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An overview of Toll-like receptors in chicken

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Abstract

Toll-like receptors (TLRs) are a class of pattern recognition receptors (PRRs) molecule of the innate immunity involved in sensing microbes. TLRs consist of an ectodomain having leucine rich repeats (LRRs) motif involved in binding microbial components referred as pathogen associated molecular patterns (PAMPs), an intracellular domain (ICD) having TIR domain involved in downstream signalling cascade and the transmembrane helix connecting both. In chicken, ten TLRs have been reported, namely chTLR1t1 & chTLR1t2; chTLR2t1 & chTLR2t2; chTLR3 binds dsRNA; chTLR4 binds LPS; chTLR5 binds flagellin; chTLR7 binds ssRNA; chTLR15 binds protease and chTLR21 binds unmethylated CpG DNA. The heterodimers chTLR2t1/chTLR1t2, chTLR2t2/chTLR1t1, and chTLR2t2/chTLR1t2 bind broad range of lipopeptides. chTLR8 is a pseudogene due to insertion of CR1 retrotransposon in its coding region. TLR dimerizes upon binding of cognate ligand and the TIR domain of it recruits adaptor proteins (MyD88, TIRAP/MAL and TRIF), which activate MyD88-dependent/ TRIF-dependent pathway resulting in production of pro-inflammatory cytokines and type 1 Interferons depending on TLR type and cell type involved. Chicken is relatively insensitive to LPS because TRAM adaptor is absent in avian genome and also might be due to absence of TRAM/ TRIF-dependent TLR4 signalling pathway to produce Type 1 Interferon. The TLR15 and 21 are specific to avian species. This review explores the structure, function and signalling mechanism of TLR in chicken. TLRs are candidate gene for studying disease resistant trait and the TLR agonist can be used as an adjuvant for vaccines to boost the immune response against various pathogens in chicken.

Keywords: *Toll-like receptor, chicken, innate immunity, signalling*

Introduction

Toll-like receptors (TLRs) are a class of the germline-encoded pattern recognition receptors (PRRs) molecules of innate immune system. TLRs are sensor of innate immunity involved in initial detection of microbial components (signal) in the environment and transduce this signal to cellular response mainly via production of cytokines and chemokines. In 1996, Jules Hoffmann and his co-workers made pioneer discovery of Toll gene function in defense against pathogens in drosophila. Similarly in 1998, Bruce Beutler and his colleagues discovered homolog of Toll gene (fruit fly) known as Toll-like receptor in mice. The Nobel Prize (2011) in Physiology or Medicine was awarded to Bruce A. Beutler and Jules A. Hoffmann for the discovery of Toll-like receptor and other half of Nobel Prize was awarded to Ralph M. Steinman for discovery of dendritic cell and its role in adaptive immunity.

TLRs are type I transmembrane protein consist of N-terminal extra cellular domain (ECD), C-terminal intracellular domain (ICD) and a transmembrane helix connecting the ECD with ICD (Fig 1). The ECD has conserved tandem 19–27 leucine rich repeats (LRR) motifs which bind specifically to microbial component referred as pathogen associated molecular patterns (PAMPs). The number of LRRs varies with different TLRs and with species. The LRRs motifs form horse shoe shaped solenoid structure. The ICD region has Toll/interleukin-1 receptor (TIR) domain named due to its homology to Interleukin-1 receptor signalling domain. The TIR domain initiates cascade of downstream signalling process and it is more conserved than ECD in TLRs.

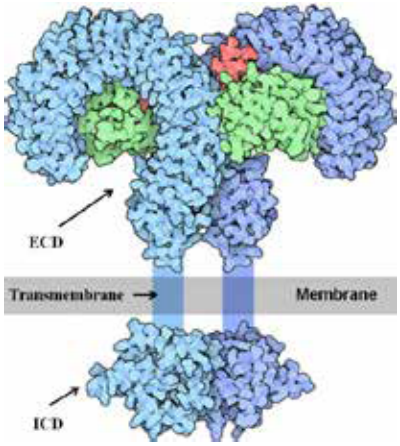

Fig 1: Structure of Toll-like receptor. ECD- Extracellular domain with LRRs motifs, ICD- Intracellular domain with TIR domain, Membrane – plasma (or) endosomal, LPS ligand in green colour. (Source: https://pdb101.rcsb.org/motm/143).

TLRs are highly conserved and found in all multicellular organisms. The number of TLRs varies with the species. The TLR repertoire in chicken consists of ten TLRs. In mammals, the TLR1, TLR2, TLR4, TLR5, TLR6 & TLR10 are located on the cell surface and TLR3, TLR7, TLR8 & TLR9 are located on the endolysosomal compartment.

TLRs of chicken

The chicken TLR (chTLR) repertoires consist of ten TLRs namely chTLR1 type 1 (chTLR1t1) or chTLR1-like protein A (chTLR1LA), chTLR1 type 2 (chTLR1t2) or chTLR1-like protein B (chTLR1LB); chTLR2 type 1, chTLR2 type 2; TLR3; TLR4; TLR5; TLR7; TLR15 and TLR21 (Temperley et al., 2008). The single chTLR1 type 1 molecule has ligand specificities of both mammalian TLR1 and TLR6 hence it is originally referred as chTLR16 (Keestra et al., 2007). The chTLR1 type 2 are truncated form of chTLR1 type 1 having shorter N-terminal LRR region. The TLR15 and TLR21 are specific to avian species and absent in mammals (Keestra et al., 2013). The chTLR21 functions as mammalian TLR9 ortholog. The chTLR8 is a pseudogene due to disruption of coding region by retroviral-like insertion element (Philbin et al., 2005). In chicken, TLR 1 and 2 underwent gene duplication to satisfy specific need, and TLR8 and 9 are lost might be due to redundancy and obsolete function. The ligand specificity of different chTLRs are mentioned (Table 1).

Table 1: Chicken TLRs and its ligands.

Chicken TLRs	Ligands
TLR2type1/ TLR1type1, TLR2type1/ TLR1type2	Triacylated lipopeptides
TLR2type2/ TLR1type1,	Diacylated lipopeptides
TLR3	dsRNA
TLR4/MD-2	Lipoploysaccharide (LPS)
TLR5	Flagellin
TLR7	ssRNA
TLR15	Protease
TLR21	DNA

The chicken TLR function was studied by inducing the immune cell of chicken with mammalian TLR agonists, which produced immune response. This supports the presence of functional TLRs and its downstream signalling pathways in chicken. Also the chTLRs were studied by recombinant expression of chTLRs in mammalian cell lines (heterologous) and also by using gene silencing method like RNA interference (RNAi) to inactivate target TLR gene.

TLR1/2

In mammals, TLR2 forms heterodimer with TLR1 or TLR6 resulting in TLR2/TLR1 or TLR2/TLR6 complex binding tri-acylated and di-acylated lipopeptides, respectively (Kang et al., 2009). Also human TLR2 dimerizes with TLR10 binds triacyl lipoproteins (Guan et al. 2010). In chicken, TLR2 expressed as two isoforms chTLR2 type 1 (chTLR2t1) and chTLR2 type 2 (chTLR2t2), and TLR1 as chTLR1t1/ chTLR1LA (chTLR16) and chTLR1t2/ chTLR1LB which are mammalian ortholog of TLR1 and TLR6 respectively. The genes of two chTLR2 isoforms and both chTLR1 like protein are present in tandem position in the chicken genome implies that its origin by gene duplication.

The chTLR2/chTLR1 heterodimer complexes chTLR2t1/chTLR1t2, chTLR2t2/chTLR1t1, and chTLR2t2/chTLR1t2 efficiently responded to both di- and tri-acylated lipopeptides and sonicated *Mycobacterium avium* extracts indicating broader ligand specificity unlike its mammalian counterparts (Keestra et al., 2007). These chTLR2/chTLR1 complexes show variation in ligand responsiveness and tissue expression implying distinct function of this individual complex in avian species. A stretch of 12 amino acids present in LRR10 of chTLR1t1 is absent in LRR10 of human TLR1 (Neerukonda and Katneni, 2020). Also swapping of LRR6-16 of chTLR1t1 to hTLR1 confers broad ligand specificity to hTLR1/ chTLR2t2 complex implying significance of this region in determining ligand specificity (Keestra et al., 2007).

TLR3

The mammalian TLR3 is an endosomal TLR that senses viral dsRNA or its analog poly (I:C) and activates downstream signalling through TRIF-dependent pathway resulting in type I IFN production. Similarly, the chTLR3 senses viral dsRNA or synthetic dsRNA analog like polyinosinic-polycytidylic acid [poly(I:C)] and produces type 1 interferon, which confers protection against viral infection. This shows the presence of TRIF-dependent signalling pathway in chicken. The mammalian ortholog of signalling components like TRIF and TBK1/IKKε have been identified in chicken genome indicating grossly similar intracellular signalling mechanism like mammal (Cormican et al., 2009).

TLR4

The mammalian TLR4/MD-2 (Myeloid differentiation protein-2) complex binds to bacterial lipopolysaccharide (LPS), which is the major component of the outer membrane of gram-negative bacteria. Functional chTLR4/MD-2 complex is formed only with chicken species specific TLR4 and MD-2 molecules. But mammalian (human or murine) TLR4 and MD-2 molecules can substitute each other to form functional complex. The chTLR4/MD-2 complex is activated by both hexa-acylated and penta-acylated LPS similar to murine TLR4/MD-2 complex unlike its human counterpart.

The mammalian TLR4/MD-2 complex activates both the MyD88/TIRAP-pathway and the MyD88-independent or TRAM/TRIF-pathway (O'Neill and Bowie, 2007). The MyD88/TIRAP-pathway leads to early NF-kB activation and pro-inflammatory cytokines production. But the TRAM/TRIF-pathway results in delayed NF-kB activation and production of type I interferons via IRF3 transcription factor resulting in endotoxic shock (Karaghiosoff et al., 2003).

Activation of chTLR4 by LPS results in increased IL-1β and IL-8, but not IFNβ suggesting chTLR4 may act only through MyD88 dependent pathway (de Zoete, et al., 2010). But the chTLR3 activation by IFN-β suggesting the presence of TRIF-dependent signalling in chicken. The chicken is markedly less sensitive to endotoxin (LPS) due to absence of TRAM in chicken genome and probably due to absence of TRAM/TRIF dependent TLR4 signalling pathway in chickens (Keestra and Van Putten, 2008a).

The CD14 molecule involved in transferring lipidated molecules to the TLR2 and TLR4 receptor complex lacks species specificity (i.e) the CD14 molecules of human and murine can enhance the chTLR2 and chTLR4 complexes activity (Keestra and van Putten, 2008a). Mammalian CD14 occurs as GPI-anchored cell surface protein can be released as soluble serum protein (sCD14) by proteolytic/ phospholipase activity. Unlike in mammal, chicken CD14 is a transmembrane protein (mCD14) which cannot form soluble serum protein (Wu et al., 2009). The sCD14 confers LPS sensitivity to cells not expressing membrane CD14 (mCD14). Hence, the absence of sCD14 and together with the absence of serum LPS binding protein (LPB) also may account for reduced sensitivity of chicken to LPS (Wu et al., 2009).

TLR5

The chTLR5 recognizes conserved regions of bacterial flagellins, which are repeat structural monomer of bacterial flagella from both gram positive and negative bacteria. In chicken, three LRR motifs of TLR5 differ from canonical motifs has different amino acid that is Ser at 135 and Leu at 137 position of LRR2, Gln at 162 position of LRR3 and Leu at 551 position of LRR10 (Keestra et al., 2008b). It plays a primary role in *Salmonella typhimurium* infection in chicken. In chicken, the nonflagellated *Salmonella gallinarum* fails to activate chTLR5 and induces immune response resulting in severe systemic infection. However, the flagellated *S. enteritidis* and *S. typhimurium* located in the gut activate chTLR5 and induce immune response (Kaiser et al., 2000).

TLR7/8

In mammal, The TLR7 family consists of TLR7/8/9 involved in intracellular recognization of nucleic acids. The chicken TLR7/8 gene locus is syntenic with mammalian TLR7/8 locus. But the chicken has only functional TLR7 gene and the TLR8 gene is non-functional due to disruption of its coding region by chicken repeat 1 (CR1). The CR1 is a 6.1 kb retroviral like insertion elements. In avian, the TLR8-CR1 insertion elements are identified in galliform birds (red jungle fowl, chicken, guinea fowl, Japanese quail, pheasant and turkey) but absent in non-galliform birds (goose, pekin duck, black swan, penguin & ostrich) (Philbin et al., 2005). The synthetic TLR7/8 agonists R848 and poly(U) elevate the chicken IL-1β and IL-8 mRNA expression and does not increase type I interferon mRNA expression. These effects are sensitive to chloroquine treatment indicating localization of chTLR7 in endosomal compartment similar to mammal TLR7 (Philbin et al., 2005).

TLR15

The TLR15 is unique to the avian and reptilian species. Functional characterization by ectopic expression in human cell line shows that chTLR15 is activated by fungal proteases and also by some bacterial proteases, which induces NF-kB pathway for cytokine production via MyD88-dependent pathway. This indicates chicken species-specific molecules are not essential for signalling (de Zoete et al., 2011). The avian TLR15 is expressed on the cell surface but the reptilian TLR15 is found intracellularly (Boyd et al., 2012).

Protease induced activation results in cleavage and homo dimerization of chTLR15 which initiates TLR signalling similar to mammalian TLR9, but chTLR15 signal response is insensitive to chloroquine treatment due to its localization in cell membrane. The highly conserved proline residue in the conserved BB-loop structure of TIR domain of chTLR15 was found. This conserved proline residue (Pro737) is needed for binding of MyD88 adaptor in the MyD88-dependent signalling in mammalian TLRs (O'Neill and Bowie, 2007).

TLR21

The expression of chTLR21 in HEK293 cells results in ER localization and activation of NFκB by synthetic CpG-ODN treatment. The chTLR21 senses unmethylated CpG DNA similar to human TLR9. It can recognizes broad range of CpG DNA molecules, different large and small DNAs, and also bacterial chromosomal DNA, unlike human and mouse TLR9. The action of chTLR21 is highly sensitive to chloroquine similar to mammalian TLR9, suggesting its localization in endolysosomes (Keestra et al., 2010). The CpG-ODN also elicits marked adjuvant effects by enhancing vaccination of chickens against bacterial and viral infections via augmenting

both humoral immune response (antibody titers) and Th1 immune responses (Gomis et al., 2007).

TLR Signalling

The binding of cognate ligand/ PAMPs to TLR results in dimerization of TLR and the cytoplasmic TIR-TIR structure recruits TIR-containing adaptor proteins like MyD88 (Myeloid differentiation factor 88); TIRAP (TIR associated protein) or MAL (MyD88 adaptor like protein); TRIF (TIR domain-containing adaptor protein-inducing IFN-β) or TICAM-1 (TIR domain-containing adaptor molecule 1) and TRAM (TRIF-related adaptor molecule) or TICAM-2 (TIR domain-containing adaptor molecule 2) in the cytoplasm and initiates downstream signalling cascade resulting in activation of nuclear factor-kB (NF-kB), mitogen-activated protein kinases (MAPKs) and interferon regulatory factors (IRFs) which produces pro-inflammatory cytokines and type1 interferons (IFNs) depending on the TLR and cell type involved. Most TLRs interact directly with MyD88 adaptor, but TLR2/ TLR4 needs TIRAP to bind MyD88 adaptor. TIRAP is a sorting adaptor that facilitates recruitment of MyD88 by anchoring itself to plasma or endosomal membrane via its lipid binding domain. The TLR3 phosphorylated cytoplasmic domain directly interacts with TRIF adaptor, whereas TLR4 needs TRAM as bridge to bind TRIF adaptor (Yamamoto et al., 2003). The TRAM adaptor is absent in avian genomes may partially explain why TRIF fails to participate in LPS–TLR4 signalling to produce type 1 IFN in chicken, unlike mammalian TLR4 which utilizes TRIF/TRAM-dependent pathway to produce type 1 IFN upon LPS activation (Brownlie and Allan, 2011).

Nucleic acid sensing chTLR3, 7 & 21 are located in the endolysosomal compartment, whereas other chTLRs are located in the plasma membrane. The downstream signalling process consists of MyD88-dependent pathway and MyD88-independent pathway or TRIF-dependent pathway (Fig 2). In chicken, the signalling of all TLRs (except TLR3) is presumed to make use of MyD88-dependent pathway. The TLR4 is unique in utilizing both MyD88- and TRIF-dependent pathways for signalling.

In the MyD88-dependent pathway, the TIR domain of TLR binds MyD88 adaptor protein directly or via TIRAP adaptor in the cytoplasm, which activates members of IRAK kinases and ubiquitin ligases (TRAF6), which in turn activates transcription factor NF-kB, which translocates into nucleus to produce various pro-inflammatory cytokines and chemokines (such as IL-1β, IL-6, and IL-8). In TRIF-dependent pathway, the TIR domain of TLR3 recruits the TRIF or TICAM-1adaptor protein, which activates interferon regulatory factor 3 (IRF3) resulting in production of INF-α/β (type I interferon response).

General conservation of TLR signalling was observed between avian and mammal. Many of the components of mammalian TLR signalling are present in avian (Cormican et al., 2009), except TRAM, few kinase, non kinases and transcription factors are absent in avian genome.

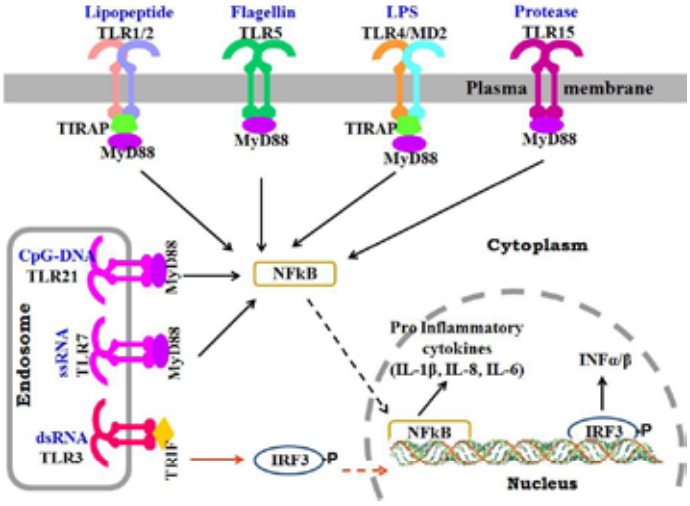

Fig 2: Schematic representation of Toll-like receptors signalling pathways in chicken.

TLR polymorphisms

The gene of immune system involved in host defense has high level of genetic polymorphism to cope up with wide range of pathogens. The chicken TLRs show on average nine times higher nucleotide diversity than human TLRs (hTLRs). Unlike hTLRs, more number of potentially functional non-synonymous variations lies in ligand binding ectodomain of chTLRs due to coevolutionary dynamics between the TLR proteins and their pathogenic counterparts. About six times more alleles was found in TLRs of chicken than in human. Also more number of alleles was shared between the breeds and allelic frequency is more equal in chicken than in human (Swiderska et al., 2018).

TLR agonist as Adjuvant

The TLR agonists contain immune modulatory activity, so these can be used as potential adjuvant with vaccines to augment chicken immune response against pathogens. The TLR3 agonists (poly I:C) enhanced the protective effects of sub-optimal HVT vaccine against Marek's disease virus (Parvizi et al., 2012). The use of bacterial flagellin as adjuvant activates TLR5 reduces *Salmonella* pathology in chicken (Genovese et al., 2007). The CpG DNA activates TLR21 and augments adaptive immune responses in chicken immunized experimentally against pathogens like fowl cholera (Herath et al., 2010), NDV vaccine (Lasota) and Low Pathogenic Avian Influenza H4N6 virus (Gunawardana et al., 2015) and pathogenic *Escherichia coli* (Gomis et al., 2007). The use of LPS-derivatives as adjuvant may be less effective due to absence of functional TRAM-TLR4 signalling pathway in chicken.

Conclusions

TLRs induce innate immune response which further activates adaptive immunity through dendritic cell. As TLR is directly involved in immune response, studying the TLRs will help to design agonist to use as vaccine adjuvants, an alternative for antibiotics and helps to understand the disease resistance trait for selection of chicken strain with increased disease resistance. In addition, the TLR variation in the degree of ligand responsiveness and specificity, and also the difference in signalling mechanism in chicken can be utilized for rational design of agonist specific and efficient to use in chicken.

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