

Original Research Article

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## TLR3 and TLR7 Cross-talk Induces Synergistic Response in the Chicken Peripheral Blood Mononuclear Cells

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### ABSTRACT

#### Keywords

Chicken, Toll-like receptor, Poly I:C, R-848, TLR-TLR cross-talk, Cytokines, Nitric oxide

#### Article Info

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Toll-like receptors (TLRs) are type of pattern recognition receptors (PRRs) greatly expressed on all immune cells. Activation of TLRs with their cognate ligand initiates the induction of innate immune responses with subsequent priming of adaptive immunity. Understanding the interaction between two different TLRs in terms of induced immune responses would help in using the TLR ligands as vaccine adjuvants. In the present study, effects of Poly I:C (TLR3 ligand) and/or R-848 (TLR7 ligand) were studied in chicken peripheral blood mononuclear cells (PBMCs). The PBMCs were isolated from White Leghorn chickens of around six weeks of age (n=6) and stimulated with Poly I:C and R-848 alone or in combination. The cells were harvested at different intervals (3, 6, 12, 24 and 48 h) post-stimulation and analyzed for the induction of different immune response genes by real-time PCR. The combination of PolyI:C and R-848 synergistically induced the up-regulation of IL-1 $\beta$ , IFN- $\beta$ , IFN- $\gamma$ , IL-4, IL-10 and iNOS transcripts as compared to the individual response in the chicken PBMCs. The results indicate that the combination of Poly I:C and R-848 would synergistically interact at cellular signaling level resulting in augmentation of pro-inflammatory, antiviral, Th1 and Th2 type of responses in the chicken.

### Introduction

First line of defense against invasion of microbial pathogen is mediated by innate or natural immunity. Innate immune cells recognize a set of conserved molecular structures on microbes through germ line encoded receptors called pattern recognition receptors (PRRs). Toll-like receptors (TLRs)

are type of PRRs and engagement of TLRs with their cognate ligands activate downstream signaling events that result in the induction of innate immune response by secreting inflammatory cytokines and type I interferons (IFNs). The secretion of inflammatory mediators not only activate innate immunity but also prime adaptive immune response which is responsible for the

clearance of pathogen (Janeway and Medzhitov, 2002). Presently, ten TLRs were identified in human and thirteen TLRs in mice (El-Zayat *et al.*, 2019). Lipopeptides are recognized by cell surface TLRs such as TLR1, 2 and 6; lipopolysaccharide (LPS) by TLR4 and flagellin by TLR5. Endosomal TLRs such as TLR3 recognize double stranded (ds) viral RNA, TLR7 and 8 identify single stranded (ss) viral RNA and TLR9 recognize CpG ODN of DNA (Akira *et al.*, 2006; Marshak-Rothstein, 2006). In chicken ten TLRs (TLR1A and TLR1B, TLR2A and TLR2B, TLR3, TLR4, TLR5, TLR7, TLR15 and TLR21) were reported (Paul *et al.*, 2013).

Chicken TLR21 is functional homologue of human TLR9 which recognize CpG ODN motifs of DNA (Brownlie *et al.*, 2009). TLR is composed of an extracellular domain containing leucine rich repeats responsible for ligand recognition and an intracellular toll/interleukin-1 (IL-1) receptor (TIR) like domain essential for signal transduction (Janssens and Beyaert, 2003; Kanzler *et al.*, 2007). Except TLR3, binding of every single TLRs to their cognate ligands make use of Myeloid differentiation primary response gene88 (MyD88) as an adapter molecule for induction of signaling events in the cell. TLR3 uses TIR domain containing adaptor protein inducing IFN- $\beta$  (TRIF) and TLR4 utilizes both MyD88 and TRIF molecule (Honda *et al.*, 2004; Kawai *et al.*, 2004; Pandey and Agrawal, 2006).

During cell surface TLR activation, interleukin 1 receptor associated kinase (IRAK) family members IRAK1 and IRAK4 interacts with an adapter molecule MyD88 to form signaling complex myddosome (Lin *et al.*, 2010). Within the signalosome or myddosome, IRAK4 activates IRAK1 which then autophosphorylate and release from MyD88 (Kollewe *et al.*, 2004; Jiang *et al.*, 2002). The released IRAK1 further associates

with TNF receptor associated factor 6 (TRAF6) which promotes polyubiquitination and activation of transforming growth factor beta activated kinase 1 (TAK1) (Chen, 2012; Ajibade *et al.*, 2013). TAK1 activates two different pathways; nuclear factor kappa B (NF- $\kappa$ B) and mitogen activated protein kinase (MAPK) pathway.

In the former, TAK1 activates catalytic subunit of IKK (inhibitor of NF- $\kappa$ B (I $\kappa$ B) kinase) complex which further phosphorylate NF- $\kappa$ B inhibitory protein I $\kappa$ B $\alpha$ . The phosphorylated I $\kappa$ B $\alpha$  is degraded and unmasked from NF- $\kappa$ B thus allows NF- $\kappa$ B to translocate into the nucleus to activate pro-inflammatory genes. In addition, TAK1 also activates MAPK family members; p38, extracellular signal-regulated protein kinase (ERK)1/2 and Jun N terminal kinase (JNK) which involve in induction of inflammatory response (Akira *et al.*, 2006; Kawai and Akira, 2010).

TLR agonists were tried by many workers both as prophylactic agents as well as adjuvants in chicken. Polyinosinic-polycytidylic acid (PolyI:C; TLR3 agonist) as an adjuvant with avian influenza virus (AIV) increased the expression of IL-6, IL-12 and IFN- $\gamma$  in chickens (Liang *et al.*, 2013). Prophylactic activity of PolyI:C was demonstrated with reduced viral shedding due to up-regulation of IFNs, IL-8 and IL-18 (Paul *et al.*, 2012a).

*In vitro* stimulation of chicken embryo fibroblast with PolyI:C reduced replication of Marek's disease virus (Hu *et al.*, 2016a). Recently, we reported the up-regulated expression of Th1 and Th2 immune response genes by R-848 (TLR7 agonist) in the chicken peripheral blood mononuclear cells (PBMCs) (Ramakrishnan *et al.*, 2015), which was also confirmed by *in vivo* study (Annamalai *et al.*, 2015). R-848 also showed potential adjuvant

activity when used with inactivated Newcastle disease virus (NDV) vaccine and enhanced the protection level as observed by challenge with virulent Newcastle disease virus with no shedding of virus in the chicken (Sachan *et al.*, 2015). Intramuscular and intranasal injection of R-848 reduced the shedding of avian influenza virus in the chicken (Barjesteh *et al.*, 2015). Simultaneous use of two different TLR ligands can cause interaction between their respective TLRs and lead to either additive, synergistic or antagonistic effect. Combination of TLR agonists can be tried to minimize the side effects, mimic the natural infection and to induce a more balanced or desired immune responses. Stimulation of porcine dendritic cells with PolyI:C and imiquimod (TLR7 agonist) along with the inactivated Porcine reproductive and respiratory syndrome (PRRS) virus antigen increased the expression of IFN- $\gamma$ , IL-12, IL-6 and IL-10 mRNA level (Hu *et al.*, 2016b).

Combination of TLR3 and TLR7 agonist boosted Th1 type of immunity in human and mice (Napolitani *et al.*, 2005). Although the combinatorial effect of TLR3 and TLR7 is reported in human and mice, it is not reported in chicken. Therefore, our present study aimed to examine the combinatorial effect of TLR3 and TLR7 agonist in the chicken PBMCs.

## **Materials and Methods**

### **Experimental birds**

Specific pathogen free (SPF) embryonated eggs were procured from Venky's India Pvt. Ltd. Pune, India and hatched at Central Avian Research Institute (CARI), Izatnagar, India. Birds were maintained following standard management practices and provided with *ad libitum* autoclaved feed and water. The total experiment was approved by the Institute

Animal Ethical Committee (IAEC), Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India 243122.

### **TLR agonists**

Commercially available TLR3 agonist, PolyI:C and TLR7 agonist, R-848 were procured from InvivoGen, CA, USA and dissolved in sterile nuclease- and endotoxin-free water.

### **Primers**

Published oligonucleotide primers specific to chicken genes viz., GAPDH, IL-1 $\beta$ , IFN- $\beta$ , IFN- $\gamma$ , IL-4, IL-10 and inducible nitric oxide synthase (iNOS) were synthesized from M/S Integrated DNA Technologies, Iowa, USA and used in the study (Table 1).

### **Chicken PBMCs isolation and stimulation**

Blood was collected in an anticoagulant containing vial (heparin, 20 IU/ml) from six weeks old specific pathogen free (SPF) White Leghorn birds (n = 6). Blood was layered over equal volume of Ficoll Hypaque (Sigma, MO, USA) with specific gravity 1.077 g/ml, centrifuged at 500  $\times$  g for 45 min. The interface containing the PBMCs was collected and washed twice in sterile phosphate buffered saline (PBS) (pH 7.2) and resuspended in RPMI-1640 medium containing 2% fetal calf serum. Cell viability was determined by trypan blue dye exclusion method and cells were adjusted to a concentration of 1  $\times$  10<sup>6</sup> cells/ml. PBMCs were stimulated with PolyI:C (50  $\mu$ g/ml) and/or R-848 (2  $\mu$ g/ml) as reported earlier (Karpala *et al.*, 2008; Barjesteh *et al.*, 2012; Bekeradjian-Ding *et al.*, 2005). The cells were incubated at 40°C with 5% CO<sub>2</sub> and harvested at 0, 3, 6, 12, 24 and 48 h post-stimulation for immune response genes analyses.

## **Analysis of immune response genes by quantitative real-time PCR**

### **RNA extraction and cDNA synthesis**

The PBMCs were centrifuged for 2 min and the resulting pellet was resuspended in 750  $\mu$ L TRIzol™ (Invitrogen, CA, USA). The resulting mixture was extracted with chloroform and precipitated with isopropanol. The RNA pellet was briefly washed with 75% ethanol and resuspended in nuclease free water (Invitrogen, CA, USA). Upon isolation of RNA, cDNA synthesis was carried out using random hexamer primers (Fermentas, MD, USA) and Revertaid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

### **Real-time PCR quantification**

Expression levels of mRNA of IL-1 $\beta$ , IFN- $\beta$ , IFN- $\gamma$ , IL-4, IL-10 and iNOS were analyzed by real-time PCR using the QuantiFast SYBR Green qPCR kit (Qiagen, CA, USA) on CFX96 real time system (Bio-Rad, CA, USA) using published gene specific primers. GAPDH was used as the reference gene. Real-time PCR was carried out in a total volume of 20  $\mu$ L consisting of 2  $\mu$ L cDNA, 10  $\mu$ L of QuantiFast SYBR Green Master Mix (Qiagen, CA, USA), and primers 0.5  $\mu$ L each. Real time PCR was carried out with the following programme: 1 cycle at 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 45 s, 70 °C for 45 s and 1 cycle of 94 °C for 30 s. The final step was to obtain a melt curve for the PCR products to determine the specificity of the amplicons. Each sample was run in triplicate on the same plate. Expression levels of the above mentioned different genes were calculated relative to the expression of the GAPDH gene and expressed as n-fold increase or decrease relative to the control samples (Pfaffl, 2001).

## **Statistical analysis**

GraphPad Prism 7.0 was used for the statistical analyses. Two way analysis of variance (ANOVA) was performed to determine the effect of agonists and their interactions. Results are expressed as Mean  $\pm$  SEM.

## **Results and Discussion**

### **Effect of polyI:C and/or R-848 on pro-inflammatory gene expression in the chicken PBMCs**

Pro-inflammatory cytokines play a pivotal role in both innate and adaptive immunity and as a crucial mediator of inflammatory response. IL-1 $\beta$  also possesses direct antiviral activity by decreasing the viral load in the respiratory mucosa of the infectious laryngotracheitis virus infected chicken (Thapa *et al.*, 2015). In our study, PolyI:C induced the expression of IL-1 $\beta$  mRNA at all the time intervals in the chicken PBMCs (Figure 1). Further, the expression level of IL-1 $\beta$  transcript (38.575 $\pm$ 12.298) was higher at 3 h post-stimulation with Poly I:C in comparison to other intervals studied.

The level of IL-1 $\beta$  transcript gradually decreased at the later time period. Similarly R-848 induced a peak IL-1 $\beta$  (36.186 $\pm$ 12.271) expression at 3 h post-stimulation in the chicken PBMCs than any other interval studied (Figure 1). Further the combination of Poly I:C and R-848 synergistically (122.853 $\pm$ 36.719) up-regulated the IL-1 $\beta$  transcripts at 3 h post-stimulation as compared to the additive effect of either of the agonist in the chicken PBMCs (Figure 1). Our study is in agreement with the earlier reports where Poly I:C amplified the expression of IL-1 $\beta$  mRNA in the microglial cells (Ifuku *et al.*, 2014). Similarly, R-848 elevated the IL-1 $\beta$  mRNA expression in

chicken splenocytes and chicken TLR7+ HD11 cell line (Philbin *et al.*, 2005). An increase in biological active IL-1 $\beta$  transcript after Poly I:C plus R-848 stimulation in dendritic cells (Napolitani *et al.*, 2005) supports the present findings.

**Effect of poly I:C and/or R-848 on antiviral gene expression in the chicken PBMCs**

Macrophages, monocytes, T cells and B cells are predominant producer of type I IFNs which in turn activate IFN stimulatory genes (Seo and Hahm, 2010). IFN- $\alpha$  and IFN- $\beta$  have well defined antiviral activity and the type I IFNs act in cis to induce dendritic cell maturation that subsequently enhance the adaptive immunity (Rahman and Eo, 2012). In our study, Poly I:C induced IFN- $\beta$  expression in chicken PBMCs with a peak increase at 6 h (3.322 $\pm$  1.487) post-stimulation (Figure 2). The expression level

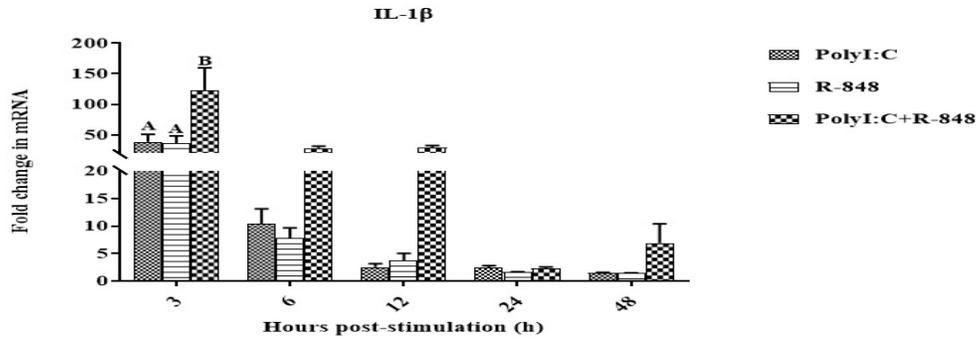
of IFN- $\beta$  was greatest at 48 h (4.058 $\pm$  1.949) post-stimulation with R-848 (Figure 2). Further, the combination of Poly I:C and R-848 stimulation synergistically enhanced IFN- $\beta$  transcript (19.017 $\pm$  11.421) at 48 h interval as compared to the additive effect of either of the agonist alone (Figure 2).

In consistent to our study when chicken cells stimulated with Poly I:C, induced strong TRIF mediated IFN-  $\beta$  response (Rothfuchs *et al.*, 2001). Oral administration of synthetic TLR7 agonist S-28828 induced type I IFNs in chicken splenocytes (Karaca *et al.*, 1996). Similarly, R-848 also induced IFN- $\alpha$  and IFN- $\beta$  in human peripheral lymphocytes (Lee *et al.*, 2003). Stimulation of dendritic cells with dual agonists, Poly I:C and R-848 enhanced IFN- $\alpha$ , IFN- $\beta$  and IL-12 cytokines which confirms the result of our study (Pearson *et al.*, 2018).

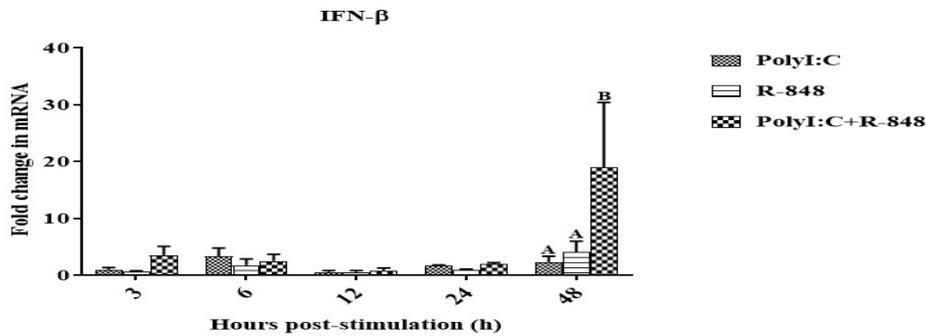
**Table.1**

Target Gene	Primer sequence (5'-3')	Product size (bp)	Reference
<b>GAPDH</b>	F: AGCACCCGCATCAAAGG R: CATCATCCCAGCGTCCA	283	Kuo <i>et al.</i> , 2017
<b>IL-1<math>\beta</math></b>	F: GGATTCTGAGCACACCACAGT R: TCTGGTTGATGTCGAAGATGTC	272	Ramakrishnan <i>et al.</i> , 2015
<b>IFN-<math>\beta</math></b>	F: GCTCACCTCAGCATCAACAA R: GGGTGTTGAGACGTTTGGAT	187	Ramakrishnan <i>et al.</i> , 2015
<b>IFN-<math>\gamma</math></b>	F: TGAGCCAGATTGTTTCGATG R: CTTGGCCAGGTCCATGATA	152	Ramakrishnan <i>et al.</i> , 2015
<b>IL-4</b>	F: GTGCCACGCTGTGCTTAC R: AGGAAACCTCTCCCTGGATGTC	82	Huang <i>et al.</i> , 2019
<b>IL-10</b>	F: CGCTGTCACCGCTTCTTCA R: TCCCGTTCTCATCCATCTTCTC	88	Zhang <i>et al.</i> , 2019
<b>iNOS</b>	F: TGGGTGGAAGCCGAAATA R: GTACCAGCCGTTGAAAGGAC	241	Zhang <i>et al.</i> , 2019

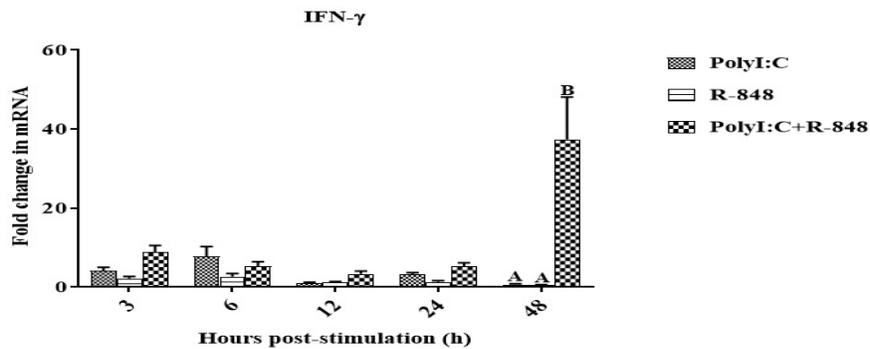
**Fig.1** Relative expression of IL-1 $\beta$  transcript in chicken peripheral blood mononuclear cells (PBMCs) stimulated with Poly I:C (50  $\mu$ g/ml) and/or R-848 (2  $\mu$ g/ml) over a period of 48 h. Bars with different uppercase indicate significant effect of TLR agonist at a time point



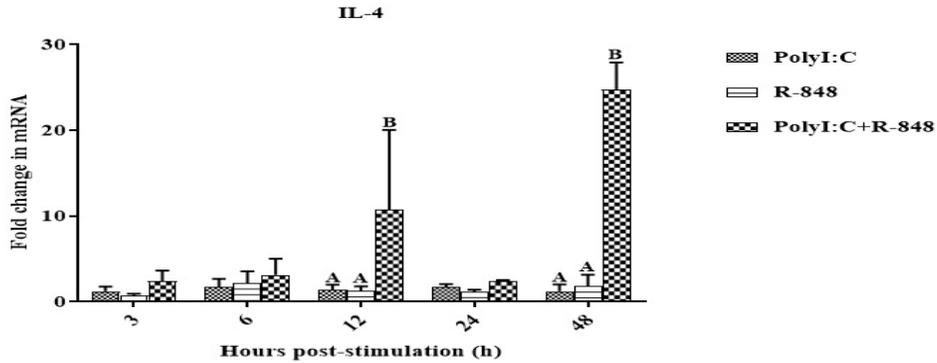
**Fig.2** Relative expression of IFN- $\beta$  transcript in chicken peripheral blood mononuclear cells (PBMCs) stimulated with Poly I:C (50  $\mu$ g/ml) and/or R-848 (2  $\mu$ g/ml) over a period of 48 h. Bars with different uppercase indicate significant effect of TLR agonist at a time point



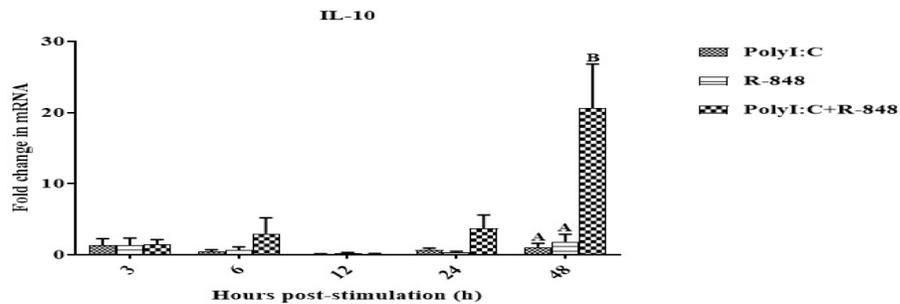
**Fig.3** Relative expression of IFN- $\gamma$  transcript in chicken peripheral blood mononuclear cells (PBMCs) stimulated with Poly I:C (50  $\mu$ g/ml) and/or R-848 (2  $\mu$ g/ml) over a period of 48 h. Bars with different uppercase indicate significant effect of TLR agonist at a time point



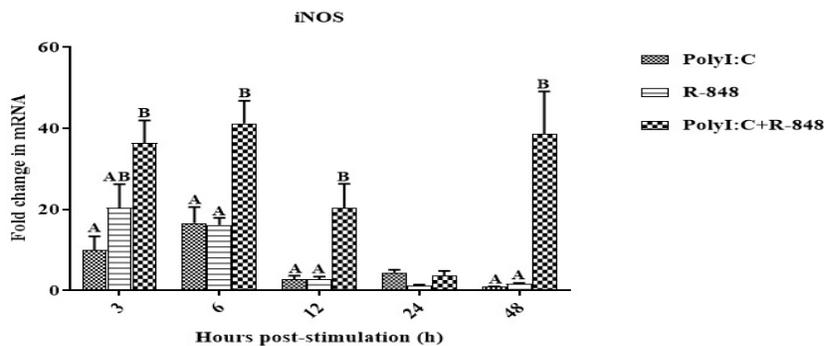
**Fig.4** Relative expression of IL-4 transcript in chicken peripheral blood mononuclear cells (PBMCs) stimulated with Poly I:C (50 µg/ml) and/or R-848 (2 µg/ml) over a period of 48 h. Bars with different uppercase indicate significant effect of TLR agonist at a time point



**Fig.5** Relative expression of IL-10 transcript in chicken peripheral blood mononuclear cells (PBMCs) stimulated with Poly I:C (50 µg/ml) and/or R-848 (2 µg/ml) over a period of 48 h. Bars with different uppercase indicate significant effect of TLR agonist at a time point



**Fig.6** Relative expression of iNOS transcript in chicken peripheral blood mononuclear cells (PBMCs) stimulated with Poly I:C (50 µg/ml) and/or R-848 (2 µg/ml) over a period of 48 h. Bars with different uppercase indicate significant effect of TLR agonist at a time point



### **Effect of polyI:C and/or R-848 on Th1 cytokine gene expression in chicken PBMCs**

IFN- $\gamma$  is a hallmark of cellular immune response (Boyoglu-Barnum *et al.*, 2014; Miceli and Parnes, 1991; Koretzky, 2010). It plays an essential role in both innate and adaptive immunity (Samuel, 2001). IFN- $\gamma$  also possesses antiviral activity by inducing CD8+ T cells, natural killer cells and interferon inducible genes (Samuel, 2001). In our current study, Poly I:C induced the expression of IFN- $\gamma$  transcript at 3, 6, 12 and 24 h post-stimulation with peak increase at 6 h ( $7.680 \pm 2.561$ ) interval (Figure 3). Similarly, R-848 induced the IFN- $\gamma$  transcript at 3, 6, 12 and 24 h post-stimulation in the chicken PBMCs. The combination of Poly I:C and R-848 showed synergistic effect on IFN- $\gamma$  transcript at 48 h post-stimulation in chicken PBMCs as compared to the additive effect of either of the agonist alone (Figure 3). In accordance to our result, Poly I:C treatment increased the expression of TLR3 and IFN- $\gamma$  transcripts in bursal cells (Paul *et al.*, 2012b). In human PBMCs, Poly I:C and R-848 strongly induced the IFN- $\gamma$  transcript and supported Th1 differentiation (Re and Strominger, 2004; Ito *et al.*, 2002; Huang *et al.*, 2006). Further, stimulation of porcine peripheral blood monocyte derived dendritic cells with dual TLR3 and TLR7 ligands synergistically enhanced mRNA and protein levels of Th1 type cytokines IFN- $\gamma$  and IL-12 through TRIF/MyD88 NF- $\kappa$ B signaling pathway.

### **Effect of poly I:C and/or R-848 on Th2 cytokine gene expression in chicken PBMCs**

Th2 cytokines such as IL-4 and IL-10 is indicative of humoral and mucosal immune response (Boyoglu-Barnum, 2014; Miceli *et al.*, 1991; Koretzky, 2010). IL-4 and IL-10 is

predominantly produced by CD4+ Th2 cells and subdues the excessive response produced by Th1 and CD8+ T cells that are responsible for immunopathology associated during infection (Gazzinelli *et al.*, 1996; Wilson *et al.*, 2005). In our present study, PolyI:C and R-848 induced IL-4 mRNA expression in chicken PBMCs at all the time intervals studied. PolyI:C plus R-848 synergistically enhanced the expression of IL-4 transcript at 12 h ( $10.782 \pm 9.271$ ) and 48 h ( $24.742 \pm 3.189$ ) post-stimulation as compared to the additive effect of either of the agonist alone (Figure 4). Further synergistic response was appreciated in the expression of IL-10 mRNA after PolyI:C plus R-848 stimulation in chicken PBMCs (Figure 5). In agreement with our study, treatment of human lymphocytes increased the expression of IL-4 mRNA through NF- $\kappa$ B pathway (Kehoe *et al.*, 2001). R-848 induced higher levels of IFN- $\gamma$  and IL-4 in mice splenocytes (Wang *et al.*, 2013), which also supports our findings. Stimulation of bursal cells with PolyI:C induced IL-10 (Paul *et al.*, 2012b) which is important for down-regulating the deleterious effects associated with the diseases. The combination of TLR3 and TLR7 ligands synergistically enhanced Th2 cytokines IL-6 and IL-10 in human dendritic cells (Hu *et al.*, 2016b) which supports the finding of our results.

### **Effect of polyI:C and/or R-848 on iNOS gene expression in chicken PBMCs**

Nitric oxide plays a major role in host defense against viral infections and tumors (Eisenstein, 2001). In our present study, Poly I:C induced the iNOS transcript at all the time interval studied with a peak increase at 6 h ( $16.628 \pm 3.968$ ) post-stimulation. Significant ( $P < 0.05$ ) increase in the level of iNOS mRNA was appreciated in chicken PBMCs at 3 h post-stimulation with R-848 as compared to Poly I:C. The combination of Poly I:C and R-848 significantly ( $P < 0.05$ ) enhanced the

expression of iNOS transcript at 3, 6, 12 and 48 h post-stimulation as compared to the individual agonists. However, the combination synergistically enhanced the iNOS mRNA at 48 h post-stimulation as compared to additive effect of either of the agonists (Figure 6). In consistent to our results, stimulation of RAW 264.7 cells with Poly I:C increased the expression of iNOS transcript and production of nitric oxide (Heitmeier *et al.*, 1998) which was able to inhibit the replication of virus *in vitro* (Djeraba *et al.*, 2000). Similarly, exposure to R-848 induced the up-regulation of iNOS mRNA in chicken macrophage like cell line (Peroval *et al.*, 2013).

In conclusion, co-stimulation of chicken PBMCs with Poly I:C and R-848 showed a synergistic up-regulation of IL-1 $\beta$ , IFN- $\beta$ , IFN- $\gamma$ , IL-4, IL-10 and iNOS transcripts than either of the agonists alone. This suggests that the combination of Poly I:C and R-848 can augment the pro-inflammatory, antiviral, Th1 and Th2 type immune responses in the chicken PBMCs. Our findings may pave way to study the adjuvant potential of Poly I:C plus R-848 with common poultry vaccines.

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