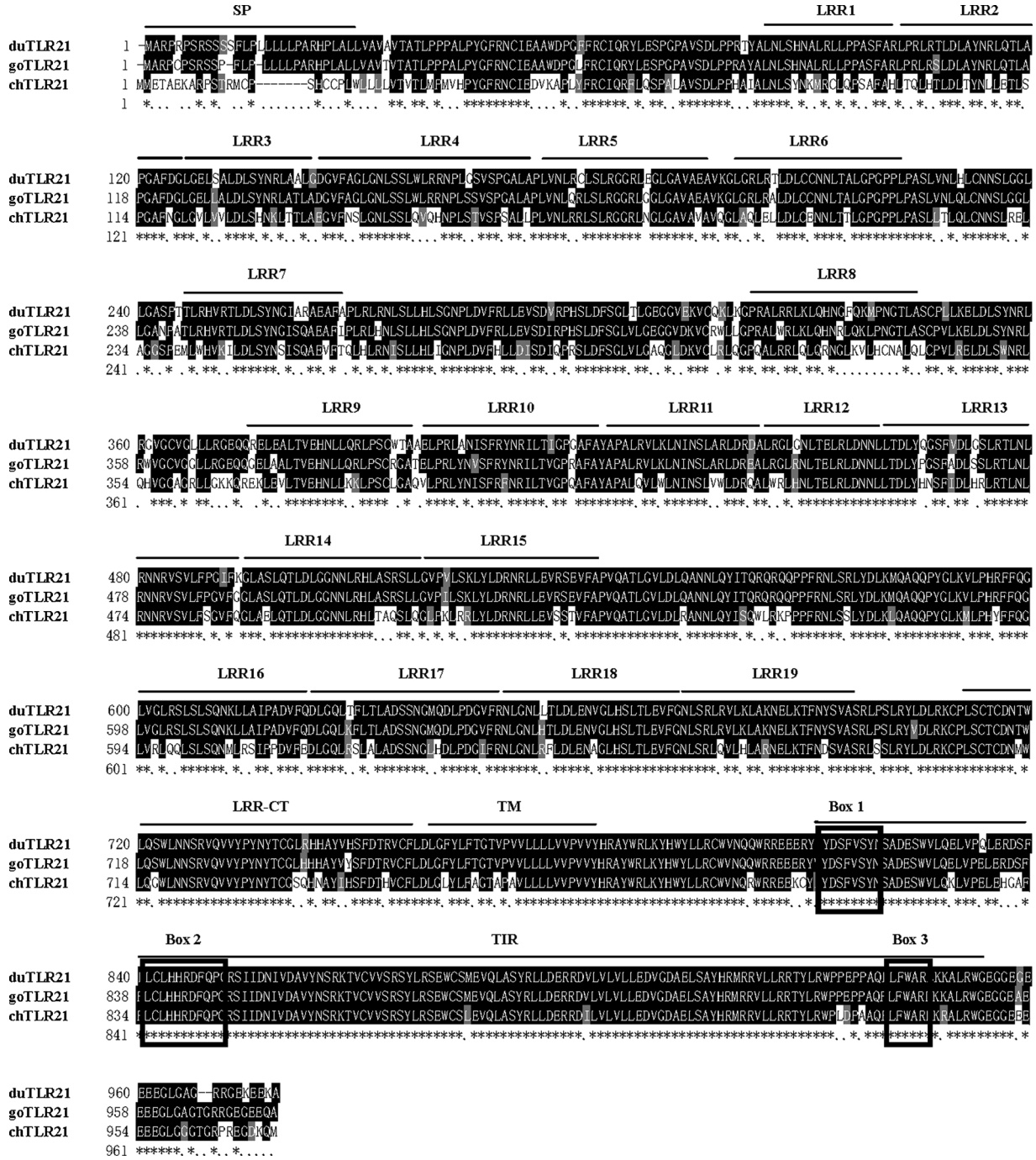

Fig. 1. Amino acid Alignment of duTLR21. Protein sequences of duTLR21 (KY829021) was aligned together with goTLR21 (AMB20882.1) and chTLR21 (AFD61602.1) for comparison. The alignment was performed using CLUSTAL W and edited in the BOXSHADE program. Identical residues were denoted by an asterisk; highly conservative substitutions, by a single dot. SP, signal peptide; LRR, leucine-rich repeat; LRR-CT, C-terminal LRR; TM, transmembrane domain; TIR, Toll/interleukin-1 receptor domain; Box1, Box2, and Box3, box 1, box 2, and box 3 in the cytosolic TIR.

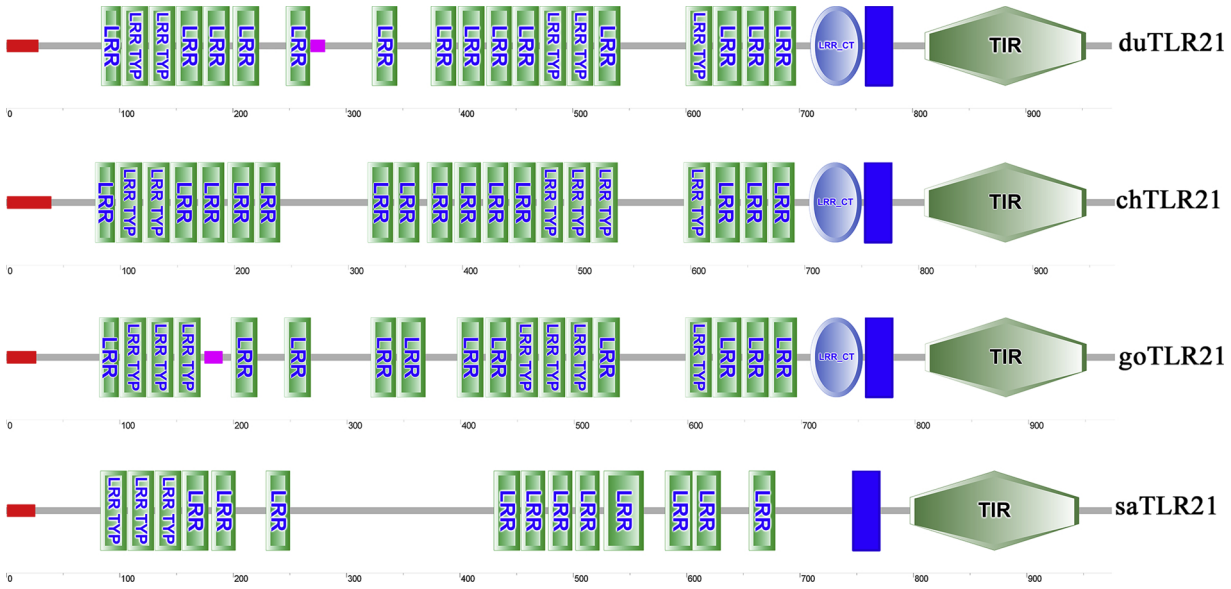

Fig. 2. Secondary protein structure analysis of TLR21. Comparison of duck, goose, chicken, and salmon TLR21 was performed by using the SMART program. The red stands for signal peptide, and the blue stands for transmembrane domain (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

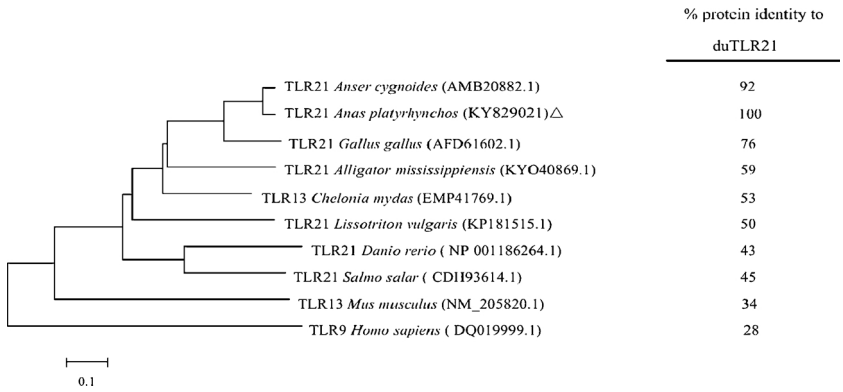

Fig. 3. Phylogenetic analysis of duTLR21. The tree was constructed by the neighbor-joining method using MEGA 7. The scale bar is 0.1, duTLR21 is denoted by △.

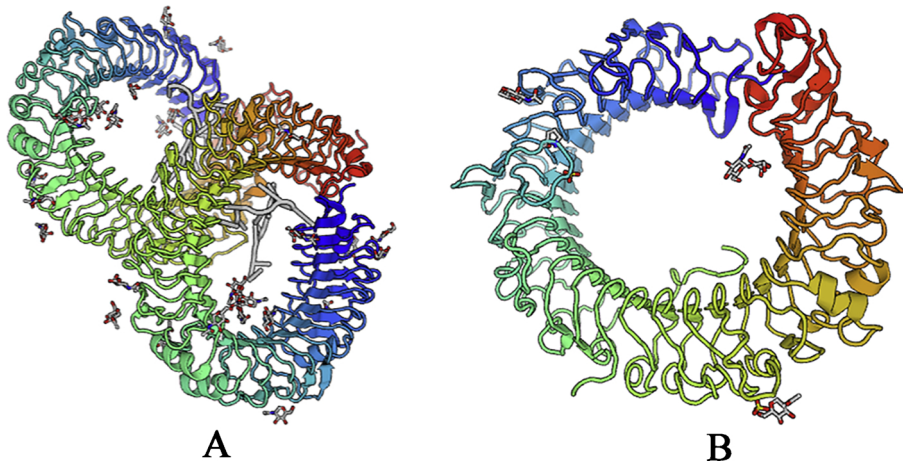

Fig. 4. Tomograph of duTLR21. Ligand-bound duTLR21 formed a symmetric TLR21-ligand complex (A), while unliganded duTLR21 was a monomer (B). The structure was predicted by using the SWISS MODEL.

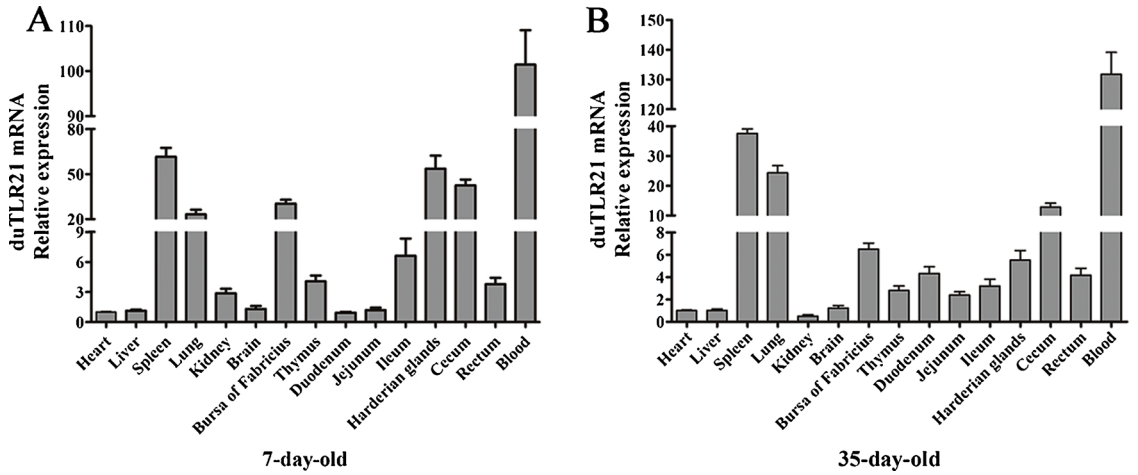

Fig. 5. DuTLR21 mRNA relative transcription levels in healthy duck tissues. Real-time RT-PCR was performed to determine the transcription levels of duTLR21 in various tissues. GAPDH was chosen as the control gene. The data were analysed by GraphPad Prism software and are presented as the mean ± SD (n = 3).

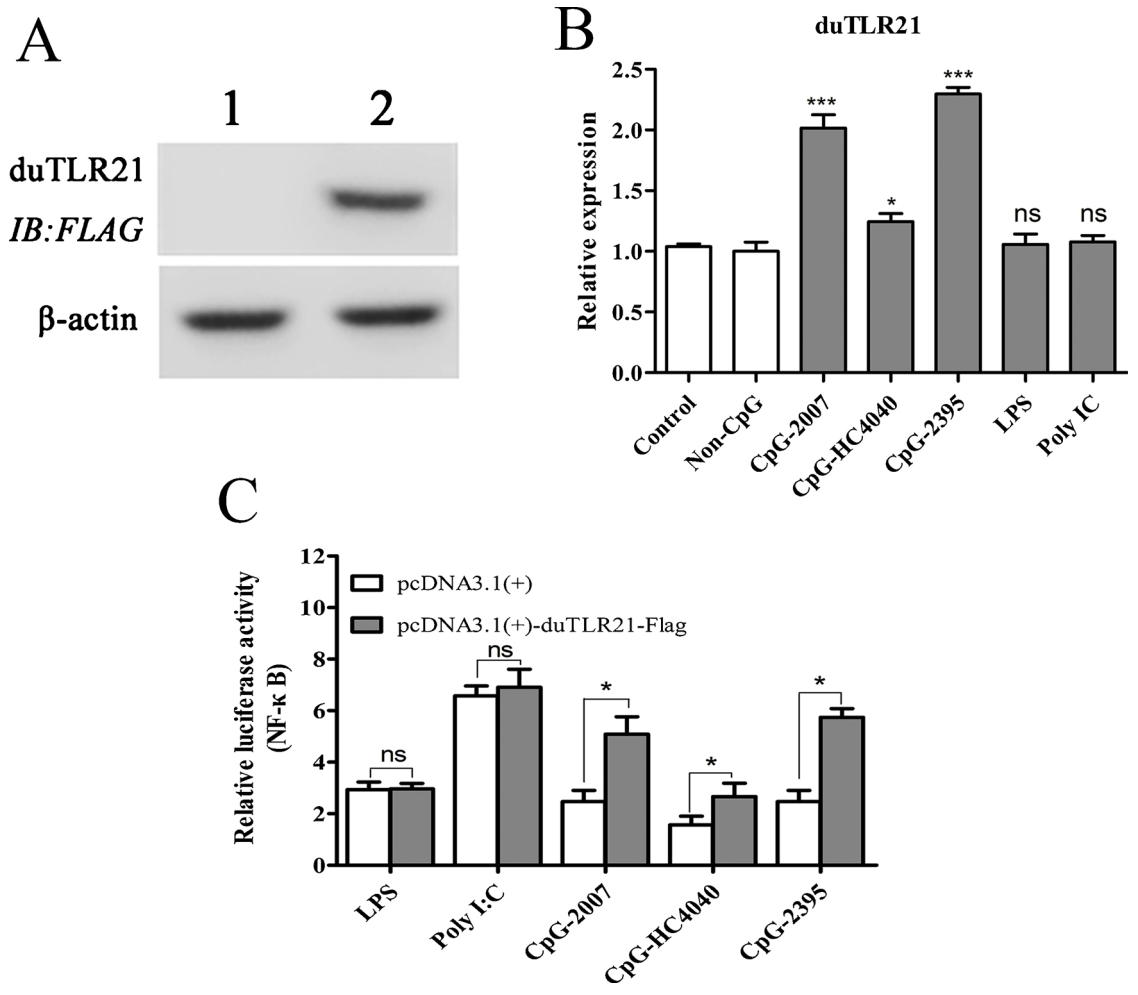

Fig. 6. The duTLR21 could activate NF-κB. (A) Western blotting analysis of expression of duTLR21 in DEFs at 48 h after transfection. Lanes 1 and 2 represent DEFs transfected with 2.5 μg/well of pcDNA3.1(+) or pcDNA3.1(+)-duTLR21-flag. DuTLR21 from the cell lysates was detected using anti-Flag tag antibodies, and β-actin served as the loading control. (B) DuTLR21 can be induced by different CpG-ODNs in DEFs. DEFs were treated with 3 μM CpG-2007, CpG－HC4040 or CpG-2395, 0.2 μg/ml LPS, and 5 μg/ml poly (I:C). The Dunnett test was used to analyze differences. Asterisks indicate values that are statistically significant between all stimulant groups and control group (*$P < 0.05$; ***$P < 0.001$). (C) DEFs were cotransfected with 470 ng/well of empty vector or pcDNA3.1(+)-duTLR21-flag along with 30 ng/well pRL-TK plasmids, as well as 500 ng/well NF-κB luciferase reporter gene, and then treated with 0.2 μg/ml LPS, 5 μg/ml poly (I:C), and 3 μM CpG-2007, CpG－HC4040 and CpG-2395. The multiple *t*-test was applied to examine the statistical significance between pcDNA3.1(+)-duTLR21-Flag and pcDNA3.1(+) at each stimulant group (*$P < 0.05$). The data are presented as the mean ± SD (n = 3).

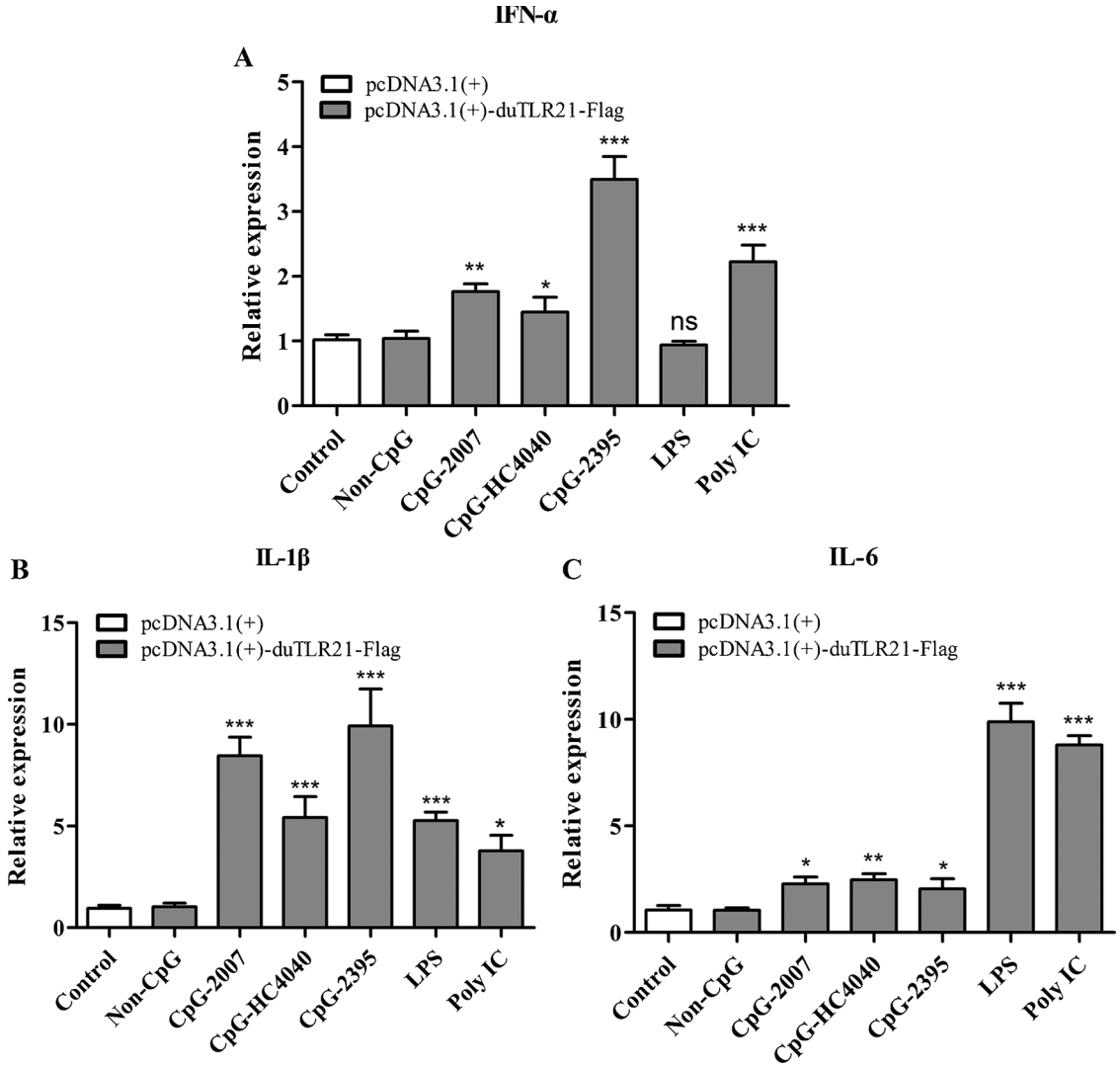

Fig. 7. Cytokine induction by different CpG-ODNs in the transfection of DEF with duTLR21. DEFs transfected with 2.5 μg/well of pcDNA3.1(+) or pcDNA3.1(+)-duTLR21-flag, and then treated with 3 μM CpG-2007, CpG-HC4040 and CpG-2395, 0.2 μg/ml LPS, and 5 μg/ml poly (I:C). Cells were harvested and analyzed for different cytokine induction by qPCR. The mRNA expression levels were normalized to the expression of the GAPDH gene. Data are presented as the mean ± SD (n = 3). The Dunnett test was applied to examine the statistical significance between all stimulant groups and Non-CpG group or control group (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

knockdown. After 24 h, the cells were collected for qPCR and Western blot (after 48 h) analysis.

2.12. Statistical analysis

All data were analysed by GraphPad Prism software. The multiple *t*-test, Dunnett test and two way ANOVA test were used to evaluate the statistical significance of differences. *P*-values < 0.05 were considered significant.

3. Results

3.1. Cloning and sequence analysis of the duTLR21 cDNA

The sequence of duTLR21 (GenBank ID: KY829021) of Cherry Valley duck (Anas platyrhynchos) that we obtained was 3009 bp in length including an ORF of 2931 bp which encoding 976 amino acid (aa) residues. Compared to the other species, the entire amino acid sequence of duTLR21 shares 92% identity with goose (Anser cygnoides) TLR21 (goTLR21), 76% identity with chicken (Gallus gallus) TLR21 (chTLR21). The deduced duTLR21 protein contains 20 LRR motifs, a signal peptide (27 aa), a TM domain (23 aa), and an intracellular TIR domain (143 aa) which contains box 1, box 2, and box 3 three conserved motifs (Fig. 1). All those elements are similar to goTLR21 and chTLR21, not too much like fishes' TLR21 (Fig. 2). A phylogenetic tree based on the duTLR21 full-length aa sequence was constructed to reveal the relationship between duck's and other species' TLR21, even other TLRs (Fig. 3). The predicted tomograph of duTLR21 protein may have two forms: unliganded and bound to ligand (Fig. 4), ligand-bound duTLR21 formed a symmetric TLR21-ligand complex, while unliganded duTLR21 was a monomer.

3.2. Expression profile of duTLR21

The duTLR21 mRNA transcript levels were detected in 15 tissue samples of ducklings and adult ducks by qPCR. As shown in Fig. 5, duTLR21 was expressed in various tissues, especially higher in the peripheral blood, spleen, bursa of Fabricius and cecum. It was more highly expressed in the tissues obtained from adult ducks when compared to those ducklings, like lung and cecum.

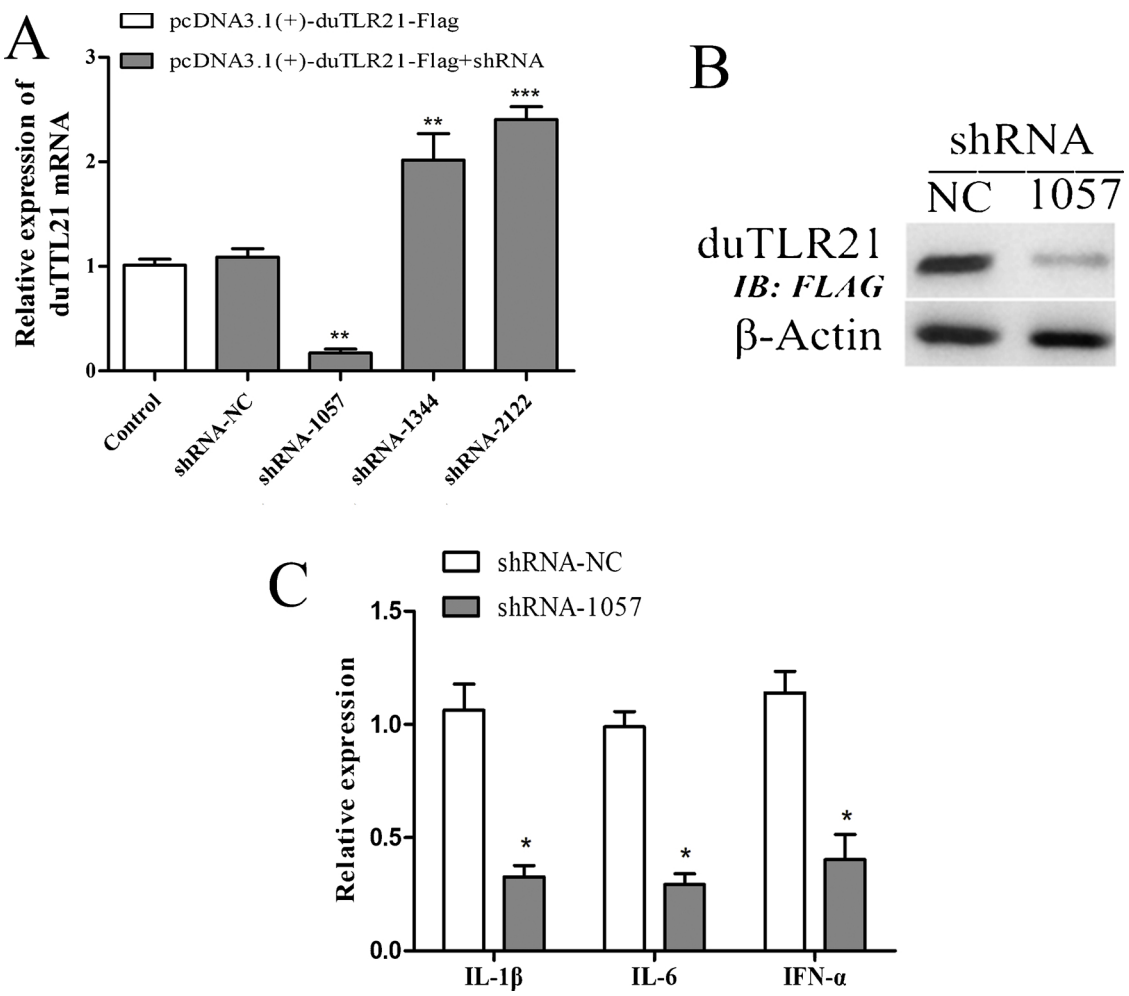

Fig. 8. The knockdown of duTLR21 by shRNA assay. (A) The knockdown efficiency of shRNA targeting duTLR21. DEFs were cotransfected pcDNA3.1(+)-duTLR21-Flag (500 ng/well) with shRNA Negative control (shRNA-NC, 500 ng/well) or shRNA-1057 (500 ng/well), shRNA-1344 (500 ng/well) or shRNA-2122 (500 ng/well). After transfection 24 h, the duTLR21 transcription levels were investigated by qPCR. Data are presented as the mean ± SD (n = 3). The Dunnett test was used to analyze differences between all shRNA groups and control group (**$P < 0.01$; ***$P < 0.001$). (B) The reduction of duTLR21 protein expression after the shRNA-1057 assay. The cell lysates transfected shRNA were detected by a western blot 48 h later. β-actin served as control. (C) The knockdown of duTLR21 reduces the host immune response. The DEFs were transfected with 1 μg/well shRNA-NC or shRNA-1057. At 48 h post transfection, the cells were stimulated with CpG-ODN 2007, the cells were collected, and the expression of IL-1β, IL-6 and IFN-α were examined by qPCR. Data are presented as the mean ± SD (n = 3). The multiple *t*-test was applied to examine the statistical significance between shRNA-1057 group and shRNA-NC group (*$P < 0.05$).

3.3. CpG-OND could activate NF-κB through duTLR21 and induces downstream innate immune factors transcription

DuTLR21 expression plasmid was constructed and transfected in DEFs, western blotting showed that the pcDNA3.1(+)-duTLR21-Flag plasmids were expressed well in the transfected DEFs at 48 h (Fig. 6A). As show in Fig. 6B, the relative expression level of duTLR21 was significantly up-regulated by CpG-ODNs stimulating DEFs. To identify whether CpG-OND could activate NF-κB through duTLR21, DEFs were transfected with duTLR21 expression plasmid together with reporter plasmid NF-κB-Luc, as well as pRL-TK, then the cells were stimulated by CpG-ONDs. As show in Fig. 6C, CpG-ONDs drastically activated NF-κB. DEFs were transfected with empty plasmid (500 ng/well) or pcDNA3.1(+)-duTLR21-Flag (500 ng/well) and stimulated with CpG-OND to investigate the change of downstream factors' transcription. The qPCR results showed that the mRNA level of IL-1β, IL-6, and IFN-α were significantly up-regulated. LPS and Poly (I:C) as control (Fig. 7).

3.4. The knockdown of duTLR21 reduces the host immune response

To further validate whether duTLR21 is involved in the NF-κB signaling pathway as a receptor for ODN stimulation, the shRNAs targeting different positions of the duTLR21 mRNA were expressed in DEFs. The result showed that duTLR21 was substantially inhibited by shRNA-1057 both at the mRNA and the protein level (Fig. 8A and B). DEFs were transfected with shRNA-1057 or with shRNA-NC, and at 48 h post-transfection, the cells were stimulated with CpG-OND 2007. As qPCR results shown, the expression of IL-1β IL-6 and IFN-α were decreased markedly (Fig. 8C).

3.5. duTLR21 is required for anti-DPV response

To investigate the duTLR21-mediated antiviral effect, we analyzed the expression of duTLR21, pro-inflammatory cytokines, and interferons during infection with DTMUV or DPV in vitro. As shown in Fig. 9, we found that DPV led to a significant up-regulation of duTLR21 mRNA levels at 24 and 48 hpi (Fig. 9A), while DTMUV cannot up-regulate duTLR21 expression (Fig. 9B). It's interesting that IL-1β expression levels had no difference in DEFs infected with DTMUV or DPV. IL-6 mRNA levels were significant up-regulated in DEFs infected with DTMUV, but not DPV. Both DTMUV and DPV can up-regulate IFN-α expression in DEFs.

Then we establish animal model of DPV infection. At three time points, duTLR21 expression levels were highly up-regulated in both

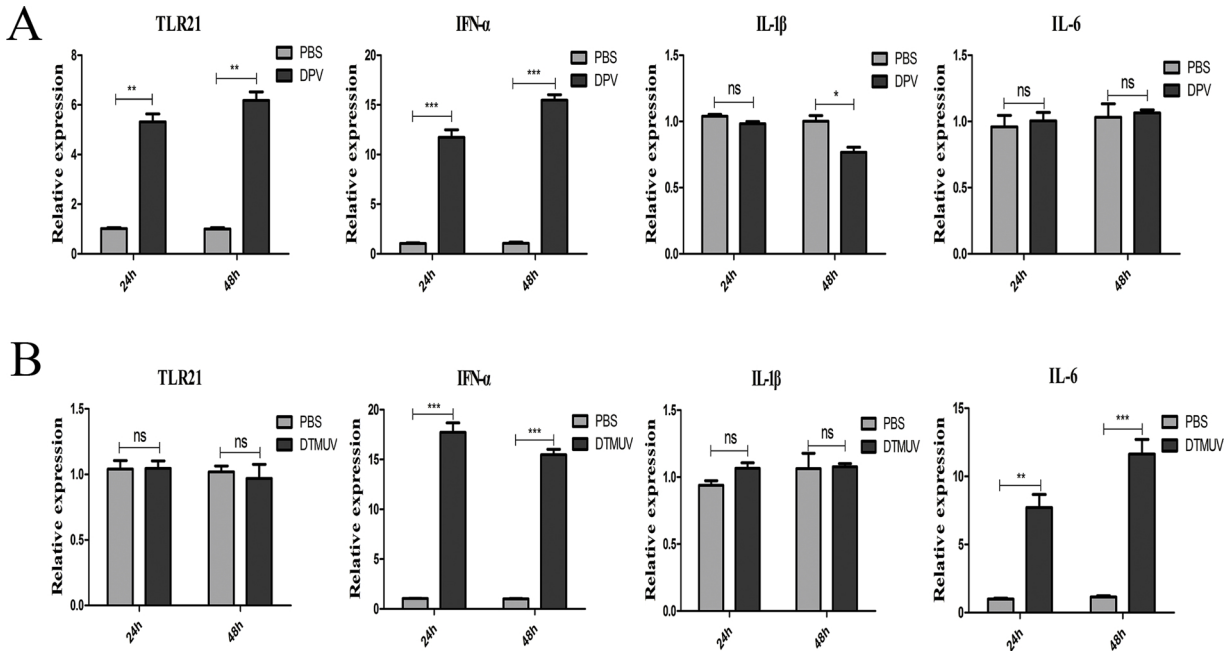

Fig. 9. The effects of DPV and DTMUV on duTLR21 and cytokines transcriptional levels in DEFs. DEF cells were challenged by DPV (10^3 $TCID_{50}$), DTMUV (10^3 $TCID_{50}$) or PBS, at the indicated time periods, the mRNA level of duTLR21, IFN-α, IL-6 and IL-1β were measured by qPCR. GAPDH was chosen as the control gene. Data are presented as the mean ± SD (n = 3). The two way ANOVA test was used to analyze the statistical significance of differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

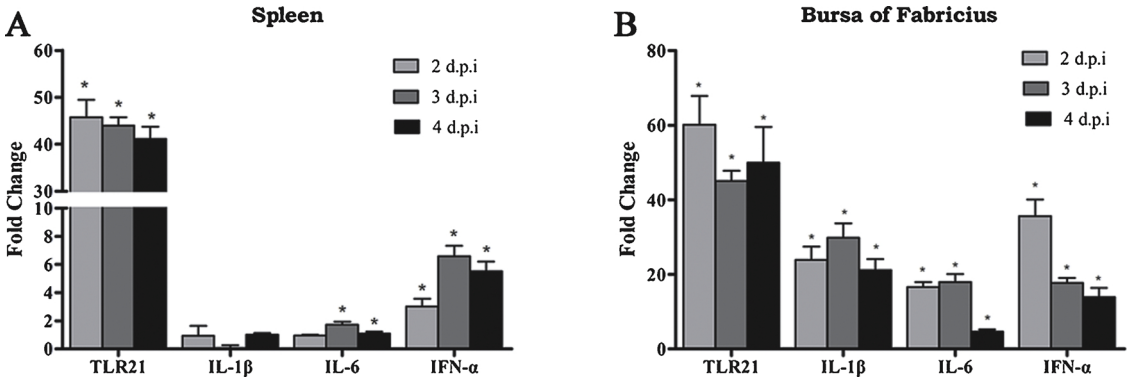

Fig. 10. The effects of DPV on duTLR21 and cytokines transcriptional levels in ducklings. 7-day-old ducklings were infected with DPV (5×10^6 $TCID_{50}$). ducklings treated with PBS as control. The spleen and bursa of Fabricius from each group were collected for RNA extraction at 2/3/4 days post-infection. The expression of duTLR21, IL-1β, IL-6 and IFN-α were measured by qPCR and were normalized to the expression of the GAPDH gene. Data are presented as the mean ± SD (n ≥ 6). The two way ANOVA test was used to analyze the statistical significance of differences (*$P < 0.05$).

bursa of Fabricius and spleen (Fig. 10). We can see the IFN-α, IL-6 and IL-1β expression levels in vivo were similar to those in DPV infected DEFs, and the IFN-α mRNA levels were up-regulated while IL-1β expression levels had no change in both tissues at each time point. But here comes something different, IL-6 mRNA levels were up-regulated in bursa of Fabricius at 2 d.p.i and 3 d.p.i.

To investigate the effect of duTLR21 modulates DPV replication in vitro, the duTLR21 expression plasmid or shRNA plasmid were transfected into the DEFs. By qPCR, the DEFs transfected with duTLR21 inhibited the DPV replication (Fig. 11A) and the knockdown of endogenous duTLR21 by shRNA significantly promoted DPV replication (Fig. 11B) at 24 and 48 hpi.

4. Discussion

Waterfowls have been known for some time to be the natural reservoir of many pathogens, particularly duck species (Juthatip et al., 2008; Long et al., 2008). TLRs, as an important part of innate immune system, play a vital role in defense against these pathogens. Now lots of researches on the duck's TLRs have been executed, but those studies are still not comprehensive enough. In this study, we cloned the cDNA of duTLR21 successfully, hoping to provide more information about this TLR. The deduced duTLR21 protein was composed of 976 aa and had the most similar amino acid sequence to that of goose (92%), followed by that of chicken (76%), but it was quite different from that of *Salmo salar* (43%) and *Danio rerio* (45%). Multiple sequences alignment showed that compared with other domains, the TIR domain, critical for TLR signaling, was more conserved. The most different part was extracellular LRR region, unlike TIR domain, this domain seems more divers, and maybe this results in different recognition profile of TLR21 between birds and fishes.

The phylogenetic analysis of TLR21 showed a similar result, but it was noteworthy that duTLR21 showed closer relationship with newt and crocodile TLR13 than fish TLR21, as we know, newt belongs to amphibia, and crocodile belongs to reptile. According to Roach' report (Roach et al., 2005), both TLR13 and TLR21 belong to the TLR11

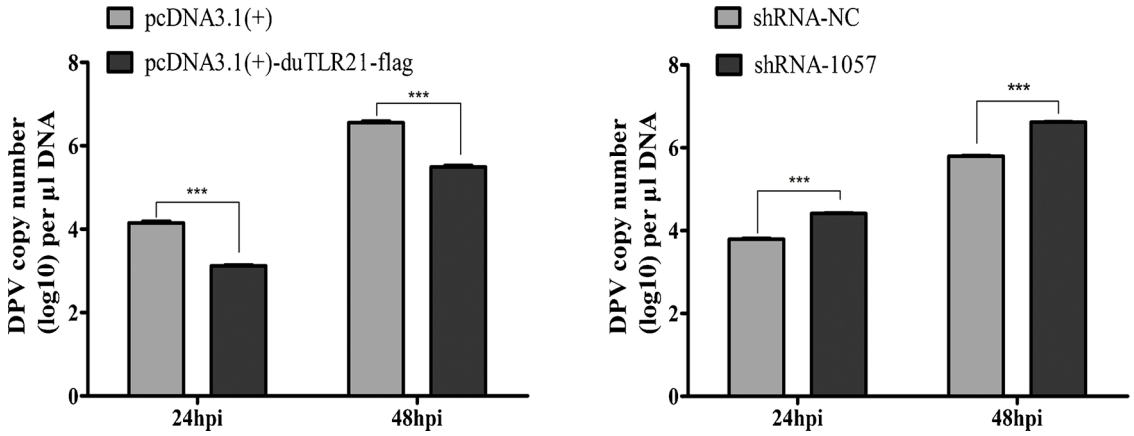

Fig. 11. Effects of the duTLR21 on DPV replication. (A) The DEFs transfected with duTLR21 inhibited the DPV replication. DEFs were transfected with 2.5 µg/well pcDNA3.1(+) or pcDNA3.1(+)-duTLR21-flag. (B) the knockdown of duTLR21 by shRNA significantly promoted DPV replication. DEFs were transfected with 1 µg/well shRNA-NC or shRNA-1057. At 48 h after transfection, the cells were infected with DPV (10^3 $TCID_{50}$). The cells were collected, and the DPV copies were examined by qPCR at 24 h and 48 h post infection. Data are presented as the mean ± SD (n = 3). The two way ANOVA test was used to analyze the statistical significance of differences (***$P < 0.001$).

family, this family is clearly very ancient, because most subclades of TLR11 have representatives from fish, frogs and reptiles. Meanwhile, TLR9 belongs to the TLR7 family, this family plays a particularly important role in protecting against viral infection, and TLR9 also senses against cell-invading bacteria (Kawai and Akira, 2010). It was demonstrated that expression of chTLR21 in HEK-293 cells could recognize exogenous ODN, chTLR21 acts as a functional homolog to mammalian TLR9 (Brownlie et al., 2009; Keestra et al., 2010). TLR21 was found in fish, amphibians and reptiles but not exist in mammal. However, TLR9 is present in both mammal and fish. We all know that fish evolved into amphibians, amphibians evolved into reptiles, and reptiles evolved into birds or mammals. Fish express both TLR9 and TLR21, while bird only express TLR21, mammals only express TLR9. So we consider due to the long-term evolution and selection, these different animals choose their own appropriate receptors that made them better adapt to their environment.

Some studies revealed that murine TLR13, an endosomal receptor can recognize the conserved motif of the large ribosome subunit of bacterial RNA (23S rRNA) and do not present in humans. Later study found that TLR13-mediated recognition of unmethylated 23S rRNA is partially required for the in vitro induction of cytokines in response to heat killed bacteria and purified bacterial RNA, but not in response to live bacteria (Signorino et al., 2014). TLR21 can recognize unmethylated CpG-DNA, we can see that TLR21 and TLR13 are both related to unmethylated nucleic acid, but what's the deeper relationship between them needs to delve into. The predicted crystal structures of duTLR21 protein had different forms, unliganded duTLR21 was a monomer, and ligand-bound duTLR21 formed a symmetric TLR21-ligand complex. Because of no available data about TLR21, both tow forms are predicted by SWISS MODEL based on TLR13 or TLR9 (Umeharu et al., 2015; Wen et al., 2015). Those predicted results require further investigation.

The tissue distribution results implied that duTLR21 was predominantly expressed in the peripheral blood, spleen, bursa of Fabricius and cecum. Those tissues possess a high content of dendritic cells, macrophages, T cells and B cells which were believe to synthesize TLR21. The relatively expression levels of duTLR21 in cecum and lung of adult ducks were higher than that of ducklings, this may be related to their development of immune system.

Studies show that TLR21 is important for regulating host defence against foreign DNA and acts as a functional homologue to mammalian TLR9 (Brownlie et al., 2009; Chrzastek et al., 2014; Han et al., 2010; Keestra et al., 2010; St Paul et al., 2011). Pohar et al. revealed huTLR9 is activated by CpG-ODNs comprising at least two CpG motifs separated by 6–10 nucleotides, and the first CpG motif is preceded by the 5′-thymidine (Pohar et al., 2015a), like CpG-2007. ZebTLR21 also could recognize CpG-2007, CpG-HC4040 and CpG-2395(Yeh et al., 2013). Mouse TLR9 (moTLR9) can be activated by an CpG-ODN comprising a single CpG motif (Pohar et al., 2015b). In this study, the CpG-2007 and CpG-2395 were found to be the most potent agonists in activating duTLR21 in DEFs, CpG－HC4040 seems weaker than CpG-2007 and CpG-2395, and they all do up-regulate proinflammatory cytokine transcripts in DEFs. Recent report indicated that the first CpG motif which preceded by the 5′-thymidine and located in ODN's 5′ end is necessary for huTLR9 (Pohar et al., 2017). So we assume that duTLR21 has broader CpG DNA ligand specificity compared with huTLR9, and this is consistent with Keestra's study in which chTLR21 could response to both human- and murine-specific TLR9 ligand as well bacterial genomic DNA while huTLR9 responsed to not all (Keestra et al., 2010). In HEK293 cells which expressed chTLR21, chTLR21 is implicated in MyD88-dependent signaling pathway and CpG DNA as ligand and activator of NF-κB and proinflammatory gene transcription, all those were remarkable resemblance to mammalian TLR9 (Brownlie et al., 2009). Our results showed similar to chTLR21, that CpG-ODN activates NF-κB through duTLR21 and the mRNA level of IL-1β, IL-6, and IFN-α were significantly up-regulated.

In vitro, DEFs were challenged with DPV and DTMUV. We found that mRNA levels of duTLR21 were significantly up-regulated in DEF infected with DPV at both 24 and 48 hpi, suggesting that duTLR21 can respond to DPV infection. Also, IFN-α mRNA levels were increased at each time point, similar results were obtained in a study about HSV (Hochrein et al., 2004). DTMUV cannot up-regulate duTLR21 expression level, this result accord with the study by Chen et al (Chen et al., 2016), it triggers melanoma differentiation-associated protein 5 (MDA5) and TLR3-dependent signaling pathways. Since TLR21 could respond to DPV in DEFs, we attempted to determine whether duTLR21 could recognize DPV in ducks. Ducklings infected with DPV were increased duTLR21 and IFN-α expression levels in both spleen and bursa of Fabricius. The expression levels of IL-6 were increased in bursa of Fabricius at 2 and 3 d.p.i, but were not significantly changed in spleen, the results in spleen were similar to the study by Li et al. (Li et al., 2016). The difference of IL-6 expression may because of the different cell types in spleen and bursa of Fabricius. Actually, when CpG-OND stimulated DEFs, mRNA expression levels of IL-1β were significantly increased, but when DPV stimulated ducklings, IL-1β has no change in both two tissues at each time point in this study. DPV belongs to the same genus as HSV. Previously studies showed that HSV can evades the host immune response (Friedman et al., 2000; John et al., 1998;

Wakimoto et al., 2003), one of those molecular mechanisms is inhibition of dendritic cells (DC) maturation, the DC infected with HSV-1 were unable to up-regulating costimulatory molecules, did not produce cytokines like IL-6, IL-10 and IL-12, and did not obtain responsiveness to chemokines which required for migration to secondary lymphoid organs, but HSV-1 did not affect mature DC (Salio et al., 1999). Another of those molecular mechanisms is that HSV is evasion from non-specific host defence mechanisms during primary infection through suppression of cytokine production in HSV-infected cells. For example, expression of IL-1β gene was enhanced by UL41-deficient HSV-1-infection compared with mock-infected cells (Tatsuo et al., 2000), this means the UL41 gene of HSV can reduce the IL-1β production in HSV-infected cells. Thus we speculate that DPV employs the similar strategies to evade immune response, resulting in DPV can activate the expression of duTLR21 significantly, but the downstream cytokines, including IL-1β and IL-6, were not dramatically changed. By overexpressing the duTLR21, we found that compared with the control group, the copy numbers of DPV were significantly reduced. And the replication of DPV was effectively increased in the endogenous duTLR21 knockdown test. We speculate that duTLR21 protects the host against DPV infection by activating the NF-κB signaling pathway. However, the further antiviral mechanism still needs to be explored experimentally.

In conclusion, our data show that duTLR21 can be activated by CpG-ODN, which mediates activation of NF-κB signaling pathway, and plays an important role in the host defence of DPV infection.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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