Resiquimod enhances mucosal and systemic immunity against avian infectious bronchitis virus vaccine in the chicken

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ABSTRACT

Adjuvant enhancing mucosal immune response is preferred in controlling many pathogens at the portal of entry. Earlier, we reported that a toll-like-receptor 7 (TLR7) agonist, resiquimod (R-848), stimulated the systemic immunity when adjuvanted with the inactivated Newcastle disease virus vaccine in the chicken. Here, we report the effect of R-848 when adjuvanted with live or inactivated avian infectious bronchitis virus (IBV) vaccines with special emphasis on mucosal immunity. Specific pathogen free (SPF) chicks (n = 60) were equally divided into six groups at two weeks of age and immunized with either inactivated or live IBV vaccine adjuvanted with or without R-848. Groups that received either PBS or R-848 served as control. A booster was given on 14 days post-immunization (dpi). R-848 enhanced the antigen specific humoral and cellular immune responses when co-administered with the vaccines as evidenced by an increase in the antibody titre in ELISA and stimulation index in lymphocyte transformation test (LTT) till 35 dpi and increased proportion of $CD4^+$ and $CD8^+$ T cells on 21 dpi in the flow cytometry. Interestingly, it potentiated the IgA responses in the tear and intestinal secretions when used with both live and inactivated IBV vaccines. The combination of IBV vaccine with R-848 significantly up-regulated the transforming growth factor beta 4 (TGFβ4) transcripts in the peripheral blood mononuclear cells (PBMCs) than that of the respective vaccine *per se*. An enhanced secretory IgA response is likely due to the up-regulation of TGFβ4, which is responsible for class switching to IgA. In conclusion, co-administration of R-848 with inactivated or live IBV vaccine enhanced the systemic as well as mucosal immune responses in the chicken.

1. Introduction

Avian infectious bronchitis (IB) is an acute, highly contagious disease of all age groups of chicken and affects primarily the respiratory system with possible infection of renal and reproductive systems [1,2]. It is a disease of global importance and listed in the world organization for animal health (OIE) diseases. Infectious bronchitis virus (IBV), the causative agent of IB, belongs to the genus Coronavirus, family *Coronoviridae*, of order *Nidovirales* [3]. Vaccination with live attenuated and inactivated vaccines is the mainstay tool to control the IB [4]. As IBV enters the host through the mucosal surfaces and replicates in the epithelial cells, vaccines inducing mucosal immunity can prevent the entry of the pathogen before establishment of infection [5]. Studies indicate that local administration of attenuated IBV vaccines is effective as compared to systemic administration implying that respiratory mucosal immunity is essential for protection [6–8]. In fact, an increase in the lachrymal-fluid IgA levels in chickens increases the resistance against IBV infection [9] and re-infection [10]. Further, in the inbred chicken lines, resistance to IBV was correlated with higher IgA levels in the tear and saliva [11].

Limited number of adjuvants is available with the capacity to enhance antigen specific mucosal immunity. Conventional mucosal adjuvants like heat-labile enterotoxin (LT) from *E. coli* and cholera toxin (CT) from *Vibrio cholerae* are toxic to the host [12]. Owing to the immunostimulatory capacity, Toll- like receptor (TLR) agonists are explored as an alternate and promising source for future adjuvant systems [13]. TLRs are evolutionary conserved germ line encoded receptors present in the different cells of various species like human, mice and

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chicken [14,15]. Emerging evidence supports the notion that the TLR agonists increase the secretory IgA (sIgA) when used with the vaccine antigens [16–19]. LPS, a TLR4 agonist, when used with inactivated Newcastle disease virus (NDV) vaccine significantly increased the mucosal and humoral immune responses [20]. Flagellin [21] and combination of CpG and poly I:C [22] increased the sIgA titres along with systemic immune responses in chicken with avian influenza virus vaccine. Resiquimod (R-848) (TLR7/8 agonist) and gardiquimod (TLR7 agonist) enhanced sIgA response besides cellular and humoral responses when used with virus like particles (VLPs) based Norwalk virus vaccine in the mice [17]. The response seen is equivalent to that seen when cholera toxin is used as adjuvant with the same antigen. Co-delivery of Norwalk VLPs with gardiquimod (TLR7 agonist) or CpG ODN (TLR9 agonist) produced strong systemic as well as mucosal immune responses in the mice [23]. Recently, we reported the adjuvant potential of R-848 in the chicken when used with inactivated NDV vaccine [24]. However, the effect of R-848 on the mucosal immune response is not explored in the chicken *hitherto*. Accordingly, we investigated the systemic as well as mucosal immune responses of live or inactivated IBV vaccine in the chicken when adjuvanted with R-848.

2. Materials and methods

2.1. Chicken

Specific pathogen free (SPF) embryonated eggs were procured from Venky's India private limited, Pune, India and hatched at Central Avian Research Institute, Izatnagar. Birds were maintained following standard management practices and provided *ad libitum* sterile feed and water. The experiment was approved by the Institute Animal Ethics Committee.

2.2. TLR agonist and vaccines

TLR7 agonist (resiquimod; R-848) was sourced from InvivoGen, California, USA. Both live and inactivated Massachusetts strain of IBV vaccines were purchased from the commercial sources.

2.3. Experimental design

Two week old SPF chicks were immunized with live or inactivated IBV vaccines in the presence or absence of R-848 (Table 1) with a booster given on 14 days post-immunization (dpi). IBV specific antibody levels were evaluated in the serum at weekly interval. Levels of sIgA were checked in the tear and intestinal secretion of the experimental birds at weekly intervals after booster dose. Cellular immune response was evaluated by lymphocyte transformation test (LTT) on 14, 21, 28 and 35 dpi and by flow cytometry on 21 dpi. Expression of TGF β4 transcripts in the peripheral blood mononuclear cells (PBMCs) of the experimental birds was quantified at 24 and 48 h post-booster dose by quantitative real time polymerase chain reaction (qPCR). The qPCR analysis of TGF β4 transcripts was also done in the spleen samples on 21 dpi.

Table 1
Immunization plan followed in the SPF chicken.

Group	Vaccine preparation	Route
1	Phosphate buffer saline (PBS)	i.m.
2	R-848 (50 μg)	i.m.
3	Inactivated IBV vaccine	i.m.
4	Inactivated IBV vaccine + R-848 (50 μg)	i.m.
5	Live IBV vaccine	i.o.
6	Live IBV vaccine + R-848 (50 μg)	i.o. (R-848: through i.m.)

i.m.- intra-muscular; i.o.- ocular instillation.

2.4. Immunization

Birds of two week age were randomly divided into six groups (n = 10/group) and immunized withlive or killed IBV vaccine (Table 1). A booster was given at the same dose and route on 14 dpi and R-848 (50μg/bird) was given through intra-muscular route (i.m.).

2.4.1. Collection of samples

Blood samples (n = 6/group on 7, 14, 21, 28 dpi and n = 3/group on 35 dpi) were collected to separate sera that was stored at −20 °C for the detection of IBV specific antibody levels on 7, 14, 21, 28 and 35 dpi. Tear samples (n = 6/group on 21, 28 dpi and n = 3/group on 35 dpi) were collected from the experimental birds as reported elsewhere [25] on 21, 28 and 35 dpi. Briefly, a pinch of molecular grade sodium chloride crystals was sprinkled on either eye that induced lachrymation within 35–45 s. The tear was aspirated with a micropipette and stored in microcentrifuge tube at −20 °C for IgA assay. Birds (n = 3/group) were humanely sacrificed on 21, 28 and 35 dpi and spleen, intestinal secretions were collected as reported earlier [26]. Briefly, around 5 cm long duodenum, jejunum and ileum were collected from each bird and pooled; the parietal surface was washed with PBS. Longitudinal strip of intestine was prepared and placed in a graduated tube to which equal volume of PBS (v/v) containing ethylene diamine tetra-acetate (EDTA) 50 mM, trypsin inhibitor 100 μg/mL and phenyl methane sulfonyl fluoride (PMSF) 0.35 mg/mL was added. The samples were vortexed briefly for 3–5 min before centrifugation at 8000 × g for 20 min at 4 °C. The clear supernatant was aspirated and kept at −20 °C until further use. The PBMCs were isolated from the experimental birds (n = 6/group) at 24 and 48 h post-booster dose using Ficoll Hypaque (Sigma, MO, USA) (1.077 g/mL) density gradient centrifugation as per the published protocol [27].

2.4.2. Evaluation of the humoral immune response

The IBV specific antibody in the serum (n = 6/group on 7, 14, 21, 28 dpi; n = 3/group on 35 dpi) was quantified using commercial IBV antibody test kit (IDEXX Laboratories, USA) following the manufacturer's instructions. An antibody titre of > 396 or 2.598 log_{10} was considered positive for IBV.

2.4.3. Evaluation of the mucosal immune response

The levels of IgA both in the tear (n = 6/group on 21, 28 dpi; n = 3/group on 35 dpi) and intestinal secretion (n = 3/group) were measured using the commercial IBV antibody test kit (IDEXX Laboratories, USA) with certain modifications using anti-chicken IgA-HRPO (Bethyl, USA) as the secondary antibody.

2.4.4. Evaluation of the cellular immune response

Cellular immune response in the experimental birds was assessed by lymphocyte transformation test (LTT) and flow cytometry as reported earlier [24].

2.4.4.1. Lymphocyte transformation test. Blood was collected in a heparinized vial containing 20 IU/mL on 14, 21, 28 and 35 dpi for LTT. The PBMCs (n = 6/group on 14, 21, 28 dpi and n = 3/group on 35 dpi) were suspended in RPMI 1640 complete medium containing 10% fetal bovine serum (FBS) and 100 IU/mL penicillin, and 50 μg/mL streptomycin. Cell viability was determined by trypan blue dye exclusion method. Cell suspension was adjusted to 1×10^7 cells/mL and 100 μL of cell suspension was plated in each well of 96 well cell culture plate. RPMI 1640 medium (100 μL) with or without ConA (20 μg/mL), IBV antigen (10 μg/well) was added to the wells in triplicate. The plates were incubated at 37 °C, 5% CO_2 for 72 h in a humidified chamber. At the end of incubation, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide; Sigma, USA] 20 μL was added from the stock (5 mg/mL). The plates were re-incubated at the same condition for another 4 h. Culture supernatant

(100 μL) was discarded from each well and the formazan crystals were dissolved by adding dimethyl sulfoxide (Amresco, USA) 100 μL to each well and optical density (OD) was taken at 570 nm in a microplate ELISA reader. Blastogenic response was calculated by dividing the mean OD of the stimulated well by the mean OD of unstimulated well and expressed as stimulation index (SI).

2.4.4.2. Flow cytometry analysis.
The proportion of $CD4^+$ and $CD8^+$ T cell subsets in the PBMCs (n = 6/group) was measured on 21 dpi by flow cytometry. For analysis, 2×10^5 cells were stained with anti-chicken CD4/CD8 R-PE and CD3-FITC labeled monoclonal antibodies (Abcam, USA) and kept overnight at 4 °C in the dark. Subsequently, the cells were washed with PBS containing 2% FBS and were not fixed. The aliquots of 1×10^4 cells were analyzed per sample by BD FACS ™ Calibur instrument (BD BioSciences, UK). The unstained cells served as the negative control.

2.4.5. Real time PCR analysis of TGF-β4
The PBMCs (1×10^6) (n = 6/group) were collected from the experimental birds as mentioned above at 24 and 48 h post-booster and one mL of RiboZol™ (Amresco, USA) was added. Similarly, one mL of RiboZol™ was added to spleen tissue collected following sacrifice (n = 3/group). Total RNA extraction was done by phenol : chloroform and isopropanol method and the purity was checked by absorbance at 260 and 280 nm in a Nanodrop UV spectrophotometer. Total RNA was used for the preparation of cDNA employing Revertaid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA), following manufacturer's instructions.

Quantification of the TGF-β4 gene was done by QuantiTect SYBR Green qPCR kit (Qiagen, CA, USA) on CFX96 Real Time System (Bio-Rad, CA, USA) following the published report [28]. β-actin served as the housekeeping gene. Published primer sequences were used for β-actin (F: 5′ TATGTGCAAGGCCGGTTT 3′, R: 5′ TGTCTTTCTGGCCCAT ACCAA 3′) [29] and TGF-β4 (F: 5′ CGGCCGACGATGAGTGGCTC 3′, R: 5′ CGGGGCCCATCTCACAGGGA 3′) [30] genes. Each sample was tested in triplicate on the same plate. Expression of TGF-β4 was calculated relative to the β-actin gene and expressed as n-fold increase or decrease relative to the control. The data of real time PCR was calculated by $2^{-\Delta\Delta Ct}$ method [31].

2.5. Statistical analysis

Each experiment was repeated twice independently and data from the first experiment was used for analysis. The treatment effect at each time point was assessed by One way analysis of variance (ANOVA) with Duncan's multiple range test as *post hoc* test to find the significance of pair-wise mean difference. The minimum level of significance was set at 95%. Results are presented as Mean ± SE. Statistical software SPSS™ 20.0 (IBM Corp., USA) was used for analysis while GraphPad prism version 5.0 was used for generating the graph.

3. Results

3.1. Humoral immune response

Effect of R-848 on vaccination induced IBV antibody titre in the sera is presented in Fig. 1. The antibody titre in the PBS control and R-848 groups was consistently negative for IBV. There was no significant (P > 0.05) difference in the antibody response between the vaccinated and control groups on 7 dpi. Only live IBV vaccine with or without R-848 induced significantly higher antibody response than that of the control group on 14 dpi. The combination of vaccine plus R-848 showed significantly higher (P < 0.05) antibody titre than that of the respective vaccine alone group after secondary immunization consistently (Fig. 1).

3.2. Mucosal immune response

Effect of R-848 on vaccination induced IgA response in the tear and intestinal secretion is presented in Figs. 2 and 3, respectively. Vaccine, either live or inactivated, induced a significantly higher IgA response than that of the control group after secondary vaccination (P < 0.01). Co-administration of R-848 with live or inactivated IBV vaccine significantly increased the IgA response in the tear and intestinal secretion from 21 dpi, which was maintained till 35 dpi as compared to the vaccine alone group (P < 0.01). The peak IgA response in the tear was observed in the live vaccine plus R-848 group followed by inactivated vaccine plus R-848, live vaccine and inactivated vaccine groups. The IgA response in the intestinal secretions was comparable with that of tear.

3.3. Cellular immune response

3.3.1. Lymphocyte transformation test
The antigen specific lymphocyte proliferation following different treatment is depicted in Fig. 4. Live as well as inactivated IBV vaccine significantly increased the SI as compared to the control at each time point studied. Further, co-administration of R-848 significantly potentiated the SI as compared to the vaccine alone groups (P < 0.01). The SI was maximum in the live vaccine plus R-848 group, which was 1.55 ± 0.022, 1.69 ± 0.021 and 1.76 ± 0.01, 1.89 ± 0.02 on 14, 21, 28 and 35 dpi, respectively (Fig. 4).

3.3.2. Flow cytometry
Both the live and inactivated vaccines significantly increased the $CD4^+$ and $CD8^+$ T cells (%) as compared to the control (P < 0.05) as depicted in Fig. 5. Co-administration of R-848 with either type of vaccine showed a significant increase in the $CD4^+$ and $CD8^+$ T cells (%) indicating a immunomodulatory role in the adaptive immunity. Combination of R-848 and live vaccine showed the highest percent increase of 25.54 ± 1.54 and 17.43 ± 0.61, respectively in $CD4^+$ and $CD8^+$ T cells.
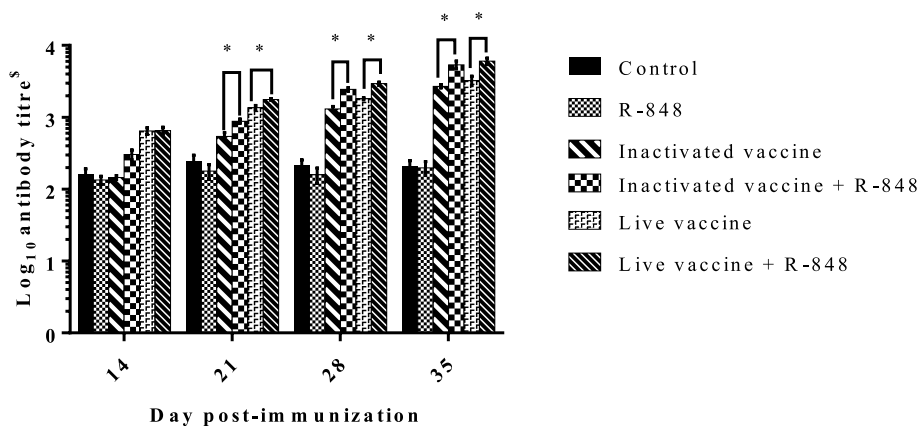
3.4. Real time PCR analysis of TGF-β4 transcripts

The groups receiving vaccine along with R-848 showed significantly (P < 0.01) higher expression of TGF-β4 than that of the respective vaccine alone groups (Fig. 6). The highest TGF-β4 transcripts was observed in the live vaccine plus R-848 group, which was 4.07 ± 0.09 and 5.58 ± 0.60 folds higher than that of the control group at 24 and 48 h post-booster immunization, respectively. The response seen in the inactivated vaccine plus R-848 group was almost equal to the live vaccine alone group. Expression of TGF-β4 transcripts was comparable in both spleen (data not shown) and PBMCs.

4. Discussion

Resiquimod, a TLR7 agonist, is an imidazoquinoline compound with tremendous immunodulatory capacity. The antiviral activity of TLR7 agonists has been reported against genital warts, herpes genitalias and molluscum contagiosum [32]. TLR 7/8 agonists like imiquimod and R-848 are FDA approved drugs for basal cell carcinoma, actinic keratosis and papilloma virus in the human [33–36]. Recently, we reported the enhanced antigen specific cellular as well as humoral immune responses in the chicken when resiquimod (R-848) was used with inactivated NDV vaccine resulting in complete protection against virulent NDV challenge [24]. Since the adjuvant potential is likely to vary with the type of vaccine, we studied the effect of R-848 with IBV vaccine. To the best of our knowledge, this is the first report on the effect of R-848 in modulating the mucosal immune response in the chicken.

Humoral immune response plays an important role in IBV infection [37]. In the present study, antibody titre was highest in the SPF chicken
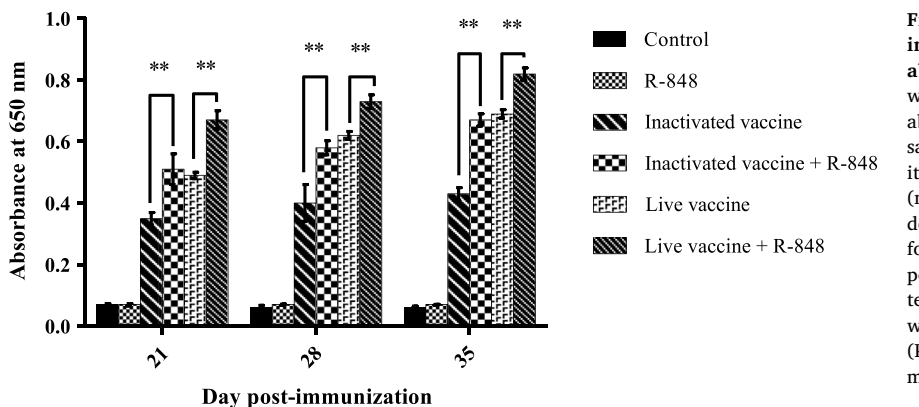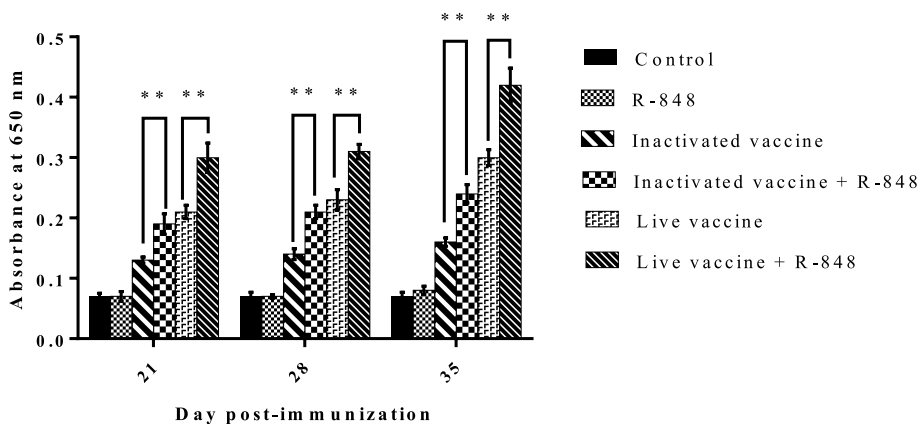
dpi: day post-immunization.

that received live vaccine with R-848 than other groups (Fig. 1). The live vaccine virus stimulates more vigorous immune response as they replicate in the host and simulate the natural infection. Adjuvanted live IBV vaccine induced higher immune responses than live vaccine alone [38], supports the concept of present study. Further, the combination of R-848 withinactivated IBV vaccine showed higher antibody response than the vaccine alone group. These findings indicate the adjuvant capacity of R-848 with live as well as inactivated IBV vaccines in increasing the antibody response, which is supported by our earlier report on inactivated NDV vaccine in SPF chicken [24].

In addition to antibodies, cell mediated immunity also plays an important role in immunity against IBV. Transfer of lymphocytes from birds on day 10 post-IBV infection to naïve chicken completely eliminates the viral infection and clinical signs after challenge [39] and viral load was reduced in the lung by increasing IBV-specific cytotoxic T cells

(CTLs) in spleen [40]. In the present study, cell mediated immunity was analyzed by LTT as well as immunophenotyping. We found that SPF chicken receiving live vaccine with R-848 mounted a strong antigen specific proliferation as compared to other groups (Fig. 4), which is supported by the concomitant increase in the proportion of $CD4^+$ and $CD8^+$ T cells (Fig. 5). Similarly, an enhanced cellular immune response with inactivated IBV vaccine recorded in the present study is supported by the findings with inactivated NDV vaccine [24].

There is a need for adjuvants that increase antigen specific mucosal immune response to curtail the infection at the entry level [41]. Despite the fact that the local CTLs are essential for the virus clearance in early infection [42], sIgA of lacrimal origin is a good indicator of protection in IBV infection [9,43,44] as mucosal antibody response resists re-infection [6]. R-848 enhanced the sIgA response in the tear (Fig. 2) as well as intestinal secretions (Fig. 3) when adjuvanted with live or
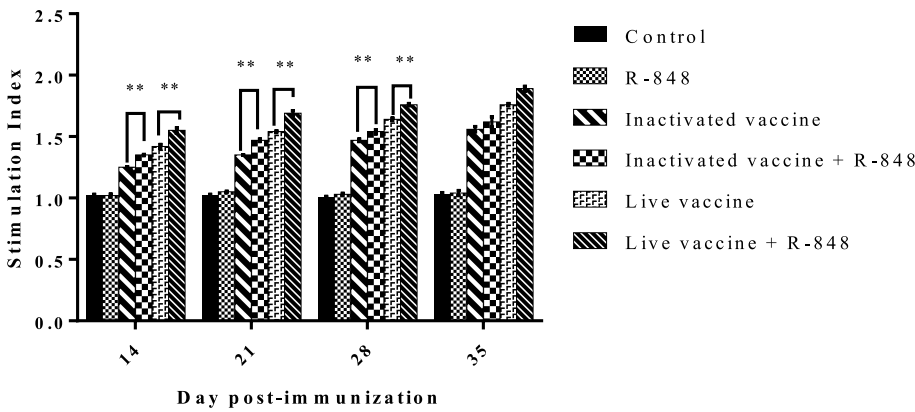
Fig. 4. Lymphocyte proliferation specific to IBV antigen in the PBMCs collected from the SPF chickens following vaccination in the presence or absence of R-848. Birds of 2 week age were immunized with live or inactivated IBV vaccine in the presence or absence of R-848 (50 μg/bird) with a booster 14 days later. PBMCs (n = 6/group on 14, 21, 28 dpi and n = 3/group on 35 dpi) were collected and stimulated with IBV antigen to assess lymphocyte proliferation using MTT dye at weekly interval from dpi 14. The experiment was repeated twice independently and data from the first experiment was used for analysis. Treatment effect was analyzed at each time point by One way ANOVA with Duncan's multiple range test to compare the pair-wise mean difference. Alpha error was set at 1%. **Indicate significant difference (P < 0.01) between

the groups. dpi: day post-immunization.

Fig. 5. CD4$^+$ and CD8$^+$ cells (%) in the PBMCs of SPF chicken following IBV vaccination in the presence or absence of R-848. Birds of 2 week age were immunized with live or inactivated IBV vaccine in the presence or absence of R-848 (50 μg/bird) with a booster 14 days later. The PBMCs (n = 6/group) were collected from the birds on 21 dpi and analyzed by flow cytometry following addition of chicken specific monoclonal antibodies. The experiment was repeated twice independently and data from the first experiment was used for analysis. Treatment effect was analyzed by One way ANOVA with Duncan's multiple range test to compare the pair-wise mean difference. Alpha error was set at 5%. *Indicate significant difference (P < 0.05) between the groups. dpi - day post-immunization.
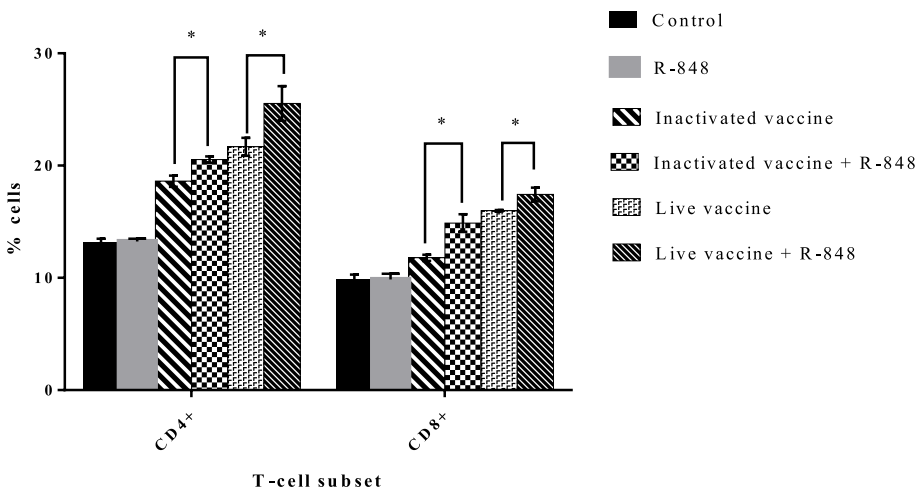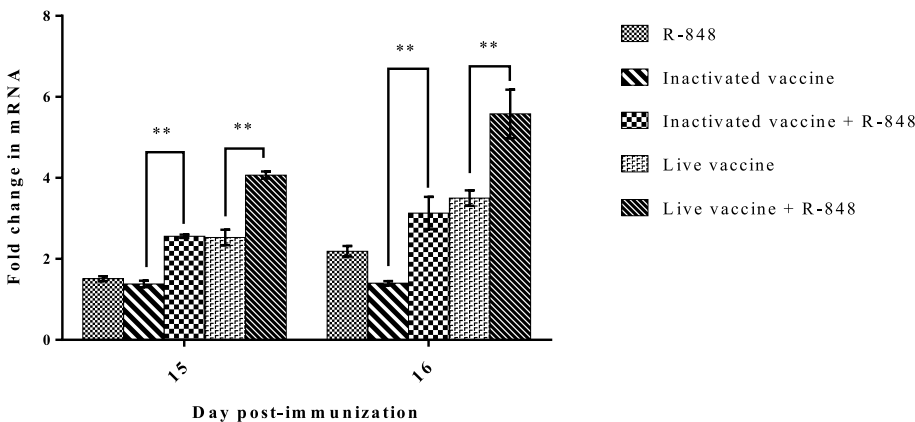
Fig. 6. Quantitative real time PCR analysis of TGF-β4 expression in the PBMCs of the SPF chicken following vaccination in the presence or absence of R-848. Birds of 2 week age were immunized with live or inactivated IBV vaccine in the presence or absence of R-848 (50 μg/bird) with a booster 14 days later. The PBMCs (n = 6/group) were collected from the birds on 15 and 16 dpi (24 and 48 h post-booster) and the TGF-β4 transcripts were analyzed by real time PCR using β-actin as the house keeping gene and expressed as n-fold increase or decrease relative to the control following $2^{-\Delta\Delta Ct}$ method (34). The experiment was repeated twice independently and data from the first experiment was used for analysis. Treatment effect was analyzed at each time point by One way ANOVA with Duncan's multiple range test to compare the pair-wise mean

difference. Alpha error was set at 1%. **Indicate significant difference (P < 0.01) between the groups.

inactivated IBV vaccine. In support of the findings, it is reported that the administration of R-848 with VLPs of Norwalk virus enhanced the mucosal IgA response in the mice [17]. To explain the impressive sIgA response, we studied the relative expression of TGF-β4 as it has a major role in the induction of IgA class switching [45]. It is well known that TGF-β1, which is the orthologue of TGF-β4, is primarily responsible for class switching of B cells to produce IgA in the mammals [46–50]. A significant increase in the relative copy number of TGF-β4 (Fig. 6) suggests a role of R-848 in IgA class switching in the groups that received live or inactivated IBV vaccine. In this study, the TGF-β4 transcripts were significantly higher in vaccine plus R-848 groups than that

of the respective vaccine alone groups. Activation of TLR7 by R-848 would initiate the signaling cascade through the MyD88-dependent pathway, which might have resulted in the up-regulation of TGF-β4 transcripts [51]. Studies by our group have shown that R-848 improves the vaccine response of NDV when adjuvanted [24] and has prophylactic potential against IBDV [29]. Thus, the adjuvant effect of R-848 with IBV is needed to be tested following challenge studies.

In conclusion, co-administration of R-848 with inactivated or live IBV vaccine enhanced the mucosal immunity by increasing sIgA which is likely mediated by TGF-β4. The potential of R-848 to enhance mucosal immunity has translational significance in poultry vaccination.

Conflicts of interest

The authors declare that there is no conflict of interest with respect to the content of the manuscript.

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