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GoTLR7 but not GoTLR21 mediated antiviral immune responses against low pathogenic H9N2 AIV and Newcastle disease virus infection

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ABSTRACT

Aquatic birds are considered the biological and genetic reservoirs of avian influenza virus and play a critical role in the transmission and dissemination of Newcastle Disease Virus (NDV). Both TLR7 and TLR21 are important for the host antiviral immune response. In an *in vivo* study, goTLR7, not goTLR21, was significantly up-regulated in the lungs of geese at 3 to 7 d after challenge with H9N2. And goOASL expression was induced in the bursa of fabricius, harderian glands and lungs. An increase in goRIG-I was detected in the lung and small intestine, whereas goPKR was increased in the lung but decreased in the thymus. In the *in vitro* study, goTLR7 and goRIG-I but not goTLR21 were highly induced by H9N2. Moreover, goOASL and goPKR were significantly induced in H9N2-treated PBMCs, whereas goMx was suppressed. The over-expression of goTLR7, not goTLR21, controlled NDV replication in DF-1 cells, resulting in a decrease in viral copies and the viral titres. Furthermore, we explored the cellular localization of goTLR7 and goTLR21 in heterologous (DF-1 and BHK21) and homologous cells (GEF) through ectopic expression of goTLRs. The antiviral functions of goTLR7 and goTLR21 during H9N2 and NDV infection and their cellular locations were reported here for the first time. These results will contribute to better understand the TLR-dependent antiviral immune responses of waterfowl.

1. Introduction

The innate immune system is the first line host defence against microbial infections. The innate immune system can recognize components of foreign pathogens called pathogen-associated molecular patterns (PAMPs) *via* pattern recognition receptors (PRRs) [1,2]. Toll-like receptors (TLRs) are a family that belongs to the PRRs and are conserved between vertebrates. To date, approximately 13 TLRs have been identified in mammals [3] and 10 TLRs have been identified in avian species [4]. TLRs can recognize a wide range of microbes, including bacteria, viruses, parasites and fungi. Some TLRs are expressed on the cell surface, whereas others exist in intracellular endosomes and lysosomes [5]. When PAMPs are recognized by TLRs, the TLRs induce inflammatory cytokine, interferon, interleukin and interferon-stimulated genes to defend against the invading pathogens [6].

H9N2 viruses commonly exist in domestic poultry. These viruses were first detected in the United States in 1966 [7]. The viruses infect hosts ranging from birds to mammals and can be transmitted to humans [8]. Avian influenza viruses (AIV) often cause lesions and even death in infected chickens [9]. Waterfowl are thought to be the natural reservoirs of avian influenza A virus and are resistant to many strains; infected aquatic birds are typically asymptomatic and experience long-lasting infections [7,10]. The immune responses of these birds differ greatly from chickens. TLRs have been reported to be involved in the induction of innate immunity. For instance, chTLR7 and chTLR15 were significantly induced in chickens infected with AIV [11]. In another study, TLR7 was elevated after an H5N1 infection in both Chicken embryo fibroblast

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(CEF) and duck embryo fibroblast (DEF) throughout the duration of the experiment [12].

Newcastle disease virus (NDV) also causes a critical disease in poultry. Unlike H9N2, NDV infection can lead to high morbidity and mortality [13]. NDV can be effectively transmitted between many avian species [14], although different responses to virulent NDV infections between species have been reported. Chickens are highly prone to NDV infection and virulent NDV strains may cause lesions and even death [15,16]. Waterfowl are reservoir hosts that have naturally high resistance to NDV [17]. Host innate immune systems such as TLR and NOD-like receptors can respond to NDV and produce antiviral factors including interferon and cytokines to help limit and inhibit viral replication [18]. Different TLR7 and TLR3 expression levels have been detected in tissues following challenge of chickens and ducks with NDV [19]. For instance, CEFs and DEFs showed different TLR7 and TLR3 expression patterns post-NDV infection in an *in vitro* study [20].

In contrast, little is known about how goose TLRs mediate the host antiviral immune response. Considering the differential susceptibility between fowl and waterfowl to both AIV and NDV, we investigated the different innate immune responses triggered by these viruses. Both H9N2 and NDV are single-strand RNA viruses that can be recognized by TLR7. This study was undertaken to investigate the goTLRs involved in the antiviral immune response against H9N2 infection *in vivo* and to detect their antiviral functions against NDV *in vitro*.

TLR functions are related not only to the recognition of PAMPs but also to their cellular localization and signal properties [21]. TLR7 was demonstrated to localize in the endosome and recognize single-stranded RNA viruses, such as avian influenza virus [22]. However, another study demonstrated that TLR7 was expressed on the surface of innate immune cells [23]. TLR21 is absent in mammals but present in birds, fish, and batrachians [4] and can modulate anti-virus innate immunity through cytokine production [24]. Interestingly, chTLR21 was localized in the endoplasmic reticulum similar to mouse TLR7 [25]. However, the localization of TLR7 and TLR21 in waterfowl is largely unknown.

Here, the cellular localization of goTLR7 and goTLR21 were shown for the first time. Collectively, our results provide insights into the goose antiviral immune system and shed light on the differential innate immune responses between fowl and waterfowl.

2. Materials and methods

2.1. Viruses, animals and cells

The avian influenza virus A/chicken/JS/C1/2008 (H9N2) was obtained from the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The virus was propagated in specific pathogen-free (SPF) chicken eggs. The H9N2 copy number was $7.14 \times 10^{12.64}$ copies/mL. The viral $TCID_{50}$ was $10^{7.375}$/0.2 mL. Newcastle disease virus (NDV) was provided by the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The copy number was $5 \times 10^{5.12}$ copies/mL.

Four healthy adult Sichuan White geese (*Anser cygnoides*) and thirty 3-day-old Sichuan white goslings were provided by the goose-breeding farm of Sichuan Agricultural University.

The DF-1 cell line was provided by our laboratory. The DF-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) at 37 °C in an atmosphere with 5% CO_2. The same culture conditions were used for the BHK21 cells. The GEFs were separated from a 9-day goose embryo.

2.2. Construction of the eukaryotic expression plasmid

Goose TLR7 was amplified by PCR from the cDNA of the goose harderian gland. Primers were designed according to the reported sequence (GenBank No. KJ022638). The primer sequences were 5′-AGTCCGGACTCAGATCTCGAGCTATGGTACCTCATGCAGAGAC-3′ (forward) and 5′-CGGGCCCGCGGTACCGTCGACCTAAA CAGTTTCCTGGAGAA-3′ (reverse). The product was cloned into the XhoI/SalI sites of PEGFP-C1 according to the instructions for the one step cloning kit (Vazyme, China) and named PEGFP-C1-goTLR7. GoTLR21 was amplified by PCR from the cDNA of the goose bursa of fabricius using primers designed according to the reported sequence (GenBank No. KT735043). The primer sequences were 5′-TCGAGCTCAAGCTTCGAATTCGCCACCATGACCGAACCAGCAGAC-3′ (forward) and 5′-ATGGTGGCGACCGGTGGATCCCGCGCCTGCTCCTC TCCCTC-3′ (reverse). The product was cloned into the EcoRI/BamHI sites of pDs-Red1-N1 according to the instructions of the one step cloning kit and named pDs-Red1-N1-goTLR21.

2.3. Animal study design, RNA extraction and cDNA preparation

To examine the mRNA level changes of immune-related genes between the control and H9N2-infected birds, thirty 3-day-old Sichuan white goslings were randomly divided into two groups. The geese of one group were infected with 0.2 mL of virus by intramuscular injection and nasal intubation. The other group was inoculated in the same manner with 0.2 mL of sterile phosphate-buffered saline (PBS). The small intestine and lung were collected 1 d and 7 d post infection(PI). The small intestine, spleen, lung, harderian gland, bursa of fabricius, and thymus were collected 3 d PI. Total RNA was extracted from the collected tissues using TRIzol (TAKARA, Japan) and then used to prepare cDNA. The 5 × All-In-One RT Master Mix (Abm, Canada) was used for first-strand cDNA synthesis. Viral nucleic acids were extracted using the TIANamp Virus DNA/RNA Kit (TIANGEN, China) according to the product manual's instructions. The HiScript 1st Strand cDNA Synthesis Kit (Vazyme, China) was used for first-strand cDNA synthesis.

2.4. PBMC cell culture and H9N2 infection in vitro

Four healthy adult Sichuan white geese provided blood by neck venipuncture. PBMCs were isolated from the blood using the goose lymphocyte separation medium kit according to the manufacturer's instructions (TBD, China). The PBMCs were cultured in RPMI 1640 medium with 10% foetal bovine serum, 100 μg/mL penicillin and streptomycin at 37 °C and 5% CO_2 with a density of approximately 2×10^6 cells/mL; each well contained a 2 mL cell suspension. The PBMCs were challenged with 0.2 mL of H9N2 virus in quadruplicate and the other four wells were infected with PBS as the control. The PBMCs were harvested 8 h PI. The RNA and cDNA preparation was performed as described above.

2.5. RT-qPCR

The RT-qPCR was performed to detect relative gene expression. The primers and PCR conditions for goMx, goPKR, and goOASL were previously reported [26]. The goRIG-I primers were designed according to the reported sequence (GenBank No. JF804977). The primer sequences were 5′-CCTCGGTGCCTTTTGTGT-3′ (forward) and 5′-CAGCACGTAGACGGGGTT-3′ (reverse). Goose GAPDH was used as an internal control [27]. The gene expression levels were measured using the Bio-Rad CFX96 Real-Time Detection System (Bio-Rad) following the manufacturer's instructions. The PCR conditions for goTLR21 and goRIG-I were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 59 °C for 1 min. For goTLR7 and NDV, the annealing temperature was 57 °C. Dissociation curves