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Differential gene expressions of NLRC5 signaling pathway in chicken macrophages cells response to *Salmonella Enteritidis* derived *lipopplysaccharides* stimulation

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ABSTRACT

As we all known, NLRC5 recognizes intracellular pathogen-associated molecular patterns (PAMPs) and provokes innate immune system. Its role in innate immune response, *NF-kB* activation and *MHC-I* expression remains controversial. In the present study, it was detected that differential gene expressions in *NLRC5* signaling pathway at 2, 4, 6 and 8 hours after exposure to LPS using qRT-PCR technology, then analyzed its roles in host defense. The results showed that, comparing to control groups, the expression of *NLRC5*, *MHC-I* and *IL-18* in LPS-treated groups were significantly up-regulated at 2 hours post stimulation (hps), *TLR4* and *NF-kB* showed conspicuously up-regulated at 4 hps, while *STAT1* was significantly down-regulated at 8 hps. Collectively, LPS did evoke inflammatory responses and *NLRC5* may negatively regulate *NF-kB* and critically regulate *MHC-I* to control intracellular PAMPs in chicken macrophage cell line but the specific role of *NLRC5* in host defense relates to cell types and species tested.

Key words: Chicken, *Lipopplysaccharides*, *NLCR5*, Signaling pathway.

INTRODUCTION

The vital factor for the initiation of proper innate immune system is recognition of bacterial and viral infections. Pattern-recognition receptors (PRRs) are keys to regulate immune response. They mediate the process by recognize PAMPs, which is the molecular structures conserved and extensively shared by microbial pathogens (Janeway 1989). A variety of PRR families have been distinguished, such as TLRs, RIG-I-like receptors, CLRs and NLR family have evolved in host cells (Janeway, 1989; Inohara *et al.*, 2005; Mohamed *et al.*, 2009). NLRs sense intracellular PAMPs, while most TLRs detect extracellular PAMPs (Philpott and Girardin, 2004; Fritz *et al.*, 2005; Rietdijk *et al.*, 2008). To date, there are at least 22 members have been distinguished in human and more and more studies and results have focused on characterizing the functions and signal pathways of NLR family members in regulating immune system.

NLR protein family shares a similar typical tripartite structure consisting of a C-terminal leucine-rich repeat (LRR) domain which in the most cases, is sensor of PAMP, a centrally-located nucleotide-triphosphatase (NTPase) (NACHT) domain (also called NOD domain) whose responsibility is self-oligomerization, and a N-terminal effector domain to trigger downstream immune signaling (Lamkanfi and Kanneganti, 2012). Recently, studies have shed light on the role of newly identified member NLCR5 in regulation of the innate immune system. Through structure prediction analysis of NLRC5, it has a unusually long stretch of 27 C-terminal LRRs and NLRC5 possesses a the predicted size of more than 200 kDa which showed it is the largest member of all known NLR family members (Neerincx *et al.*, 2010; Benko *et al.*, 2010). In recent years, researches have showed that *NLRC5* highly expressed in immune tissues and immune cells (e.g. lymphocytic and macrophage cell lineages) (Benko *et al.*, 2010; Davis *et al.*, 2011). In spite of these noticeable progresses in understanding of the structure and the expression of *NLRC5*, the characters in regulating immune responses and the inflammasome signaling pathway remain controversial. Lots of researches also focused on the relationship between *NLRC5* and *MHC class I* and *NF-kB*, and the specific relationship between them is also controversial. Nuclear transcription factor STAT1 and STAT3 are suggested to play a vital role in *NLRC5* expression regulation (Staehli *et al.*, 2012; Li *et al.*, 2014).

In the current study, the role of *NLRC5* in the regulation of *NF-kB*, *MHC-I*, *IL-6*, *IL-18*, *STAT1* and *STAT3* by utilizing stimulation with *lipopplysaccharides* (LPS) from *Salmonella Enteritidis* (SE) in chicken macrophage cell line (HD11 cell) was investigated. To elucidate the association

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of *NLRC5* with these genes involved in *NLRC5* signaling pathway, we detected the expression changes of these genes over time. We reported *NLRC5* related genes expression changes over time and discussed and analyzed its roles in host defense. This is the first study that covering so many genes related in *NLRC5* signaling pathway (LPS membrane-binding receptor TLR4, pro-inflammatory cytokine IL-6 and IL-18, antigen presenting pathway gene *MHC class I* and nuclear transcription factors NF-kB, STAT1 and STAT3) in chicken. It's novel and of significant scientific value for a better understanding of the role *NLRC5* plays in immunity response in chicken.

MATERIALS AND METHODS

Cell culture and stimulation with LPS: HD11 cell line (Beug *et al.*, 1979) was provided by Professor Susan J. Lamont, Iowa State University, USA. HD11 cells were cultured in RPMI1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 5% chicken serum (Gibco), 10 mM HEPES (WISENT INC., Canada), 2 mM L-glutamine (Gibco), 0.1 mM non-essential amino acids (WISENT INC.), 1 mM sodium pyruvate (Gibco) and 0.05 mM β-mercaptoethanol (Sigma Aldrich, USA) at 37°C with an atmosphere of 5% CO_2 and 60-70% relative humidity.

For LPS stimulation, HD11 was seeded in 12-well plates and cultured overnight. The cells were treated with SE-LPS (Sigma Aldrich) at concentration of 10 μg/ml. At 0, 2, 4, 6, and 8 hours post stimulation, the cells were collected for RNA extraction. Three biological triplicates were used in each time point group.

RNA isolation and quantitative reverse transcriptase-PCR: RNA was isolated from each sample by using Trizol extraction method (Connolly *et al.*, 2006) and detected the concentration and quality of RNA by Nanodrop 1000 (Thermo Fisher, USA). Complementary DNA (cDNA) was synthesized using FastQuant RT Kit(with gDNase) (Tiangen, China) following the manufacturer's instructions and stored at -20°C until the time of real-time PCR testing.

The primers for *NLRC5*, *IL-6*, *MHC class I* have been previously reported (Kaiser *et al.*, 2003; Lian *et al.*, 2012).The primers specific for other genes were designed with Oligo7.0 software according to sequences of NLRC5 pathway-related and housekeeping genes published on the GenBank and synthesized by Shanghai sangon biotechnology company (Shanghai, China). All sets of primers are listed in Table 1. Real-time PCR was performed on an ABI 7500 Real-time PCR Detection System (Applied Biosystems, Carlsbad, CA) with UltraSYBR Mixture reagent (With ROX) (CoWin biotechnology company, Beijing, China). Each reaction was run in triplicate and in a final volume of 20 μl with 1 μg total RNA, 10 μl 2X UltraSYBR Mixture (With ROX), 0.5 μM of each primer and 2 μl cDNA. The following PCR cycling profile was used: one single step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, ending with a melting curve analysis of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°Cfor 15 s.

Data analysis: Data were analyzed with the SPSS 13.0 software. The multiple comparisons among different time points were determined by LSD test. Differences were considered to be statistically significant when the *P* value was less than 0.05 when compared with respective controls. Results were described as fold-change determined by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

Expression changes of TLR4, NLRC5, NF-kB, MHC-I, IL-6, IL-18, STAT1 and STAT3 post LPS stimulation:

Table 1: Primers used for Real-time PCR

Gene symbol	Primer sequences(5'-3')	Accession no.	Amplification/bp	Annealing temperature/C
TLR4	F : GGATCTTTCAAGGTGCCACA R : CAAGTGTCCGATGGGTAGGT	KP410249.1	199	60
NF-kB	F : TGAACGGATCCGCACCAATA R : CGTTCACCCACACCTGGAAG	NM_205129.1	107	60
MHC-I	F : ACAAGTACCAGTGCCGCGTG R : CGCGATGTTGTAGCCCTTCC	NM_001097530.1	197	60
IL-6	F : GCTACAGCACAAAGCACCTG R : GACTTCAGATTGGCGAGGAG	AJ250838	112	60
IL-18	F : AGGTGAAATCTGGCAGTGGAAT R : ACCTGGACGCTGAATGCAA	AJ276026	94	60
STAT1	F : CCGATACACATGGCAATGATAA R : TGCATCAAGCTCCTTCTGTTTA	NM_001012914	147	60
STAT3	F : TAGTGCTGCTCCGTATCTGAAG R : CAGGTCAATGGTATTGCTGAAG	NM_001030931.1	73	60
NLRC5	F : TGAGCTACACGTCAGGAAGGA R : GCTCTGCAGAATGGACACAA	JQ044414.1	193	60
GAPDH	F : CGATCTGAACTACATGGTTTAC R : TCTGCCCATTTGATGTTGC	NM_204305	151	60

LPS activates *NLRC5* pathway-related genes expression change. We characterized those genes expression changes with 10 μg/ml LPS treatment at different points. As known by Real-time PCR, in comparison with control groups, the expression of *NLRC5*, *MHC-I* and *IL-18* in LPS-treated HD11 cells groups was significantly up-regulated at 2 hours post stimulation (hps), *TLR4* and *NF-kB* genes expression showed conspicuously up-regulated at 4 hps, while *STAT3* showed a decreasing trend and *STAT1* gene expression was significantly down-regulated at 8 hps (Fig. 1g and 1h). *TLR4*, *NLRC5* and *IL-18* genes expression showed similar trends, up-regulated conspicuously at first and then decreased to the normal expression level (Fig. 1a, b and f). The expression of *TLR4* after LPS treatment at 4 hps was significantly higher than 6 and 8 hps (Fig. 1a). *NLRC5* gene expression at 2 hours post LPS stimulation was conspicuously higher than 4, 6 and 8 hps (Fig. 1b). *IL-18* gene expression was significantly higher at 6 hours post LPS stimulation than these at other time points (Fig. 1f). The expression of *NF-kB*, *MHC class I* and *IL-6* kept significant increase and got the peak at 8 hps (Fig. 1c,d and e).

Model of transcriptional regulation of NLRC5 relevant genes: We made a model of *NLRC5* relevant genes transcriptional regulation post LPS treatment based on results in this research and combining previous research findings (Lian *et al.*, 2012; Qiu *et al*., 2015; Qiu *et al*., 2016; Chang *et al*., 2011) (Fig. 2.). LPS stimulation activated TLR4-NF-kB pathway and induced *NLRC5* expression. On the one hand, NF-kB as a trans-activator induces downstream genes expression and generates pro-inflammatory effects. On the other hand, NF-kB up-regulates *NLRC5* expression and NLRC5 repressed *NF-kB* expression by inhibiting NF-kB-dependent responses forming a negative feed-back loop (Cui *et al.*, 2010). NLRC5 induces *MHC class I* expression by binding its promoters directly (Meissner *et al.*, 2010).

To date, many functional TLRs have been identified and each TLR can detect unique PAMPs. LPS, Gram-negative bacteria cell wall components, can be recognized by TLR4 (Akira *et al.*, 2006), which have been extensively used in host innate immune response studies as an agonist of TLR4 to mimic bacterial infection and provoke inflammatory responses. And it has also been confirmed to strongly stimulate a high level transcription and expression of *NLRC5* (Cui *et al.*, 2010; Yao *et al.*, 2012). Similar results were obtained in the current study: SE-LPS stimulation induced the *TLR4*, *IL-6* and *NLRC5* expression remarkably up-regulated at 4, 6 and 2 hps respectively. NLRC5 transcription changes in Lian et al. (2012) researches in HD11 cell line with 1 μg/ml ST-LPS treatment had similar trends but time points of IL-6 gene expression differed. (Lian *et al.*, 2012), Li *et al*. (2014) reported that, in RAW264.7 cells, with 200 *n*g/ml LPS treatment, NLRC5 peaked at both mRNA and protein levels at 10 hps. Cui *et al* (2010). demonstrated that, in RAW264.7 cells, LPS stimulus up-regulated *NLRC5* at both mRNA and protein levels and reached the expression peak at 6 hps (Cui *et al.*, 2010), while Benko et al. (2010) reported that LPS treatment did not change the *NLRC5* transcription in murine bone marrow-derived macrophages (BMDMs) (Benko *et al.*, 2010). Thus, we deduced that it might be the LPS dose and sources lead to the differences.

Additionally, in the present research, the expression of *NF-kB* gene in chicken HD11 macrophage cells was up-regulated significantly at 4 hours post LPS stimulus and peaked at 8 hps while *NLRC5* expression was up-regulated remarkably and peaked at 2 hps then down-regulated significantly at 4 hps. These results indicate that *NLRC5* may regulate *NF-kB* negatively similar to what Cui et al. (2010) and Benko *et al*. (2010) reported in HEK293T and RAW264.7 (Cui *et al.*, 2010; Benko *et al.*, 2010; Li *et al.*, 2014). And recently, a series of studies based on overexpression and shRNA-mediated knockdown of *NLRC5* and *NLRC5*-deficency mice or cell lines showed *NLRC5* as a negative modulator of inflammatory pathways and antiviral immunity. Nevertheless, similar experiments came into opposite conclusions in HelaS3 cells and THP-1 (Neerincx *et al.*, 2010; Kuenzel *et al.*, 2010). It is confirmed in primary human monocytic cells that *NLRC5* knockdown repressed *IL-18* response to bacterial infection (Davis *et al.*, 2011). Similar results showed in current study, *IL-18* gene expression showed similar trend with *NLRC5* and up-regulated sharply at 2 hps and peaked at 6 hps then down-regulated over time. These results conflicted with some studies in HelaS3 cells and THP-1 (Neerincx *et al.*, 2010; Kuenzel *et al.*, 2010). Moreover, overexpression of *NLRC5* failed to trigger inflammatory responses such as the *NF-kB* in HEK293T cells (Neerincx *et al.*, 2010). Kumar et al. (2011) reported that the expression of *IL-6*, *IFNB* and *IFNA* showed no difference between *NLRC5*-deficient mice and wild type after whether viruses or bacteria infection (Kumar *et al.*, 2011). These varied results revealed that the effect of *NLRC5* in downstream pathways may rely on the cell types tested even the specific species (Davis *et al.*, 2011).

In addition, we detected the expression of *MHC-I* in current study, it showed similar expression tendency with that of *NLRC5* which backed up the point of Meissner *et al*. and conflicted with the results of Benko *et al*. (2010) Meissner *et al* (2010). reported that *NLRC5* positively regulate *MHC-I* expression in lymphoid and epithelial cell lines by binding *MHC-I* promoter region, while Benko et al. (2010) observed that *NLRC5* knockdown enhanced *MHC-I* expression on the surface of RAW 264.7 cells (Meissner *et al.*, 2010; Benko *et al.*, 2010). Lian *et al.* observed that in chicken HD11 cells, where knockdown of *NLCR5* have no effect on *MHC-I* expression with LPS stimulation and they deduced that chicken macrophages have another regulator

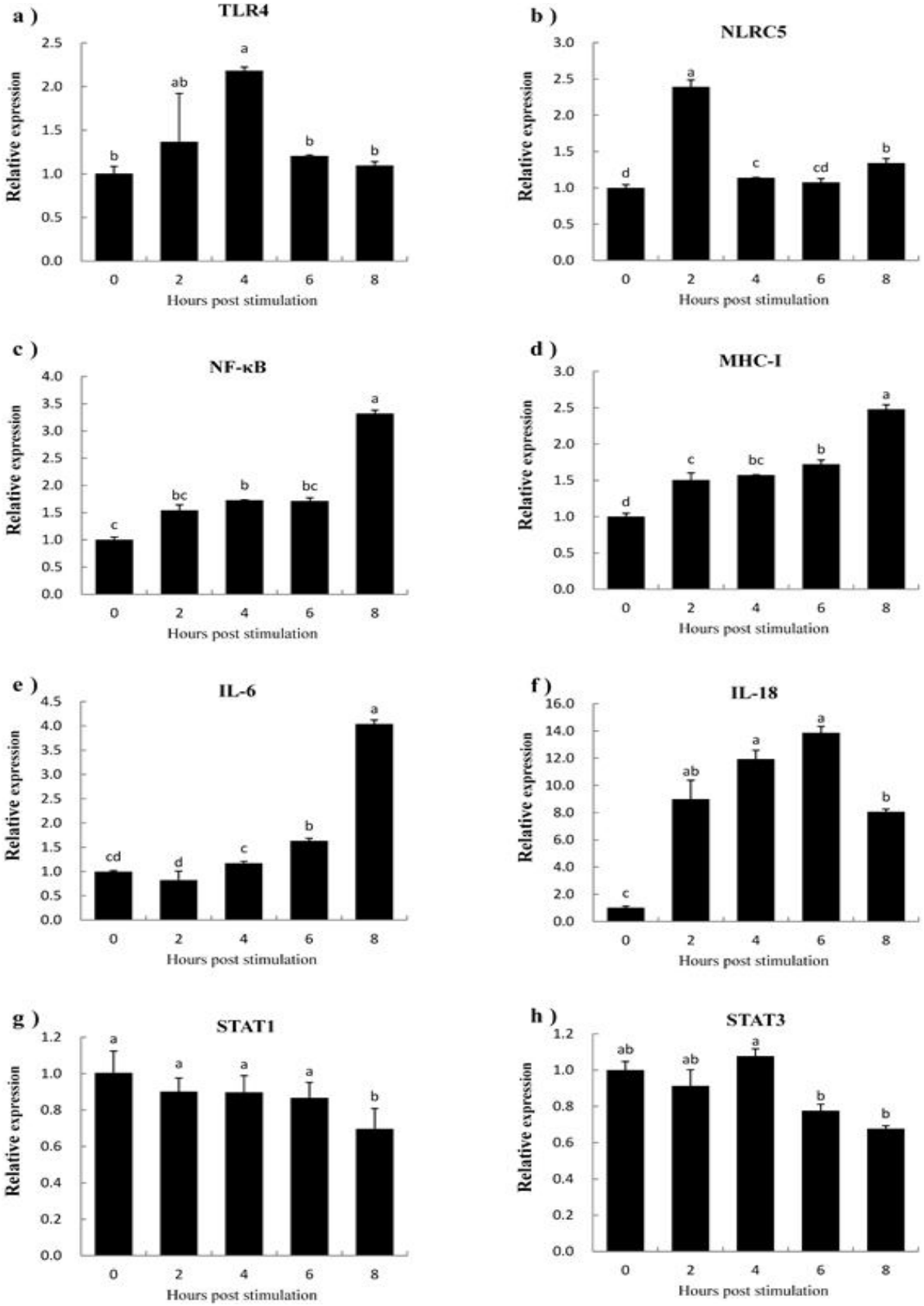

Fig 1: Gene expression of *TLR4*, *NLRC5*, *NF-κB*, *MHC-I*, *IL-6*, *IL-18*, *STAT1* and *STAT3* after LPS treatment in chicken HD11 macrophages. HD11 macrophages were stimulated for 2, 4, 6 and 8 hours with 10 μg/ml LPS. Different letters in the same figure mean significant difference (*P* values are lower than 0.05).

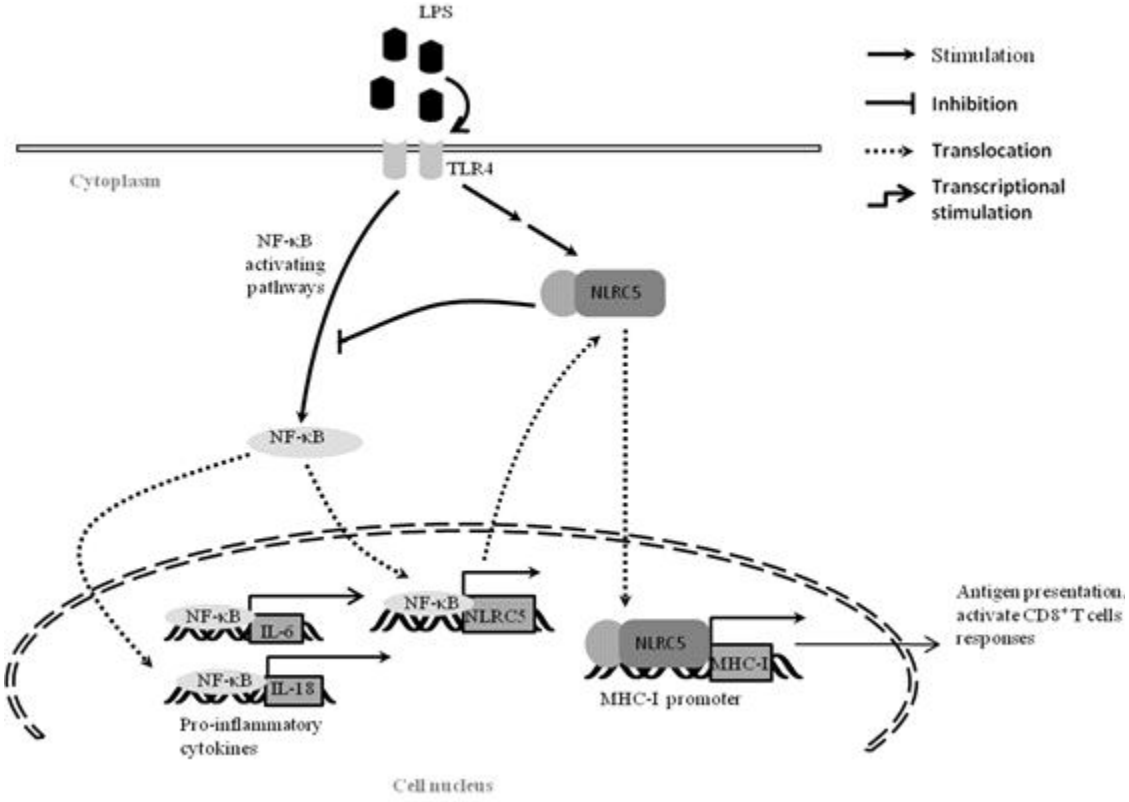

Fig 2: Model depicting the role of NLRC5 in transcriptional regulation of *NLRC5* relevant genes.

of *MHC-I*. Application of emerging technologies and methods make studies easier but controversies are far from over. Different dosages, LPS sources, cell types tested even the specific species influence results.

Collectively, these studies and results suggested that LPS did evoke inflammatory responses in chicken HD11 cell line but the effects of stimulation are dependent upon dosages and the cell types tested. Besides, *NLRC5* may negatively regulate the *NF-kB* and critically regulate *MHC-I* to control intracellular PAMPs in chicken HD11 macrophage cell line. Specific role of *NLRC5* involved in innate immune response associates with cell types and species tested.

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