Poultry CRC

IMMUNOGENOMICS IN THE CHICKEN: INNATE IMMUNE MOLECULE ANALYSIS

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SUMMARY
Recent outbreaks of avian influenza have highlighted the need to understand the molecular mechanisms associated with immune response to virus in the chicken. Viral infection results in the production of foreign molecular signatures, in particular foreign nucleic acid, which allows the host an opportunity to detect the presence of a pathogen. This results in the activation of the innate immune system, which in turn triggers an array of immune responses that effectively limits the replication and spread of the virus. This detection is largely the responsibility of the Toll-like receptors (TLR) which provide a front line of defense. In mammals, TLR are known to recognise various viral motifs including double- and single-stranded RNA. With this in mind, approaches directed at utilizing the chicken genome to identify crucial molecules of the immune system, such as the TLR, will allow the development of new strategies directed at enhancing immune response. We report here the identification and cloning of the chicken orthologues of mammalian TLR3 and TLR7. At the predicted amino acid level, both receptors showed considerable similarity to their human counterparts, TLR3 showing 61% and TLR7 64% identity. Furthermore, the predicted protein domains of these TLR show secondary structures expected for TLR. These findings will provide insights into the pathways used by the innate immune cells in the recognition of viral pathogens in the chicken.

INTRODUCTION
As birds phylogenically diverged from mammals some 300-350 million years ago, access to the genome sequence of the chicken has the potential to boost our information with regard to this divergence. Additionally, from a phylogenic standpoint, the chicken genome, being from a non-mammalian vertebrate, provides a tool for comparative and developmental immunology. Comparative genomics studies utilizing the chicken genome sequence information are a potent means of identifying conserved, and therefore presumably significant, components of the immune system. Therefore, the accessibility of the chicken genome now provides the prospect of identifying the immune components important in the response to virus common to birds and mammals, and, in some respects more importantly, those components unique to chickens.

Viral infections continue to have major impacts on productivity in the poultry industry. Moreover, the continued ability of viruses to escape vaccine-induced immune response means there is a requirement to develop a new phase of protection strategies against viral infection. Resistance to infection by pathogens, such as viruses, involves the engagement of innate mechanisms of host defense. Historically, it was believed that these mechanisms were non-specific and did not involve the use of antigen-specific receptors. Recent advances in understanding of the innate immune system have shown that innate recognition involves the identification of highly conserved and widely distributed structures of pathogens (Janeway & Medzhitov, 2001). This first line of defence detects the presence of invading pathogens via a family of receptors known as the TLR. In mammals, TLR are known to

recognise various viral motifs including double and single-stranded RNA, these are the proposed ligands for TLR3 and TLR7, respectively. The recognition of nucleoside-based molecules is likely to be important for uninfected cells as an early-warning system of imminent viral infection, particularly when RNA is released from lysed cells (Lund *et al*., 2004). Furthermore, intracellular anti-viral mechanisms are known to be activated by nucleosides (Dunne & O'Neill, 2005).

Undoubtedly, the initial approaches in understanding the role of TLR in nucleic acid-induced protective immune responses in the chicken will involve the identification and development of reagents to assess these molecules. This will involve the cloning and characterization of these molecules, the identification of which cells express which TLR and respond to agonists of these TLR. Furthermore, this will allow an investigation of the signaling pathways induced by TLR-ligand interactions. With this in mind, access to the information provided by the chicken genome sequence has the potential to hasten enhancement of this area of research with implications for chicken health, welfare and an overall benefit of increased food safety. As the TLR involved in the innate response to viral infection were of importance, we focused on the characterisation of TLR3 and TLR7 and using immunogenomics approaches, we have identified and cloned the chicken orthologues of TLR3 and TLR7. In this study, we show the homology of these molecules to their human counter parts and identify a similarity in their stimulation to mammalian molecules by the production of appropriate anti-viral responses when stimulated by their ligands.

MATERIALS AND METHODS

Sequence identification. Protein sequences of the human TLR were retrieved from GenBank and used as queries in a tblastn search of the draft chicken genome. The identified TLR orthologues were amplified from kidney cDNA (TLR3) and spleen cDNA (TLR7) using PCR primers based on the predicted sequence.

In vitro **splenocyte stimulation with TLR ligands.** For TLR7 ligand (loxoribine) stimulation, splenocytes from 3-5 week-old SPF chickens were prepared for cell culture. The cells were resuspended in Dulbecco's Modified Eagle's media (DMEM) and various concentrations of TLR7 ligand (loxoribine; Sigma, Germany) were added. After 24 h of stimulation, the supernatants from these cultured cells were harvested and production of the cytokine interleukin-6 (IL-6) was measured by bioassay. For TLR3 stimulation the TLR3 ligand, polyinosinic-polycytidyllic acid (poly I:C), was used. A chicken macrophage cell line (HD11) was cultured with polyI:C (Sigma) at the following concentrations (10 μg/ml, 50 μg/ml, 100 μg/ml).

RNA isolation and RT-PCR. Cells were harvested and total RNA was isolated using an RNeasy minikit (Qiagen). Cytokine transcript levels for IL-6 were measured using semi-quantitative RT-PCR. Amplification of IL-6 targets and the endogenous normaliser GAPDH was performed in a MicroAmp Optical 96 well format (Applied Biosystems). qRT-PCR data was analysed according to the ABI Prism 7700 instructions (Applied Biosystems) using a comparative method.

RESULTS AND DISCUSSION

A comparison of known human TLR nucleotide sequences with the chicken genome data base enabled us to identify a number of chicken TLR, including chicken TLR3 and TLR7 (Table 1). The

predicted protein sequences of chicken TLR 7 and 3 were developed based on the identification of open-reading frames within the nucleotide sequence. Analysis of the chicken genomic sequences shows a similarity of the chromosomal location of the chicken TLR as compared to human TLR. Comparison of the identities of these predicted protein sequences with those of human TLR showed considerable similarity: TLR3 showing 61% and TLR7 64% identity (Table 2). Furthermore, prediction of the protein domains shows secondary structures expected for TLR (Figure 1).

Table 1. Identification and chromosomal location of proposed chicken TLR orthologues

TLR	Cell type	Ligand	Accession	Chromosome	E value
HuTLR1	leukocytes	lipoprotein	NM00326	un	0
HuTLR2	monocytes, granulocytes	lipoprotein	BC033756	4	0
HuTLR3	dendritic cells	dsRNA	NM003265	4	0
HuTLR4	monocytes	LPS	NM138554	17	0
HuTLR5	leukocytes, prostate, ovary	Bacterial flagellin	NM003268	3	0
HuTLR6	leukocytes, ovary, lung	lipoprotein	NM006068	un	0
HuTLR7	spleen, placenta,	ssRNA	BC033651	1	0
HuTLR8	leukocytes, lung	ssRNA	NM016610	1	0
HuTLR9	leukocytes	CpG DNA	NM017442	1	e^{-17}
HuTLR10	Lung, B cells	unknown	AF29667	un	0

Table 2. Similarity comparison of proposed chicken TLR orthologues, TLR7 and TLR3, to their human counterparts

Chicken TLR	Compared to human	% Identical aa
TLR7	TLR7	64
	TLR8	41
	TLR9	37
TLR3	TLR3	61

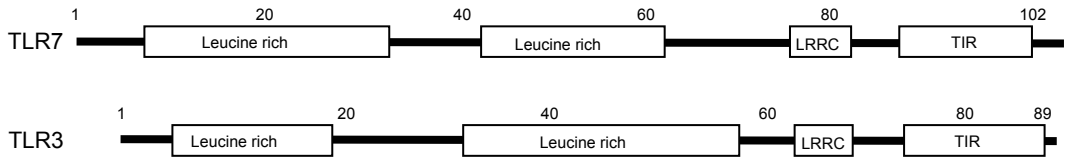

Figure 1. Putative secondary structures of chicken TLR7 and TLR3. Leucine-rich repeat areas flanked by cysteine regions carboxy-terminal region (LRRCT) are indicated, as are other leucine-rich repeat areas. The Toll/IL-1 receptor domain (TIR) is also indicated.

In order to examine the innate immune response in chickens, we have activated lymphocytes with various stimulatory ligands and analysed the resultant response. Two nucleoside-based molecules, loxoribine and polyinosinic-polycytidylic acid (poly I:C), were used to mimic the stimulatory capacity of single- and double-stranded RNA and hence to determine their ability to induce a cytokine response. These molecules induced cell proliferation and the production of IL-6 in a dose-dependant manner (Figure 2). These findings are analogous to those reported in mammals which are attributed to signalling events via the TLR family of receptors.

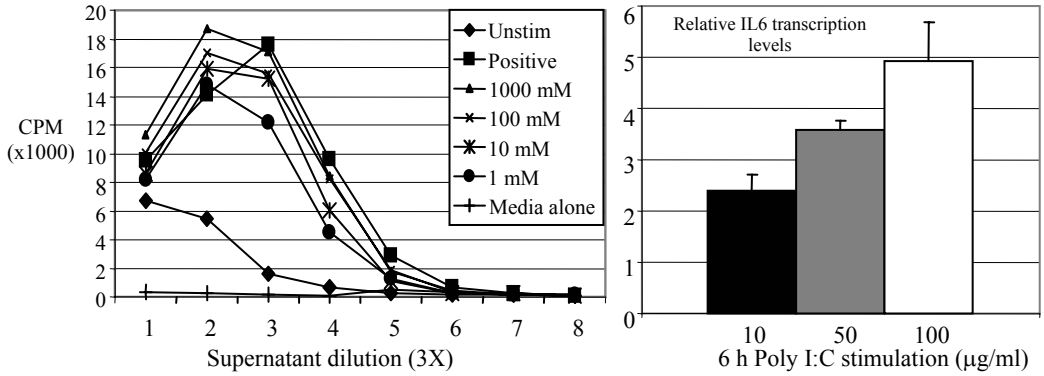

Figure 2. TLR7- and TLR3-ligand induction of IL-6. The left panel shows the measured levels of 3H-T incorporation, a reflection of cell proliferation, of 7TD1 cells (an IL-6 dependant cell line) in response to cell culture supernatants from TLR 7 ligand (loxoribine)-stimulated chicken splenocytes. Increased proliferation indicates the presence of higher levels of IL-6 in the culture supernatant as a correlate of increased stimulation by the TLR7 ligand (loxoribine). TLR7 ligand (loxoribene) stimulates splenocytes to produce IL-6 in a dose-dependant manner. The right panel indicates the relative level of IL-6 transcription by cultured HD11 cells (a chicken macrophage-like cell line), as measured by real-time PCR, in response to TLR3 ligand (poly I:C) stimulation. TLR3 ligand (poly I:C) stimulates IL-6 production in a dose-dependant manner.

Previous studies on chicken have identified that birds differ in their resistance/susceptibility to pathogens (Leveque *et al*., 2003). With this in mind, polymorphisms in TLR could in some way contribute to these differences which may correlate with disease resistance/susceptibility profiles. This area of research will be of great importance to the poultry industry.

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