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Flagellin and R-848 Co-stimulation induces early synergistic and delayed attenuated immune responses in the Chicken peripheral blood mononuclear cells

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Abstract
Understanding the interaction between distinct Toll-like receptors (TLRs) is crucial for optimizing combinatorial TLR ligands as vaccine adjuvants. In this study, we investigated the crosstalk between TLR-5 and TLR-7 in the chicken peripheral blood mononuclear cells (PBMCs) using their respective agonists, flagellin and R-848. The PBMCs were isolated from two-week-old specific pathogen-free chickens (n = 6) and stimulated with flagellin and/or resiquimod (R-848). Levels of gene activity governing key immune responses (IFN-β, IL-1β, IFN-γ, iNOS, IL-13) was measured at 3 h, 6 h, 12 h, 24 h, and 48 h post-stimulation using qRT-PCR, and nitric oxide (NO) levels were quantified at 24 and 48 h using the Griess assay. The combination of flagellin and resiquimod resulted in a synergistic up-regulation of IL-1β, IFN-β, IFN-γ, IL-13, and iNOS transcripts at 3h post-stimulation (P<0.05). This early peak was followed by attenuated expression profiles at later time points, with no corresponding increase in NO production, despite elevated iNOS transcripts. In conclusion, co-stimulation with flagellin and R-848 induced a potent, early-phase immune activation in the chicken PBMCs, highlighting its potential as an immunomodulatory strategy.

Keywords: Chicken, toll-like receptor, flagellin, resiquimod, TLR-TLR cross-talk, cytokines, nitric oxide

Introduction
Vaccination alone falls short in controlling many pathogenic organisms due to insufficient and delayed immune response. New approaches involving toll-like receptor (TLR) agonist enhance host defence and vaccine efficacy by modulating the host innate immune responses (Gutjahr *et al*., 2016; Bhadouriya *et al*, 2019) [26, 9]. TLRs are type I transmembrane protein present in immune cells such as macrophages, mast cells, dendritic cells (DCs), eosinophils, neutrophils and B lymphocytes. The extracellular region of TLRs, characterized by leucine-rich repeats, enables the identification of pathogen-associated molecular patterns (PAMPs) (O'Neill, 2004) [48]. Engagement of a cognate ligand activates the Toll/IL-1 receptor domain in the cytoplasm, which in turn induces inflammatory cytokine and chemokine production and enhances the expression of surface molecules and MHC-II (Zhang *et al*., 2015; Kalaiyarasu *et al*., 2016) [80, 34]. In chickens, ten distinct TLRs have been described: TLR1A, TLR1B, TLR2A, TLR2B, TLR4, TLR5, and TLR15 are expressed on the cell membrane, whereas TLR3, TLR7, and TLR21 are localized within endosomal compartments (Keestra *et al*., 2013) [36]. TLR15 is a receptor exclusive to chickens, while TLR21 serves as the avian counterpart of mammalian TLR9 (Paul *et al*., 2013) [49]. Lipids and lipopeptides are detected through TLR2, lipopolysaccharide is recognized by TLR4, flagellin is sensed by TLR5, and nucleic acids are recognized by TLR3, TLR7, and TLR9 (Akira *et al*., 2006; Marshak-Rothstein, 2006) [3, 42]. Stimulation of individual TLR can skew the immune response towards Th1, Th2, or mixed profiles depending on the ligand. For instance, CpG ODNs (TLR21 ligand) promote Th1 responses in neonatal chicks, Stimulation with Pam3CSK4 (TLR2 ligand) elicits a mixed cytokine profile involving Th1 as well as Th2 mediators (St. Paul *et al*., 2013) [49]. Among these, the TLR7 agonist R-848 has shown prophylactic and adjuvant

potential in chickens, upregulating key immune response genes such as IFN-γ, IL-1β, IFN-α and iNOS, and enhancing the protection against viruses including NDV and IBDV when used with their respective vaccines (Annamalai *et al*., 2016, Gupta *et al*., 2020) [4, 24]. Similarly, flagellin (TLR5 agonist) have shown to reduce mortality due to Salmonella and improve mucosal responses to avian influenza (Genovese *et al*., 2007) [22], while also modulating cytokines such as IFN-α and IL-1β in chicken immune cells (Kogut *et al*., 2006; St. Paul *et al*., 2012) [37, 50]. Pathogens often release multiple PAMPs simultaneously, leading to co-activation of multiple TLRs. The resulting TLR-TLR cross-talk can produce synergistic, additive, or antagonistic responses, depending on the combination and timing involved (Underhill, 2007) [76]. Previous studies in chickens have demonstrated synergistic immune responses upon dual stimulation of TLR3 and TLR21 (He *et al*., 2007) [29], TLR4 and TLR21 (Kim *et al*., 2018) [27], and TLR4 and TLR7 (Ramakrishnan *et al*., 2015) [58]. These interactions resulted in enhanced cytokine production and modulated immune response.

In mammals, co-stimulation of TLR5 and TLR7 has been reported to increase IL-6, IL-12p70 production, and chemokines in human macrophages and dendritic cells (Mäkelä *et al*., 2009) [41]. However, this interaction has not been investigated in chickens to date. Given that viral infections in poultry are frequently followed by secondary bacterial infections, understanding how TLR5 and TLR7 co-activation modulates immune responses may provide critical insight for developing effective immunostimulants or adjuvant combinations. Hence, the present study aimed to investigate the interaction between TLR5 and TLR7 signaling in the chicken peripheral blood mononuclear cells (PBMCs), using their respective agonists flagellin and R-848. The co-stimulation with flagellin and R-848 was followed by the analysis of immune responses through gene expression analysis of key cytokines and iNOS, along with nitric oxide production.

2. Materials and methods

2.1. Experimental birds

Specific pathogen-free (SPF) embryonated White Leghorn eggs were obtained from Venky's India Pvt. Ltd., Pune, and incubated at the Central Avian Research Institute (CARI), Izatnagar. The hatched chicks were reared under standard husbandry conditions with unrestricted access to autoclaved feed and water. Experimental procedures were carried out with prior approval from the Institute Animal Ethical Committee (IAEC), Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh (Approval No. F. 26-1/2015-16/J.D (R), dated 25 October 2016).

2.2. TLR agonists

The TLR5 ligand flagellin and the TLR7 agonist R-848 were purchased from InvivoGen (CA, USA), prepared in sterile endotoxin-free water, and stored at -20 °C.

2.3. Primers

Published oligonucleotide primers specific to chicken genes *viz.*, GAPDH, β-actin, IL-1β, IFN-γ, IFN-β, IL-13 and iNOS were synthesized from M/S Integrated DNA Technologies, Iowa, USA and used in the study (Table 1).

2.4. Effect of flagellin and/or R-848 on immune response genes in the chicken peripheral blood mononuclear cells (PBMCs)

Immune response genes expression induced by flagellin and R-848 alone and in combination were studied in the chicken PBMCs.

2.4.1. Chicken PBMCs isolation and stimulation with TLR agonist (s)

Peripheral blood was collected from two-week-old SPF White Leghorn chickens (n = 6) using sterile heparinized syringes (20 IU/ml of blood). Samples were overlaid on an equal volume of Ficoll-Histopaque 1.077 (Sigma, MO, USA) and subjected to density gradient centrifugation at 500 × g for 45 minutes to isolate PBMCs. The separated cells were washed twice with sterile PBS (pH 7.2) and resuspended in RPMI-1640 complete medium supplemented with 2% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cell viability was assessed by trypan blue exclusion, and the suspension was adjusted to a concentration of 1×10^7 cells/ml. PBMCs were stimulated with flagellin (1 µg/ml) and/or R-848 (2 µg/ml) following previously described protocols (Bekeredjian-Ding *et al*., 2005; Peroval *et al*., 2013; Radomska *et al*., 2016; Ramakrishnan *et al*., 2015) [32, 51, 56, 58]. Cultures were maintained at 37 °C in a 5% CO_2 atmosphere, and cells were collected at 0 h, 3 h, 6 h, 12 h, 24 h, and 48 hours post-stimulation for downstream immune gene expression analysis.

2.4.2. Isolation of total RNA from the PBMCs

Total RNA was extracted from both treated and control PBMCs. Briefly, cells were pelleted by centrifugation (5 min) and lysed in 750 µl of QIAzol reagent (Qiagen, CA, USA). Phase separation was achieved by adding 250 µl of chloroform, followed by vortexing for 30 s and centrifugation at 12,000 × g for 20 min at 4 °C. The aqueous phase containing RNA was transferred to a fresh tube, and RNA was precipitated with 400 µl of isopropanol by centrifugation at 12,000 × g for 15 min. The resulting pellet was washed once with 1 ml of 70% ethanol, centrifuged at 12,000 × g for 10 min at 4 °C, and air-dried on sterile filter paper for approximately 10 min to remove residual ethanol. Finally, the RNA pellet was dissolved in 20 µl of RNase-free water. RNA concentration and purity were determined using a Nanodrop spectrophotometer (Thermo Scientific, USA) by recording absorbance at 260 and 280 nm.

2.4.3. Preparation of complementary DNA (cDNA)

Complementary DNA (cDNA) was synthesized from 2 µg of total RNA using random hexamer primers and the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's protocol. In brief, 2 µg RNA and 1 µl of random hexamer primer were combined with nuclease-free water to a final volume of 12.5 µl and incubated at 65 °C for 5 min. Subsequently, 4 µl of 5× reaction buffer, 0.5 µl Ribolock RNase inhibitor, 2 µl of 10 mM dNTP mix, and 1 µl RevertAid reverse transcriptase were added. The mixture was gently vortexed and incubated at 25 °C for 10 min, followed by 50 °C for 50 min for reverse transcription. The reaction was terminated by heating at 85 °C for 5 min, and the synthesized cDNA was stored at -20 °C until further use.

2.4.4. Quantitative Real-Time PCR (qPCR)

Relative transcript levels of IL-1β, IFN-β, IFN-γ, IL-13, and iNOS were measured by quantitative real-time PCR (qPCR) using the QuantiFast SYBR Green kit (Qiagen, CA, USA) on a CFX96 Real-Time PCR System (Bio-Rad, CA, USA). Gene-specific primers reported previously were employed, with β-actin and GAPDH used as internal reference genes for normalization. Each 20 µl reaction consisted of 2 µl cDNA, 10 µl QuantiFast SYBR Green Master Mix, 0.5 µl of each primer (10 pmol), and RNase-free water. Thermal cycling conditions were: initial denaturation at 95 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 60 °C for 45 s, and 70 °C for 45 s, with an additional denaturation step at 94 °C for 30 s. Melt curve analysis was performed to verify the specificity of the amplification products. All samples, including controls, were analyzed in duplicate on the same plate. Data normalization was carried out using the geometric mean of β-actin and GAPDH expression (Boeglin *et al*., 2011; Berzi *et al*., 2014) [10, 8]. The cycle threshold (Ct) was defined as the point at which fluorescence exceeded the preset threshold. Relative changes in mRNA levels were calculated using the 2^-ΔΔCt method (Pfaffl, 2001) [52].

2.5. Effect of flagellin and/or R-848 on nitric oxide production:

PBMCs (2 × 10⁶ cells/ml) obtained from two-week-old SPF White Leghorn chickens (n = 6) were suspended in RPMI-1640 medium supplemented with 5 mM L-arginine. Cells were stimulated with flagellin (1 µg/ml) and/or R-848 (2 µg/ml), while lipopolysaccharide (LPS; 10 µg/ml) served as a positive control. Culture supernatants were harvested at 24 and 48 h post-stimulation and stored at -20 °C until analysis. Nitric oxide (NO) production was quantified indirectly by assessing nitrite levels using the Griess reaction. For this, 50 µl of sample or standard was mixed with 50 µl of Griess reagent (Sigma, MO, USA) and incubated at 37 °C for 30 min. Absorbance was recorded at 550 nm using a spectrophotometer. Sodium nitrite standards of known concentrations were used to generate a calibration curve, from which nitrite levels in the samples were calculated.

2.6. Statistical analysis

Peripheral blood mononuclear cells (PBMCs; 2 × 10⁶ cells/ml) isolated from two-week-old SPF White Leghorn birds (n = 6) were cultured in RPMI-1640 medium supplemented with 5 mM L-arginine. The cells were exposed to flagellin (1 µg/ml) and/or R-848 (2 µg/ml), with lipopolysaccharide (LPS; 10 µg/ml) included as a positive control. Supernatants were collected at 24 and 48 hours after stimulation and stored at -20 °C until further analysis. Nitric oxide (NO) levels were determined by measuring nitrite accumulation using the Griess assay. Briefly, 50 µl of each sample or standard was combined with 50 µl of Griess reagent (Sigma, MO, USA) and incubated at 37 °C for 30 minutes. Absorbance was then read at 550 nm using a spectrophotometer. A standard curve prepared with sodium nitrite solutions of known concentration was used to calculate nitrite levels in the test samples.

3. Results

3.1. Effect of flagellin and/or R-848 on immune response genes in chicken PBMCs

3.1.1. Solitary effect of flagellin

Flagellin up-regulated the IL-1β transcripts at every time interval with a peak fold change at 3 h (5.85±0.60 folds) post-stimulation in the chicken PBMCs (Fig. 1). It also up-regulated the expression of IFN-β transcripts with a peak increase at 48 h (30.39±2.21 folds) interval (Fig. 2). There was also a gradual increase in IFN-γ transcripts and its expression reached a maximum (64.28±6.43 folds) at 48 h post-stimulation (Fig. 3). Similarly, the maximum expression of IL-13 was 185.52±13.55 folds at 48 h time interval (Fig. 4). Further, it also up-regulated the expression of iNOS at all the intervals studied with a peak expression (20.00±2.02 folds) at 24 h post-stimulation (Fig. 5).

3.1.2. Solitary effect of R-848

R-848 up-regulated the IL-1β transcripts at every time interval with peak expression of 7.49±1.50 folds at 3 h time period (Fig. 1). IFN-β transcript was up-regulated at the all-time intervals with a significant (P<0.05) up-regulation (56.66±3.48 folds) at 48 h post-stimulation (Fig. 2). Similarly, R-848 induced significant (P<0.05) expression of IFN-γ transcripts (126.40±15.23 folds) at 48 h interval (Fig. 3). Further, R-848 induced the up-regulation of IL-13 transcripts at all the intervals and a significant (P<0.05) expression of 242.09±23.76 folds was found at 48 h post-stimulation (Fig. 4). The iNOS transcript was also up-regulated by R-848 with a peak expression (28.34±2.58 folds) at 48 h interval (Fig. 5).

3.1.3. Co-stimulatory effect of flagellin and R-848

The combination of flagellin and R-848 induced the up-regulation of IL-1β transcripts at the all-time intervals (Fig. 1) with a significant (P<0.05) fold change at 3 h (36.29±5.82 folds) interval. Further, the combination showed a synergistic effect on (P<0.05) IL-1β transcripts at 3 h interval as response exceeds the arithmetic sum of individual agonist effect. Although at 6 h post-stimulation the combination showed higher expression but no synergy was observed at later time intervals. Flagellin and R-848 in combination synergistically (P<0.05) up-regulated the IFN-β transcripts with a relative fold change of 99.14±15.87 and 62.66±10.27 folds at 3 and 6 h post-stimulation, respectively. (Fig. 2). Simultaneous exposure of chicken PBMCs to flagellin and R-848 resulted in synergistic up-regulation in IFN-γ transcripts with a biphasic trend (Fig. 3). A synergistic peak was observed at 3 h post-stimulation (P<0.05), followed by a transient reduction at 6 h. However, expression progressively increased again over later time points, culminating in a second peak at 48 h post-stimulation. This response suggests that TLR5-TLR7 synergy not only induces early immune activation but may also prime cells for sustained cytokine expression over time. IL-13 had expressions similar to IFN- γ (Fig. 4). Further, the combination also synergistically (P<0.05) induced the iNOS transcripts (187.12±22.44 folds) at 3 h post-stimulation (Fig. 5) when compared to the additive effect of either flagellin or R-848. At later time points expression returned near basal levels.

3.2. Effect of flagellin and/or R-848 on nitric oxide production:

The concentration of NO in chicken PBMCs treated with LPS, flagellin, R-848 and flagellin plus R-848 reached a peak at 48 h interval, which was 19.32±1.033, 2.83±0.14, 28.33±0.67 and 21.10±0.45 µM, respectively (Fig. 6). The production of NO at 24 h was not detectable in flagellin stimulated chicken PBMCs. R-848 was found to induce significantly (P<0.05) higher level of NO than that of the medium control and flagellin stimulated cells at both the intervals studied. Co-stimulation of chicken PBMCs with flagellin and R-848 significantly increased NO production at 24 and 48 h intervals. However, the NO concentration in the combination treatment did not exceed the additive effect of the individual agonists.

4. Discussion

In a host, simultaneous activation of multiple Toll-like receptors (TLRs) can occur not only due to the presence of diverse pathogen-associated molecular patterns (PAMPs) within a single pathogen but also as a consequence of co-infections or viral disease followed by secondary bacterial invasion. Based on this rationale, we evaluated the combined influence of flagellin and R-848 on immune gene expression in chicken PBMCs. Dual stimulation, targeting cell-surface TLR5 with flagellin and endosomal TLR7 with a synthetic ssRNA analogue, produced an augmented cytokine response. Insights into such receptor interactions could contribute to the development of improved disease control strategies and enhancement of vaccine efficacy.

Our data demonstrated that exposure to flagellin and/or R-848 rapidly induced IL-1β transcripts, with maximal expression observed as early as 3 h post-stimulation. IL-1β is a key pro-inflammatory cytokine that plays an essential role in host defense across various bacterial and viral infection models (Sahoo *et al*., 2011) [63]. Its early induction likely contributes to faster bacterial clearance, thereby preventing pathogen establishment. Previous work showed that flagellin and R-848 upregulated IL-1β expression in the uterus and vaginal tissues of laying hens (Abdel-Mageed *et al*., 2014) [1]. Similarly, TLR5 engagement by flagellated *Pseudomonas aeruginosa* was reported to trigger IL-1β release, promoting endosomal acidification and bacterial clearance by alveolar macrophages. In contrast, the unflagellated strain failed to stimulate IL-1β, rendering it resistant to macrophage-mediated killing (Descamps *et al*., 2012) [19].

Consistent with our results, De Silva Senapathi *et al*. (2020) [18] observed elevated IL-1β, IFN-γ, and iNOS transcript levels in the lungs of day-old chicks following resiquimod (R-848) treatment. Likewise, flagellin-adjuvanted inactivated Newcastle disease virus (NDV) vaccine enhanced expression of pro-inflammatory mediators (IL-6 and TNF-α) in PBMCs and elicited stronger cellular and antibody responses compared with inactivated NDV alone or a commercial montanide-based vaccine. Oral delivery of R-848 has also been shown to increase IL-1β and IL-8 transcripts in the duck intestine (Volmer *et al*., 2011) [78], while studies in chicken splenocytes (Philbin *et al*., 2005) and PBMCs (Ramakrishnan *et al*., 2015) [58] further support its role in IL-1β induction.

In the present study, co-stimulation with flagellin and R-848 produced a synergistic increase in IL-1β expression at 3 h post-treatment, although this effect was not sustained at later time points. Comparable results have been documented in human immune cells, where combined stimulation of monocyte-derived dendritic cells and macrophages with flagellin and R-848 enhanced IL-6 and TNF-α expression at 24 h but failed to exhibit synergism.

Type I interferons (IFNs) are primarily secreted by macrophages, monocytes, T cells, B cells, and natural killer (NK) cells, and subsequently drive the activation of interferon-stimulated genes (Seo and Hahm, 2010; Sengupta and Chattopadhyay, 2024) [69, 68]. IFN-α and IFN-β are well established for their antiviral properties, while type I IFNs also act in an autocrine manner to promote dendritic cell (DC) maturation, thereby strengthening adaptive immune responses (Rahman and Eo, 2012) [57]. In the current study, stimulation of chicken PBMCs with either flagellin or R-848 led to increased IFN-β transcription, and co-stimulation with both agonists produced a synergistic rise at 3 and 6 hours post-treatment. Since type I IFNs are among the earliest mediators produced during infection, their rapid induction is crucial for limiting viral spread. Supporting evidence shows that flagellin activates TLR5 in murine bone marrow-derived macrophages to induce IFN-β (Ha *et al*., 2008) [27], while TLR7 signaling enhances type I IFN production via viperin in plasmacytoid DCs (Saitoh *et al*., 2011) [64]. Our previous studies also demonstrated that R-848 triggered IFN-α and IFN-β expression in chicken spleen (Sachan *et al*., 2015) [62] and PBMCs (Annamalai *et al*., 2016) [4], consistent with the present findings.

Type II interferon (IFN-γ) is produced by $CD4^+$ Th1 cells, $CD8^+$ T cells, NK cells, macrophages, and DCs, and is further regulated in an autocrine loop (Schroder *et al*., 2004) [67]. This cytokine is central to antiviral immunity by activating cytotoxic $CD8^+$ T cells, NK cells, and interferon-inducible genes, thereby bridging innate and adaptive responses (Romagnani, 1997; Samuel, 2001) [61, 65]. In our study, flagellin moderately enhanced IFN-γ expression in PBMCs, which mirrors earlier observations where murine NK cells and DCs released only low amounts of IFN-γ following flagellin exposure (Tsujimoto *et al*., 2005) [74], and human PBMCs also showed weak induction of IFN-γ and IL-12 upon flagellin stimulation (Merlo *et al*., 2007) [44]. In contrast, R-848 acted as a stronger inducer of IFN-γ and IFN-β in human PBMCs (Re and Strominger, 2004) [59] and promoted Th1 cytokine production (IFN-γ, IL-12) in murine B lymphocytes (Shen *et al*., 2008) [70]. Our earlier work in chickens confirmed R-848-driven induction of IFN-γ in both spleen and PBMCs (Sachan *et al*., 2015; Annamalai *et al*., 2016) [62, 4]. Consistently, in this study, R-848 significantly upregulated IFN-γ expression in chicken PBMCs, especially at later time intervals. Notably, co-stimulation with flagellin and R-848 produced a strong synergistic rise in IFN-γ transcripts at 3 h post-treatment, followed by a transient decline, and then a gradual resurgence from 12 h onwards.

IL-13, a Th2 cytokine functionally similar to IL-4, is mainly produced by Th2 cells but can also originate from $CD8^+$ T cells, mast cells, eosinophils, and basophils. In the current study, flagellin treatment gradually increased IL-13 expression in chicken PBMCs, peaking at 48 h, consistent with earlier reports where antigen-specific $CD4^+$ T cells induced IL-4 and IL-13 after flagellin exposure in mice (Didierlaurent *et al*., 2004) [20]. Recombinant flagellin stimulation also enhanced IL-4 expression in chicken PBMCs (Gupta *et al*., 2013) [25], further supporting our findings. Similarly, R-848 elicited IL-13 expression at all

tested time points, with the highest fold change observed at 48 h. This agrees with our earlier results showing IL-4 induction in the spleen following R-848 administration in chickens (Sachan *et al*., 2015) [62]. Interestingly, studies in mice demonstrated that R-848 administered with *Leishmania major* antigen promoted Th1 responses while dampening Th2 responses (Zhang and Matlashewski, 2008) [81], suggesting possible species-specific differences.

In this investigation, co-stimulation with flagellin and R-848 synergistically enhanced both IFN-γ and IL-13 expression at 3 h, with a transient decline at 6 h. From 12 h onwards, expression of these cytokines increased progressively, peaking at 48 h post-stimulation. Among the tested treatments, R-848 alone proved to be a more potent inducer of IFN-γ and IL-13 at later stages compared with flagellin or the combined stimulation.

To evaluate the influence of TLR stimulation on macrophage activity, we analyzed both inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) release in the supernatants of chicken PBMCs treated with flagellin and/or R-848. iNOS is a critical enzyme responsible for generating NO, a molecule with recognized antimicrobial and antiviral properties (Uehara *et al*., 2015) [75]. In our study, both agonists enhanced iNOS transcription, with R-848 producing the highest levels at 48 h post-stimulation. R-848 also promoted greater NO production at 24 and 48 h compared with LPS, flagellin, or the combined stimulation, consistent with our earlier observations (Ramakrishnan *et al*., 2015) [58]. Interestingly, co-exposure to flagellin and R-848 synergistically increased iNOS expression at 3 h, with elevated NO release observed at 24 and 48 h. However, this combination did not result in synergistic NO production overall, which correlated with reduced iNOS transcript levels at 6 and 48 h in co-stimulated PBMCs.

Previous work has shown that TLR5 engagement by flagellin triggers the production of inflammatory cytokines, chemokines, NO, and other host defense proteins in mice (Vijay-Kumar *et al*., 2008). In chickens, flagellin has similarly been reported to upregulate iNOS and NO in monocytes (He *et al*.) and induce NO in PBMCs (Gupta *et al*., 2013) [25]. Mechanistically, TLR5 signaling can proceed via MyD88-dependent and MyD88-independent pathways. The MyD88-dependent route recruits adaptor proteins to activate NF-κB and MAPK signaling cascades, while the MyD88-independent pathway involves TRIF through a TLR5/TLR4 heterodimer, leading to IFN-β production, STAT1 activation, and subsequent induction of iNOS and NO (Mizel *et al*., 2003) [46]. Consistent with this, several studies have reported R-848-mediated induction of iNOS and NO in chickens (Peroval *et al*., 2013; Barjesteh *et al*., 2014; Annamalai *et al*., 2015) [51, 6, 5]. Activation of TLR7 by R-848 in HD11 cells was shown to drive NF-κB and MAPK signaling, resulting in increased IL-1β, IL-6, and iNOS transcripts alongside enhanced NO production (Peroval *et al*., 2013) [51].

These findings highlight the importance of timely modulation of the innate immune response. Early activation may enhance defense against viral-bacterial co-infections, while controlled downregulation at later stages could help avoid hyperinflammation or cytokine storm. Supporting this, combined use of antibiotics with flagellin was reported to be more effective than antibiotics alone in controlling secondary pneumococcal infection following influenza in mice (Porte *et al*., 2015) [54]. Overall, the interaction of flagellin and R-848 in chicken PBMCs produced an early synergistic immunostimulatory effect, marked by induction of pro-inflammatory cytokines, Th1/Th2 cytokines, and iNOS, along with increased NO release. At later stages, R-848 alone displayed stronger activity, suggesting its role in sustaining immune priming. Since TLR7 and TLR5 agonists mimic viral and bacterial PAMPs, respectively, their combined use may help in designing strategies to strengthen host immunity against viral-bacterial co-infections.

Collectively, our results demonstrate that co-stimulation of chicken PBMCs with flagellin and R-848 produces a pronounced synergistic effect during the early phase of activation, particularly evident at 3 h in the expression of IL-1β, IFN-β, IFN-γ, IL-13, and iNOS. At subsequent time points, the combined treatment attenuated the expression of IFN-β, IFN-γ, IL-13, and iNOS, which may represent an intrinsic mechanism to control hypercytokinemia and inflammatory damage. Understanding the molecular basis of TLR5-TLR7 interaction provides valuable opportunities for applying dual TLR agonists in the design of improved immunotherapeutic and vaccination approaches in poultry.

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Table 1: Primers used for quantitative real time PCR

Target Gene	Primer sequence (5'-3')	Product size (bp)	Reference
β-Actin	F: TATGTGCAAGGCCGGTTTC R: TGTCTTTCTGGCCCATACCAA	110	Ramakrishnan *et al*., 2015 [58]
GAPDH	F: GTGGTGCTAAGCGTGTTATCATC R: GGCAGCACCTCTGCCATC	269	Huo *et al*., 2016 [33]
IL-1β	F: GGATTCTGAGCACACCACAGT R: TCTGGTTGATGTCGAAGATGTC	272	Ramakrishnan *et al*., 2015 [58]
IFN-β	F: GCTCACCTCAGCATCAACAA R: GGGTGTTGAGACGTTTGGAT	187	Ramakrishnan *et al*., 2015 [58]
IFN-γ	F: TGAGCCAGATTGTTTCGATG R: CTTGGCCAGGTCCATGATA	152	Ramakrishnan *et al*., 2015 [58]
IL-13	F: CTGCCCTTGCTCTCCTCTGT R:CCTGCACTCCTCTGTTGAGCTT	123	Liu *et al*., 2010 [40]
iNOS	F: AGGCCAAACATCCTGGAGGTC R: TCATAGAGACGCTGCTGCCAG	371	Ramakrishnan *et al*., 2015 [58]

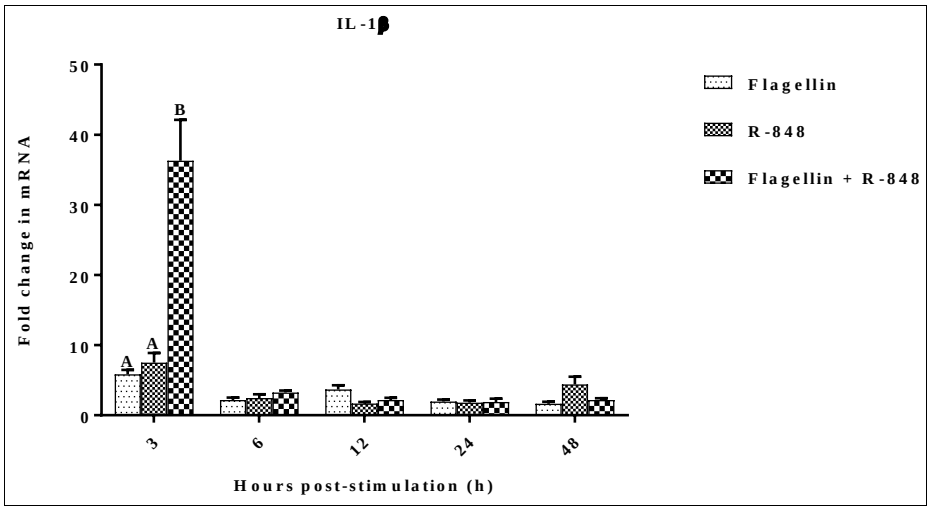

Fig 1: Relative expression of IL-1β transcript in the chicken peripheral blood mononuclear cells (PBMCs) (n=6) stimulated with flagellin (1 µg/ml) and/or R-848 (2 µg/ml) over a period of 48 h. Bars (Mean± SEM) indicate the representative data of a single experiment with different uppercase indicate significant effect of TLR agonist at a time point.

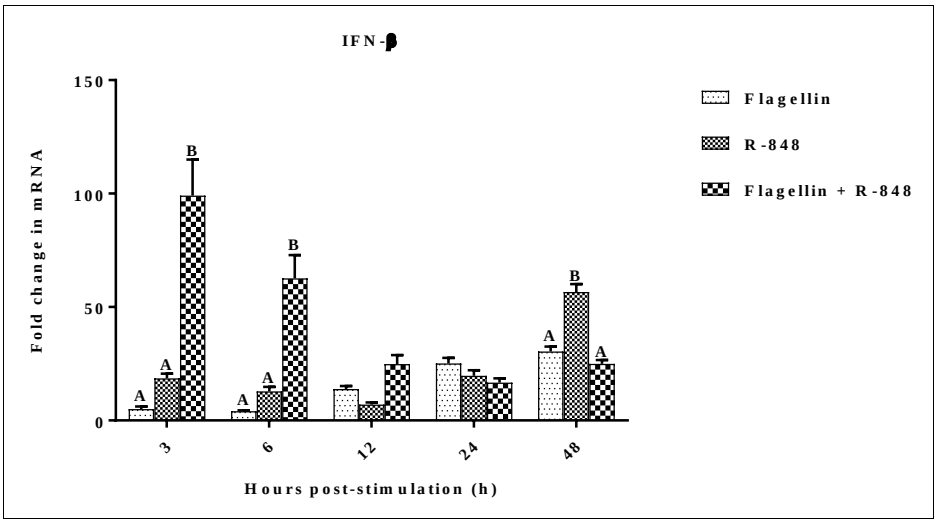

Fig 2: Relative expression of IFN-β transcript in the chicken PBMCs (n=6) stimulated with flagellin (1 µg/ml) and/or R-848 (2 µg/ml) over a period of 48 h. Bars (Mean± SEM) indicate the representative data of a single experiment with different uppercase indicate significant effect of TLR agonist at a time point.

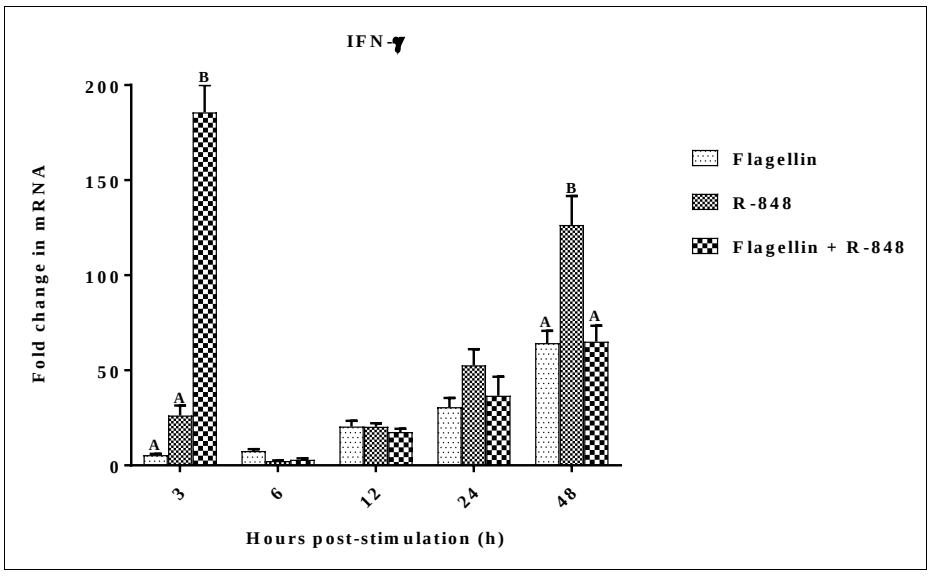

Fig 3: Relative expression of IFN-γ transcript in the chicken PBMCs (n=6) stimulated with flagellin (1 µg/ml) and/or R-848 (2 µg/ml) over a period of 48 h. Bars (Mean± SEM) indicate the representative data of a single experiment with different uppercase indicate significant effect of TLR agonist at a time point.

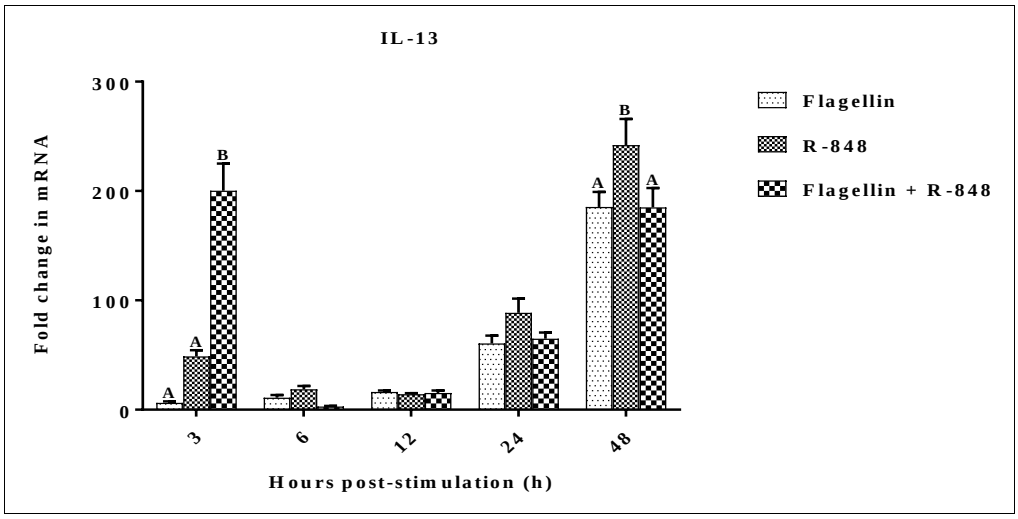

Fig 4: Relative expression of IL-13 transcript in the chicken PBMCs (n=6) stimulated with flagellin (1 µg/ml) and/or R-848 (2 µg/ml) over a period of 48 h. Bars (Mean± SEM) indicate the representative data of a single experiment with different uppercase indicate significant effect of TLR agonist at a time point.

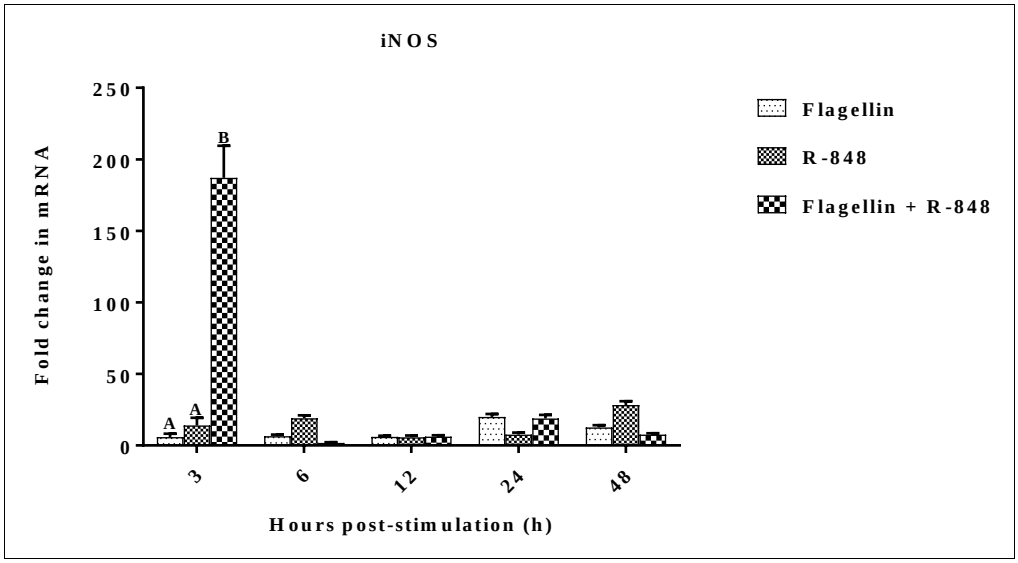

Fig 5: Relative expression of iNOS transcript in the chicken PBMCs (n=6) stimulated with flagellin (1 µg/ml) and/or R-848 (2 µg/ml) over a period of 48 h. Bars (Mean± SEM) indicate the representative data of a single experiment with different uppercase indicate significant effect of TLR agonist at a time point.

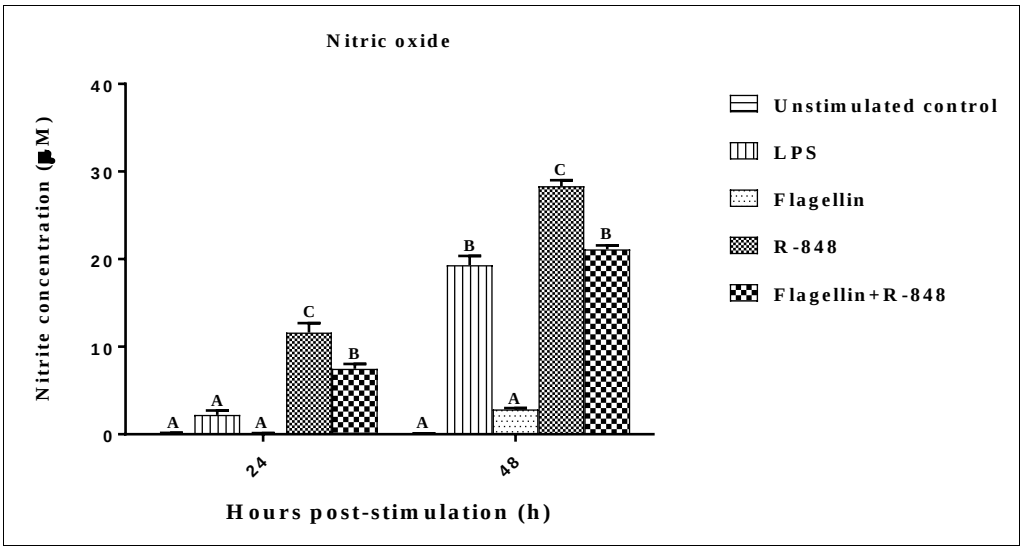

Fig 6: Production of nitric oxide (µM) in the chicken PBMCs (n=6) stimulated with flagellin (1 µg/ml) and/or R-848 (2 µg/ml) at 24 and 48 h with LPS (10 µg/ml) as a positive control. Bars (Mean± SEM) indicate the representative data of a single experiment with different uppercase indicate significant effect of TLR agonist at a time point.

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