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Research paper

Goose toll-like receptor 3 (TLR3) mediated IFN-γ and IL-6 in anti-H5N1 avian influenza virus response

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ABSTRACT

Induction of the innate immune pathways is critical for early anti-viral defense. How geese recognize viral molecules and activate these pathways is not well understood. In mammals, Toll-like receptor 3 (TLR3) recognizes double-stranded RNA. Activation of TLR3 induces the activation of NF-κB and the production of type-I interferon. In this study, the goose TLR3 gene was cloned using rapid amplification of cDNA ends. Goose TLR3 encoded an 896-amino-acid protein, containing a signal secretion peptide, 14 extracellular leucine-rich repeat domains, a transmembrane domain, a Toll/interleukin-1 receptor signaling domain, and shared 46.7–84.4% homology with other species. Tissue expression of goose TLR3 varied markedly and was highest in the pancreas and lowest in the skin. Human embryonic kidney 293 cells transfected with goose TLR3 and NF-κB-luciferase-containing plasmids responded significantly to poly i:c. The expression of TLR3, IL-6 and IFN-γ mRNA, but not IL-1 mRNA, was significantly upregulated after poly i:c or high pathogenic avian influenza virus (H5N1) stimulation in goose peripheral blood mononuclear cells cultured *in vitro*. Furthermore, geese infected with H5N1 showed significant upregulation of TLR3, especially in the lung and brain. We conclude that goose TLR3 is a functional TLR3 homologue of the protein in other species and plays an important role in virus recognition.

1. Introduction

Toll-like receptors (TLRs) are a major component of the pattern recognition receptor (PRR) repertoire in vertebrates. TLRs can recognize conserved pathogen-associated molecular patterns of bacteria, fungi, viruses, parasites and also some self-ligands such as host DNA in immune complexes and hemozoin (Medzhitov and Janeway, 1997; Sikora et al., 2010; Takeshita et al., 2005). Upon activation by an agonist, TLRs induce the expression of a wide range of immunoregulatory molecules (Remer et al., 2003; Thoma-Uszynski et al., 2001; Yoshimura et al., 2014), which regulate immune cell maturation and clearance of pathogenic microorganisms (Banchereau and Steinman, 1998; Brightbill and Modlin, 2000; Hertz et al., 2001; Thoma-Uszynski et al., 2001).

TLR3 plays an important role in defense against viral invasion by upregulating the expression of antiviral-type-I IFN (Alexopoulou et al., 2001), especially in recognizing double-stranded RNA (dsRNA), which is formed during viral genome replication or transcription, and is localized exclusively in intracellular vesicles such as the endosome and endoplasmic reticulum where viruses undergo uncoating during infection (Kawai and Akira, 2010). Interestingly, low expression of TLR3 leads to an unexpected survival advantage – efficient viral replication and decreased lung lesions – as observed in TLR3-deficient mice challenged with H5N1 virus (Le Goffic et al., 2006). This suggests that the decrease in TLR3 in the lungs during influenza infection is an important protective host mechanism to prolong survival against influenza.

Chicken TLR3 (chTLR3) has approximately 48% amino acid identity with human TLR3 (Kogut et al., 2005), and displays a broad tissue

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distribution, yet is only moderately expressed in bone marrow, skin, and muscle, similar to the expression profile of mammalian TLR3 (Barjesteh et al., 2012). The Muscovy duck (*Cairina moschata*) TLR3 (MdTLR3) shares 87.3% amino acid identity with chTLR3, and 62.0% and 60.2% identity with human TLR3 and mouse TLR3, respectively (Jiao et al., 2012). It should be noted that the sequence differences between the Muscovy duck and chicken are primarily located in the TIR domain of TLR3, which is among the most conserved TLR sequences. The expression of MdTLR3 is significantly upregulated in the brain reaching a maximum at 48 h post-infection with high pathogenic avian influenza virus (HPAIV) H5N1, which is similar to chTLR3 (Jiao et al., 2012). In contrast, downregulation of MdTLR3 was observed in the spleen and lung after HPAIV H5N1 infection, while chTLR3 is significantly upregulated in both tissues during infection (Chen et al., 2013). The expression of pigeon (Columbidae) TLR3 [GenBank accession No. AB618533] is upregulated in the brain of HPAIV-H5N1-infected pigeons, whereas TLR3 expression was inversely correlated with viral replication in the lungs (Hayashi et al., 2011; Le Goffic et al., 2006).

Although TLR3 genes have been identified in mammalian and non-mammalian vertebrates including chicken and duck (Barjesteh et al., 2012; Hayashi et al., 2011; Jiao et al., 2012; Kogut et al., 2005), it is not known whether the goose, an important waterfowl species in China, also has a homologue of mammalian TLR3 for recognizing pathogens, and the molecular mechanism of avian TLR3 action requires further investigation. In this study, the goose TLR3 sequence, and its expression in several tissues and its poly i:c-mediated signals in Human embryonic kidney (HEK)-293T cells, were investigated. The expression of TLR3 and pro-inflammatory cytokines in goose tissues following challenge with HPAIV H5N1 was also studied.

2. Material and methods

2.1. Animal

Magang geese (*Anser domesticus*) were purchased from Qingyuan Livestock Co. (Guangdong, China). For the TLR3 clone experiments, and those that required peripheral blood mononuclear cells (PBMCs), 30-week-old geese were used. For infection experiments, 1-day-old geese were used and allowed to acclimatize for approximately 3 weeks. All geese were housed in isolators with access to water *ad libitum* and fed a diet according to the recommended nutrient allowances for this breed. The geese were serologically negative for H5N1 by agar gel precipitation test and hemagglutinin inhibition assay. All animal experiments were conducted under the guidance of the Centers for Disease Control's Institutional Animal Care and Use Committee in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. This study was approved by the Guangdong Ocean University Animal Ethics Committee (Permit No. 201–1225).

2.2. Virus

The A/Duck/Guangdong/212/2004 (H5N1) virus (DK212) was isolated from ducks in the Guangdong Province of China in 2004 and identified as H5N1 avian influenza A virus by the hemagglutination inhibition and neuraminidase inhibition tests. The virus was purified and propagated in the allantoic cavity of 10-days-old specific-pathogen-free chicken egg embryos. Allantoic fluid pooled from multiple eggs was clarified by centrifugation and aliquots frozen at −70 °C. All experiments were carried out in Animal Biosafety Level 3 facilities.

2.3. Cells culture

HEK-293 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Goose PBMCs were isolated by density gradient centrifugation using LTS-1077 according to the manufacturer's protocol (density = 1.077 g ml^{-1}; TBD, Tianjin Haoyang Biological Manufacture Co., Tianjin, China). Briefly, 2 ml blood was diluted with 2 ml Hank's medium and layered over LTS-1077, and centrifuged at 225*g* for 15 min at room temperature. PBMCs were collected from between the LTS-1077 layer and the serum, washed twice in Hank's medium, re-suspended, and counted. PBMCs were re-suspended in culture medium (RPMI 1640, GIBCO) at a concentration of 2×10^5 cells ml^{-1}, and 0.5 ml of cell suspension was added to 24-well tissue culture plates and incubated at 37 °C in a carbon dioxide incubator containing 95% air and 5% CO_2 at a humidity of 100%.

2.4. Molecular cloning of goose TLR cDNAs

Total RNA was extracted from the spleens of three Magang geese using RNAiso Plus (Takara Bio Inc., Dalian, China) according to the manufacturer's instructions. To avoid contamination with genomic DNA, total RNA samples were treated with RNase-free DNase I (Takara). To clone the goose TLR3 (gTLR3) gene, primers for polymerase chain reaction (PCR) were designed (Table 1) based on the relatively well-conserved nucleotide sequences of TLR3 from chicken (*Gallus gallus*; GenBank: NM204278), duck (*Anas platyrhynchos*;

Table 1
Polymerase chain reaction primers used in this study.

Primer name	Primer sequence(5′-3′)	Annealing temp. (°C)	Product size (bp)
gTLR3F	CAAAGAATAACTAATACAACATTTTTA	54	1277
gTLR3R	GTCACAACAAAAATAATCTTCCTGC		
gTLR3qF	CTCTCTGGAAAA AAATAAGCAATTTG	58	270
gTLR3qR	CTCAAGCAAAGTGCATGATTGC		
β-actin F	CGGCAACGAGCGGTTCAGGT	65	171
β-actin R	GGGTACATGGTGGTGCCGCC		
gIL-1qF	CCTGCCTCTGTCTTCAGAAGAAGCCTCGTC	71	257
gIL-1qR	CCTCCTCCAGGAGAGCGCTCAGGTCGCT		
gIL-6qF	AGCAAAAAGTTGAGTCGCTGTGC	63	223
gIL-6qR	TAGCGAACAGCCCTCACGGT		
gIFN-γqF	ACATCAAAAACCTGTCTGAGCAGC	62	94
gIFN-γqR	AGGTTTGACAGGTCCACGAGG		
gTLR3F(ORF)	GCCATGGGAAGTGATATTCTTTGTTGGAAC	60	2691
gTLR3R(ORF)	GCACCGTGCTTTACTATTAGATTTA		
3′RACE-PCR	GGGTTTGATGAGATTCCAGTTCAGG	68	897
5′RACE-PCR	CAACATAATCAATAAGAAACATTCA	70	1748

Abbreviations: F, forward; R, reverse; RACE, rapid amplification of cDNA ends; UPM, Universal Primer A Mix; qF, forward primer for qRT-PCR; qR, reverse primer for qRT-PCR.