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Interferon-γ influences immunity elicited by vaccines against very virulent Marek's disease virus

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ABSTRACT

Vaccination of chickens with herpesvirus of turkey (HVT) confers only partial protection against challenge with a very virulent Marek's disease virus (MDV). Here, we evaluated the ability of recombinant chicken interferon-gamma (rChIFN-γ) to enhance protective efficacy of HVT against the very virulent MDV strain, RB1B. The bioactivity of IFN-γ expressed by a plasmid expression vector was confirmed by its ability to stimulate a chicken macrophage cell line (HD11) to produce nitric oxide (NO) *in vitro*. The administration of HVT with 5 μg of pcDNA:chIFN-γ plasmid reduced the incidence of tumor development significantly when compared to vaccinated birds (77.7% in the HVT + empty vector group and 80% in HVT group versus 33.3% in the HVT + chIFN-γ group) and significantly increased IFN-γ expression in the splenocytes of the protected group, suggesting that rChIFN-γ increases the potency of HVT against MDV. Further analysis demonstrated that the protected birds that received HVT vaccine and/or plasmid had lower MDV genome load and lower amounts of transcripts for meq and vIL-8 than in the birds without lesions. Similarly, lower expression of IL-10, IL-18 and IL-6 was observed in the chickens without lesions compared to the chickens that had lesions, suggesting an inverse association between up-regulation of these cytokines and vaccine-induced immunity. In conclusion, IFN-γ can positively influence immunity conferred by HVT vaccination against challenge with a very virulent Marek's disease virus (vvMDV) in chickens.

1. Introduction

Marek's disease (MD), which is caused by a highly contagious cell-associated alphaherpesvirus named Marek's disease virus (MDV), is a lymphoproliferative and immune suppressive disease of chickens (Biggs, 2001; Baigent et al., 2006; Calnek, 2001). There are three serotypes of MDV: serotype 1 (also known as *Gallid herpesvirus-2*, GaHV-2), which includes oncogenic virus strains such as RB1B and GA, serotype 2 (also known as *Gallid herpesvirus-3*, GaHV-3), which includes non-oncogenic strains such as SB-1, and serotype 3 (*Meleagrid herpesvirus-1*, MeHV-1) which includes non-oncogenic viruses of turkey also known as herpesvirus of turkeys (HVT). Early reports revealed that HVT, which is non-oncogenic for chickens and antigenetically related to MDV, can be used as a vaccine against MD (Witter et al., 1970; Kawamura et al., 1969). MD has since been controlled by vaccinating chick embryos or chickens with different vaccine formulations using HVT alone, HVT in combination with non-oncogenic strains as bivalent or trivalent vaccines, or with attenuated serotype 1 strains (Churchill et al., 1969; Okazaki et al., 1970; Rispens et al., 1972; Witter and Schat, 2003). Although these vaccines do not prevent chickens from becoming infected with MDV, they have varying degrees of efficacy against disease caused by viruses of different pathotypes. For instance, while monovalent HVT vaccines are efficacious against virulent (v) MDV strains, these vaccines are not highly protective against very virulent (vv) viruses (Witter, 1997). Despite the widespread use of vaccines, MD outbreaks are still occurring due to a variety of factors including the emergence of strains with increasing virulence (Baigent et al., 2006). Evolution of MDV virulence may be related to factors such as constant administration of vaccine which tends to exert evolutionary pressure on the virus leading it to evolve into more virulent pathotypes, a feature known as vaccine-driven evolution (Schat and Baranowski, 2007). Co-administration of avian immunomodulatory molecules, including cytokines, have been proven to enhance protective host immune responses to a variety of pathogens such as *Eimeria acervulina* (Min et al., 2001), duck hepatitis B virus (DHBV) (Long et al., 2005), infectious bursal disease virus (IBDV) (Hsieh et al., 2006), *Escherichia coli* (Janardhana et al., 2007), and infectious bronchitis virus (IBV) (Wang et al., 2009). Analysis of host responses to MDV infection has led to identification of a few cytokines that play a role in immunity against the virus, hence may be included in vaccines for enhancing their immunogenicity and efficacy (Parvizi

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et al., 2010; Kaiser et al., 2003; Xing and Schat, 2000b; Kano et al., 2009; Haq et al., 2010b).

Interferon-gamma (IFN-γ) is a type II interferon and an important regulator of immune responses including antiviral defenses. Chicken IFN-γ (chIFN-γ) was first cloned by Digby and Lowenthal (1995), and since then has been shown to activate macrophages (Lowenthal et al., 1995; Lowenthal et al., 1997), neutralize viral replication (Digby and Lowenthal, 1995; Song et al., 1997), enhance major histocompatibility complex (MHC) class II antigen expression (Song et al., 1997; Weining et al., 1996) and other genes in chickens (Mallick et al., 2010). The expression of IFN-γ is associated with protection against MD (Xing and Schat, 2000b; Kano et al., 2009; Haq et al., 2010a; Jarosinski et al., 2005; Abdul-Careem et al., 2007), making this cytokine a candidate for inclusion in Marek's disease vaccines to enhance their protective efficacy. Recombinant chIFN-γ (rChIFN-γ) may be delivered via the use of liposomes (Kedar et al., 1997), recombinant viral vectors (Johnson et al., 2000; Yong-Ke et al., 2005), and plasmid DNA (Haygreen et al., 2005). The immune enhancing effect of rChIFNγ has been well studied in the coccidiosis challenge model, demonstrating that the treatment of birds with chIFN-γ reduces the oocyst production and improves resistance to *E. acervulina* (Min et al., 2001; Lowenthal et al., 1997; Lillehoj and Choi, 1998). A recent study also showed that a DNA vaccine in combination with plasmid encoded chIFN-γ protected birds from coccidiosis, which was marked by prevention of weight loss and oocyst excretion (Shah et al., 2010). The immune enhancing effects of IFN-γ in other avian species have also been recorded. For instance, administration of recombinant IFN-γ inhibited duck hepatitis B replication in hepatocytes and improved vaccine efficacy against the disease in duck (Long et al., 2005; Schultz and Chisari, 1999). In this study, we hypothesized that administration of IFN-γ, via the use of a plasmid expressing chIFN-γ, will enhance the protective efficacy of HVT vaccine against challenge with a very virulent strain of MDV in chickens.

2. Materials and methods

2.1. Cell lines

An immortal chicken fibroblast cell line, DF-1, was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS), 1% chicken serum (CS) and 100 μg/mL of penicillin and streptomycin at 37 °C and 5% CO_2. Chicken macrophage cell line, HD11, was maintained in RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% FBS, 2 mM glutamine, 100 μg/mL penicillin and streptomycin at 41 °C and 5% CO_2.

2.2. Experimental animals

Specific-pathogen free (SPF) eggs were obtained from the Animal Disease Research Institute, Canadian Food Inspection Agency (Ottawa, ON, Canada) and hatched at the Arkell Poultry Research Unit, University of Guelph. Hatched chicks were housed in the animal isolation facility at the Ontario Veterinary College, University of Guelph during the experimental period. All animal experiments were approved by the Animal Care Committee, University of Guelph.

2.3. Virus and vaccine stains

Very virulent MDV strain RB1B (passage 9) was provided by Dr. K.A. Schat (Cornell University) and was used for infecting chickens. Chickens were vaccinated subcutaneously on the day of hatch using ¼ of the recommended dose of cell-free HVT purchased from Fort Dodge Animal Health, Division of Wyeth, IA 50501 USA.

2.4. Construction of plasmid containing chIFN-γFLAG

The chIFN-γ coding sequence was PCR amplified from cDNA prepared from concavalin A (ConA) stimulated primary splenocytes using primers designed to span the predicted chIFN-γ coding sequence (CDS) obtained from Ensembl database (ENSGALT00000016105). The primer sequences were: Forward 5′-GA ACTGAGCCATCACCAAGAA-3′, Reverse 5′-CGTCTTATGACCTCCTGT GCT-3′. Briefly, the PCR conditions were as follows: one cycle of 95 °C for 10 min, 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min using High fidelity (HiFi) platinum Taq polymerase (Invitrogen Canada Inc., Burlington, ON, Canada). The amplified product and a 1× FLAG fusion tag fused at the 3′ end was cloned into the pcDNA3.1(+) expression vector (Invitrogen Canada Inc., Burlington, ON, Canada), and the resulting recombinant plasmid was designated as pcDNA:chIFN-γFLAG. Plasmid DNA was prepared using a GenElute™ plasmid purification kit (Sigma–Aldrich, St. Louis, MO, USA). Recombinant plasmid pcDNA:chIFN-γFLAG was sequenced bi-directionally to verify correct insertion and sequence fidelity (Laboratory Services Division, University of Guelph).

2.5. DNA and RNA extraction

Spleen tissues collected on 21 days post-infection (dpi) were preserved in RNA later (Qiagen Inc., Mississauga, Ontario, Canada) prior to DNA and RNA extraction using Trizol reagent (Invitrogen Canada Inc. Burlington, Ontario, Canada) as described previously (Abdul-Careem et al., 2006b).

2.6. Reverse transcription

Complementary DNA (cDNA) was prepared from 2 μg of DNase-treated total RNA by reverse transcription using MMLV reverse transcriptase and Oligo$(dT)_{12-18}$ primers (SuperScript™ First-Strand Synthesis System, Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions.

2.7. Primers

The absolute MDV genome load in the spleen was quantified using primers specific for the MDV-*meq* gene. The relative quantification of MDV-meq and MDV-vIL-8 transcripts was done using primers targeting MDV-*meq* and MDV-*vIL-8* genes and normalized against expression of the chicken β-actin gene. The primers used to amplify MDV-meq, MDV-vIL-8, IFN-γ, IL-10, IL-6, IL-18 and β-actin have been published previously (Abdul-Careem et al., 2006a,b; Abdul-Careem et al., 2007). All primers were synthesized by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

2.8. Real-time PCR and RT-PCR

All the DNA and cDNA were tested in real-time PCR and RT-PCR assays, respectively, along with a dilution series of the standard plasmids that served as the calibrator as well as a no template control. Real-time RT-PCR and PCR quantifications were performed in a LightCycler 480 Instrument (Roche Diagnostics, Laval, QC, Canada) in a reaction volume of 20 μl using LightCycler 480 SYBR Green 1 Master Mix (Roche Diagnostics). In addition, the reaction consisted of 0.25–0.5 μM of each primer and 5 μl of 1:10 dilution of cDNA or 100 ng of DNA extracted from spleen as template and PCR grade water. The optimal PCR parameters for all genes have been described previously (Abdul-Careem et al., 2007; Abdul-

Careem et al., 2006a). Briefly, the cycles included an initial heat-denaturing step at 95 °C for 10 min, 45 cycles at of amplification at 95 °C for 10 s, annealing for 5 s, and elongation at 72 °C for 10 s. Following amplification, melting curves were determined at 95 °C for 10 s, 65 °C for 30 s and 97 °C for 0 s continuous acquisition. Fluorescent acquisition was done at 72 °C and expression levels were normalized to β-actin expression.

2.9. rChIFN-γFLAG expression & bioactivity

The recombinant plasmid, pcDNA:chIFNγFLAG, was used to transfect DF-1 cells. DF-1 cells ($1x10^6$) were incubated 24 h prior to transfection with pcDNA:chIFN-γFLAG or an empty vector (pcDNA3.1) as a negative control. Stock solution of polyethyleneimine (PEI) (PolySciences, Eppenheim, Germany) was prepared in water at a final concentration of 1 mg/ml, and plasmid DNA was diluted to required concentrations. Prior to transfection, DF-1 cells were rinsed twice with PBS to remove any residual cell culture medium and 200 μL of the DNA-PEI solution was added and incubated overnight (16–18 h). At this point, the transfection mix was removed, cells were washed and complete DMEM was added to all wells. Cells and culture supernatant samples were collected 24, 48 and 72 h post-transfection for RNA extraction and protein expression analysis. Culture supernatant was separated by 12% SDS–PAGE and electrophoretically transferred to nitrocellulose membrane for western blotting using an anti-FLAG® monoclonal antibody (Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) and detected using BCIP/NBT assay.

The bioactivity of the rChIFN-γFLAG was tested by a nitric oxide (NO) assay and by assessing the antiviral activity of the recombinant chIFN-γFLAG. Nitrite assay was used to determine NO production in HD11 cells after stimulating with rChIFN-γFLAG using the Griess Reagent System (Promega Corporation, Madison, WI, USA). HD11 cells (1×10^5 cells in 200 μL) were cultured overnight in sterile 96 well plates. Fifty microliters of supernatant was aspirated and replaced by 50 μL of transfected DF-1 culture supernatant or purified rChIFN-γFLAG, as a positive control, for 48 h. The concentration of nitrite was then determined by adding 50 μL of experimental samples to 100 μL of Griess reagent (1:1 mixture of 1% sulphanilamide, 2.5% phosphoric acid and 0.1% naphthylethylenediamine), and absorbance was measured with a filter between 520 and 550 nm with a spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). The concentration of nitrite in test samples was determined by preparing a 0–100 μM nitrite standard reference curve. All experiments were done in triplicate.

2.10. Experimental design

There were two challenge trials in this study to assess the ability of chIFN-γ expressed by a plasmid to enhance protection conferred by HVT vaccination. In the first trial, which was conducted as a pilot experiment, day old chicks (n = 5–6/group) were vaccinated with HVT and injected with two plasmid doses (1 and 5 μg). The trial included five groups of 5–6 birds each. One group served as an uninfected control, one group was infected with 250 plaque forming units (PFU) of RB1B intra-abdominally (i.a) at 5 days of age, another group was vaccinated on day 1 of hatch with HVT vaccine (Fort Dodge Animal Health, Division of Wyeth, IA 50201, USA) and infected i.a on day 5 of hatch with 250 PFU of RB1B, and the other two groups were vaccinated, MDV-infected and administered two different doses of plasmid (1 or 5 μg) subcutaneously in the leg.

In the second trial, each group had a larger number of birds (n = 9–10/group) and based on the results from the pilot trial, an optimal plasmid dose (5 μg) was selected and an additional control group was added. The doses and administration day were similar to the first trial. One group served as uninfected control and another group as the infected only (RB1B). The other three groups consisted of vaccinated MDV-infected (R+H), and vaccinated MDV-infected administered with 5 μg of pcDNA:chIFN-γFLAG (R + H + 5 μg Plasmid) or an empty vector (R + H + 5 μg EV). The chickens were then monitored daily for any clinical signs over a 3-week period. At 21 days post-infection, all birds were weighed, necropsied and presence of gross lesions was recorded. Based on the presence or absence of gross lesions characteristic of Marek's disease, chickens were further categorized as with or without lesions. To this end, all major organs including liver, heart, spleen, bursa, kidney, proventriculus, sciatic nerve, gonads, muscles and eyes were observed for presence of tumors and gross lesions. In addition, the weights of spleen and bursa of Fabricius were recorded, and the ratios of spleen/bursa of Fabricius to body weight were determined. Analysis of the viral load and gene expression profiling from the second trial is presented.

2.11. Data analysis

Quantification of MDV genome load and transcripts by real time PCR and RT-PCR was done as described previously (Abdul-Careem et al., 2006a; Abdul-Careem et al., 2006b). Briefly, absolute number of MDV genome per 100 ng of DNA was calculated based on an external standard curve. Expression of MDV-meq, vIL-8, IFN-γ, IL-10, IL-6 and IL-18 was calculated relative to the expression of β-actin gene. Results are presented as with lesions and without lesions in each group. For tumor incidence data, Fisher's exact test was used, whereas for all other data a two-tailed t-test was used to identify differences among groups. Comparisons were considered significant at $P \leqslant 0.05$.

3. Results

3.1. Construction of plasmid, expression of rChIFN-γFLAG in vitro and biological assay

The first aim of this study was to obtain a biologically active rChIFN-γ protein expressed using a eukaryotic expression system. In order to confirm the expression of cloned IFN-γ, chicken embryo fibroblast (DF-1) cells were transfected with pcDNA:chIFN-γFLAG or empty vector (mock). RNA was extracted at 24, 48 and 72 h post-transfection and transcripts were detected by real time qRT-PCR (Fig 1a). Results confirmed the expression of significantly higher chIFN-γ mRNA compared to empty vector and medium-treated cells at the three time points ($P \leqslant 0.05$). To confirm the identity of the protein expressed by pcDNA:chIFN-γFLAG, culture supernatants of transfected cells were analyzed by Western blotting (Fig. 1b). This analysis confirmed the presence of a band with a molecular weight of approximately 17 kDa, the position corresponding to the monomeric, non-glycosylated form of rChIFN-γ (Lillehoj and Choi, 1998; Lambrecht et al., 1999; Takehara et al., 2002). In addition, another faint band was present above the major band which most likely represents the glycosylated form of the cytokine (Fig 1b). The results confirmed expression of rChIFN-γFLAG at both the transcript and protein level. Biological activity of the recombinant protein was tested by treating HD11 cells with the supernatants from pcDNA:chIFN-γFLAG transfected DF-1 cells and measuring NO production using the Griess assay. Purified recombinant protein (positive control) and culture supernatants of pcDNA:chIFN-γFLAG transfected DF-1 cells induced high levels of nitrite production by HD11 cells (Fig. 1c). Supernatants from DF-1 cells transfected with an empty vector (mock transfected) or medium produced undetectable levels of nitrite. The average amounts of nitrite produced by HD11 cells after incubation of these

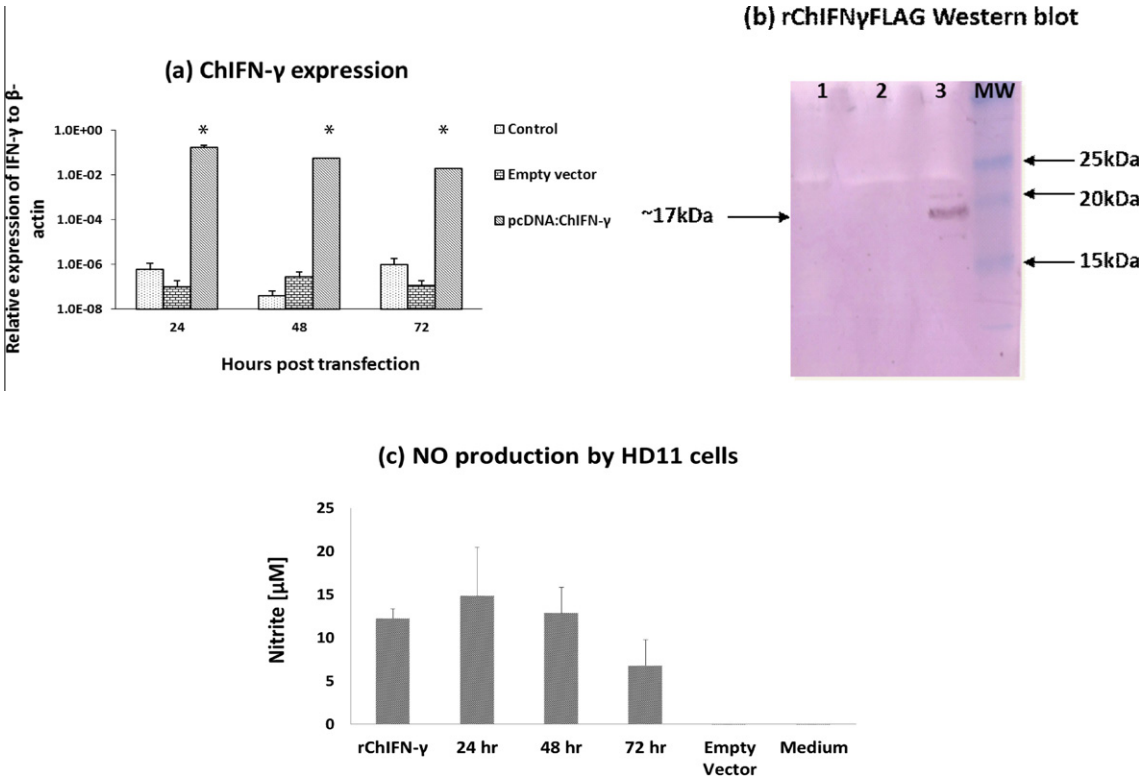

Fig. 1. Expression of recombinant chicken IFN-γ and assessment of its bioactivity (a) real-time RT-PCR analysis of chIFN-γ expression in negative control cells, mock-transfected cells (transfected with empty vector) or cells transfected with the recombinant plasmid (pcDNA:chIFN-γFLAG) at three different time points; 24, 48 and 72 h post-transfection. Data shown are representative of three replicates. *Significantly ($P \leq 0.05$) higher compared to the gene expression in control or empty vector transfected groups. (b) Western blot analysis of FLAG tagged rChIFN-γ using anti-FLAG M2 mAb. Culture supernatant from control DF-1 cells (Lane 1), transfected with pcDNA 3.1 empty vector (Lane 2) and pcDNA:chIFN-γFLAG was analyzed (Lane 3). M_W is the molecular weight marker (c) NO synthesis in HD11 macrophage cells treated with supernatants of DF-1 cells transfected with pcDNA:chIFN-γFLAG. Triplicate HD11 samples were treated with positive control (1.25 μg purified rChIFN-γ expressed using a baculovirus), culture supernatants from DF-1 transfected cells, culture supernatant of empty vector transfected DF-1 cells, or negative control (culture medium). Cell activation was measured by nitrite accumulation in culture supernatant and concentration (μM) was determined by comparisons against a standard curve. Error bars represent standard error of the mean.

cells with cell culture supernatants from transfected DF-1 cells ranged from 6.77-14.8 μM. The higher expression of rChIFN-γ mRNA seen in DF-1 transfected cells correlated with the presence of the rChIFN-γ protein in the culture supernatants from three time points (24, 48, and 72 h), as confirmed by the Griess assay. Cells transfected with an empty vector and the negative control neither had an increase in IFN-γ transcript expression nor did their supernatant stimulate the production of detectable levels of nitrite.

3.2. Effect of pcDNA:chIFN-γFLAG on enhancing HVT conferred immunity against MDV-RB1B challenge

Four days after HVT vaccination and administration of the plasmid encoding chIFN-γ, chicks were challenged with MDV-RB1B virus and monitored daily for development of clinical signs until 21 days post-infection. The incidence of grossly visible tumors and observable changes in organ size and condition were recorded in all the experimental groups during necropsy. Based on the presence or absence of tumors, chickens were classified as with lesions or without lesions, respectively. Table 1 summarizes the incidence of tumors among various groups in the pilot and main experiment. In the pilot study (Trial 1), the number of chickens treated with pcDNA:chIFN-γFLAG combined with HVT and developed tumors after MDV-RB1B challenge was lower compared to those that only received HVT. In the HVT+IFN-γ group, the incidence of MD tumors was 20% compared to 66.6% in the group that had only received HVT. In Trial 2, the unvaccinated RB1B-infected group, the tumor incidence was 100% whereas in the infected groups that received HVT only or HVT + pcDNA 3.1 (empty vector, EV), tumor incidence was 80% and 77.7%, respectively. However, in the group that received HVT + pcDNA:chIFN-γFLAG prior to MDV infection, the tumor incidence was reduced to 33.3%. None of the birds in the unvaccinated uninfected control group developed tumors or clinical signs.

Table 1
Tumor incidence affected by MDV-RB1B at 21 days post-infection. *n*, number of birds/group. EV, empty vector.

Groups	Trial 1		Trial 2	
	Tumor Incidence%	*n*	Tumor Incidence%	*n*
RB1B	100	6/6	100	10/10
RB1B + HVT	66.6	4/6	80	8/10
RB1B + HVT + pcDNA (EV)	–		77.7	7/9
RB1B + HVT + 1 μg pcDNA:chIFNγ	33.3	2/6	–	
RB1B + HVT + 5 μg pcDNA:chIFNγ	20	1/5	33.3	3/9

The mean ratio of bursa of Farbricius and spleen to body weight from both trials at 21 dpi is presented in Fig. 2. The spleens of the birds in the MDV-infected groups were either enlarged or had tumors and resulted in increased spleen to body weight ratios, whereas the bursa to body weight ratios were lower in the MDV-infected groups compared to controls due to bursal atrophy. The

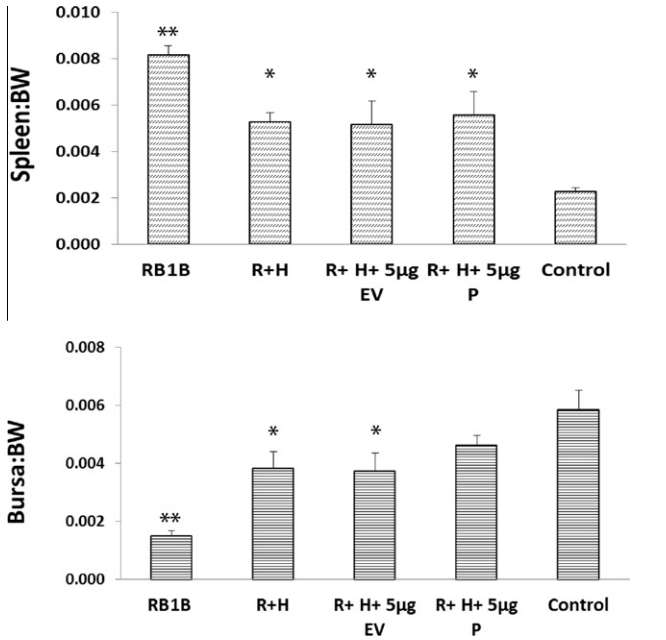

Fig. 2. Ratios of spleen or bursa of Fabricius to body weight (S:BW and B:BW, respectively), among the various treatment groups from both trials. Treatments groups include infected only (RB1B), vaccinated MDV-infected (R + H), vaccinated MDV-infected with pcDNA:chIFN-γFLAG (R + H + 5 µg P) and vaccinated MDV-infected with empty vector (R + H + 5µg EV). The error bars represent the standard error of the mean. *Significant difference in ratio when compared to uninfected controls ($P \leqslant 0.05$), **significant difference when comparing to uninfected control ($P \leqslant 0.001$).

spleen:body weight ratio was significantly higher in vaccinated birds compared to unvaccinated uninfected birds ($P \leqslant 0.05$). However, the ratio in unvaccinated MDV-infected group was significantly higher when compared to vaccinated MDV-infected and unvaccinated uninfected control ($P \leqslant 0.05$ and $P \leqslant 0.001$, respectively). The bursa:body weight ratio was significantly lower in the unvaccinated MDV-infected group when compared to all vaccinated treatments and unvaccinated uninfected control group ($P \leqslant 0.05$ and $P \leqslant 0.001$, respectively). The bursa:body weight ratio was also significantly lower in vaccinated MDV-infected and empty vector treated groups when compared to unvaccinated uninfected group ($P \leqslant 0.05$); however, the ratio in the rChIFN-γ administered group was not significantly lower when compared to uninfected controls ($P > 0.05$).

3.3. MDV genome load & MDV replication in spleens of chickens

Total cellular DNA was isolated from spleen tissue and MDV genome load was quantified in spleen samples and compared between birds with and without lesions within each treatment group. Initial screening of spleen DNA from the uninfected control group determined that all controls were MDV-free. DNA originating from infected birds was further analyzed by real-time PCR and the data are shown in Fig 3a. MDV genome was quantifiable in all groups, however significantly less genome load was observed in the groups without lesions ($P \leqslant 0.05$) when compared to chickens belonging to the unvaccinated MDV-infected group. The birds with lesions in the vaccinated groups had a higher MDV genome load when compared to groups without lesions ($P > 0.05$).

MDV replication was assessed by measuring the expression of MDV-*meq* and vIL-8 genes in the spleen tissue. Expression of MDV-*meq* and MDV-vIL-8 was quantified relative to β-actin expression, and is illustrated in Fig. 3b and c, respectively. Among all the birds without lesions in the vaccinated groups, *meq* transcripts were significantly lower compared to the unvaccinated MDV-infected group ($P \leqslant 0.001$). The birds with lesions in the vaccinated MDV-infected group also had significantly lower level of meq expression when compared to the unvaccinated MDV-infected group ($P \leqslant 0.05$). MDV-vIL-8 also had a trend similar to that of MDV-*meq*, being expressed at lower levels in the chickens with-

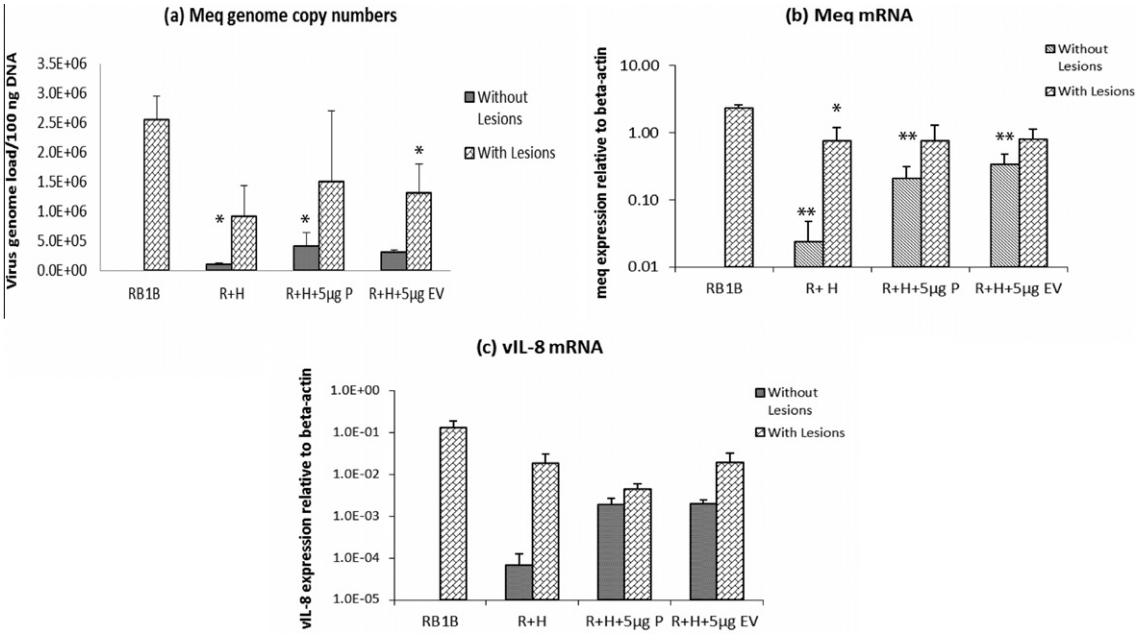

Fig. 3. MDV genome load and viral gene transcripts in the spleen of chickens from the second trial at 21 dpi. Mean MDV genome load (a), meq mRNA (b), and vIL-8 mRNA (c) expression relative to β-actin mRNA expression are presented and the error bars represent standard error of the mean. MDV genome copies were calculated based upon 100 ng of spleen tissue. *Significantly lower expression when compared to unvaccinated MDV infected group (RB1B) ($P \leqslant 0.05$). **Significantly lower expression when compared to unvaccinated MDV-infected group (RB1B) ($P \leqslant 0.001$).

out lesions compared to those with lesions, but unlike MDV-*meq*, the lower expression was not statistically significant.

3.4. Cytokine expression in spleen of chickens

The expression of IFN-γ, IL-10, IL-18 and IL-6 in spleens of all the treatment groups was analyzed and is illustrated in Fig. 4a–d, respectively. There was significant up-regulation in the expression of IFN-γ in all the chickens with lesions compared to the control group (unvaccinated uninfected) (Fig. 4a). There was also a significant increase in IFN-γ expression in spleens of birds without lesions in the pcDNA:chIFN-γFLAG administered chickens that were vaccinated and MDV-infected compared to uninfected control birds ($P \leqslant 0.05$). Between the infected groups, however, there was no statistically significant difference in the expression of IFN-γ. Unvaccinated MDV-infected chickens had significantly higher expression of IL-10 in comparison to all vaccinated and control groups ($P \leqslant 0.05$) (Fig. 4b). The birds with lesions from the empty vector treated group also had significantly higher expression of IL-10 when compared to the control group ($P \leqslant 0.05$). However, all the vaccinated groups, irrespective of presence or absence of tumors, had a lower expression of IL-10 compared to the unvaccinated challenged group. The expression of IL-18 mRNA was somewhat suppressed in the vaccinated groups that displayed no lesions compared to the groups with lesions with a significantly lower expression in the pcDNA:chIFN-γFLAG treated birds ($P \leqslant 0.05$). The birds without lesions in the pcDNA:chIFN-γ treated group also had significantly lower expression of IL-18 when compared to unvaccinated MDV infected group (Fig. 4c). IL-6 mRNA levels followed a somewhat similar pattern to that of IL-18, with low expression seen in the birds without lesions when compared to with lesions and an up-regulation in the infected only group (Fig. 4d), however, the differences were not statistically significant ($P > 0.05$).

4. Discussion

It is widely known that some host cytokines, such as IL-1, IL-2 and IFN-γ, act as an adjuvant and enhance immune responses elicited by vaccine formulations. One of the most widely used cytokines in adjuvant studies is IFN-γ, which activates antigen presenting cells (APC), up-regulates MHC class I and II expression on the surface of APC, promotes differentiation of naive T cells to become Th1 cells and, in general, is a main contributor to cell-mediated immune responses (Tovey and Lallemand, 2010). In the present study, the effects of recombinant chicken IFN-γ on enhancing immunity elicited by HVT vaccine against the very virulent MDV-RB1B strain were examined. While HVT is effective against virulent MDV strains, it is less protective against very virulent strains, such as RB1B. Therefore, it is important to develop novel vaccines that provide better protection against very virulent strains of MDV. The results of the present study indicate that co-administration of a plasmid encoding IFN-γ and HVT vaccine can provide enhanced protection as demonstrated by a reduction in the occurrence of tumor development following infection with vvMDV. In addition, we have demonstrated that the immunity conferred by the vaccine was associated with lower viral replication and significant reduction in MDV genome load.

IFN-γ is a pleiotropic cytokine that plays key roles in modulating immunity to infectious diseases. The mature chicken IFN-γ is a 16.8 kDa protein and has been well characterized and shown to be functionally similar to its mammalian counterpart (Digby and Lowenthal, 1995; Lowenthal et al., 1995). In the present study, when chickens were co-administrated with pcDNA:chIFN-γFLAG and HVT, a significant reduction in tumor incidence was observed, signifying the protective role of IFN-γ in immunity against MD. Given the experimental constraints, we could not extend the challenge trial beyond day 21 post-infection. Therefore, we cannot formally rule out the possibility that the birds with no lesions

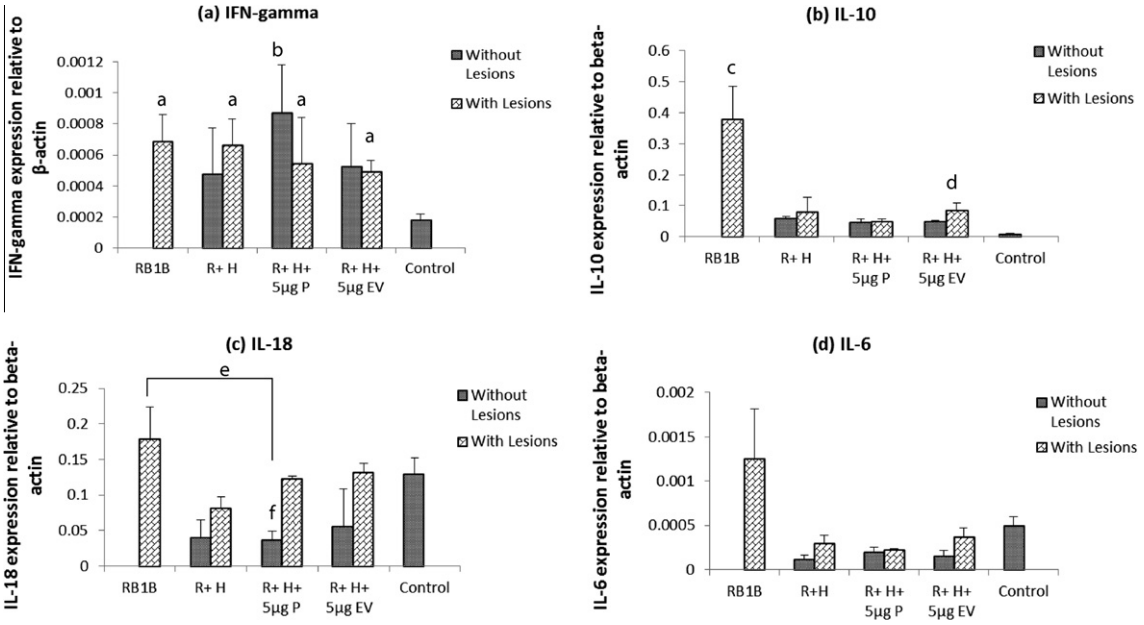

Fig. 4. Expression of various cytokine genes in the spleen tissue of chickens infected, vaccinated and administered recombinant plasmid from the second trial at 21 dpi: IFN-γ (a), IL-10 (b), IL-18 (c), and IL-6 (d). Target and reference gene expression was quantified by real-time RT-PCR and expression is presented relative to β-actin expression and normalized to a calibrator. Error bars represent the standard error of the mean. a = Significant up-regulation of IFN-γ mRNA in birds with lesions when compared to negative control birds ($P \leqslant 0.05$). b = Significant up-regulation of IFN-γ mRNA expression in birds without lesions from the vaccinated recombinant plasmid-treated group (R + H + P) compared to negative control birds ($P \leqslant 0.05$). c = Significant up-regulation of IL-10 mRNA when compared to unvaccinated uninfected birds ($P \leqslant 0.05$). d = Significant up-regulation in birds with lesions in the empty vector treated group (R + H + EV) when compared to unvaccinated uninfected group ($P \leqslant 0.05$). e = Significant down-regulation of IL-18 seen in the plasmid-treated group without lesions when compared to unvaccinated MDV-infected group. f = Significant down-regulation when comparing birds with lesions and without lesions within the plasmid-treated group (R + H + P).

would have remained tumor-free, had we kept them longer. Therefore, the combination of IFN-γ and HVT may have only delayed the onset of tumor formation. However, based on the data presented here and considering the highly virulent nature of the virus used in this study, it is plausible to suggest that addition of IFN-γ to the vaccine had significantly reduced morbidity and, perhaps, severity of disease.

There are at least two possible mechanisms that may be involved in immunity conferred by HVT + IFN-γ: (1) IFN-γ stimulates immune system cells and expands antivirus adaptive responses elicited by the vaccine formulation. For example, it has been demonstrated that administration of IFN-γ in the form of plasmid DNA enhances immunity against viruses and tumors by activation cytotoxic T cells and steering the response towards a T helper (Th)1 like response (Iida et al., 2004; Ohlschlager et al., 2009). IFN-γ can also up-regulate expression of MHC on the surface of tumor cells, hence rendering them more susceptible to recognition by $CD4^+$ or $CD8^+$ T cells (Kennedy and Celis, 2008). (2) IFN-γ can directly or indirectly inhibit MDV replication. In support of this scenario, IFN-γ has been shown to activate macrophages to produce NO (Lillehoj and Li, 2004), which in turn inhibits replication of various viruses including MDV (Djeraba et al., 2000; Xing and Schat, 2000a; Lee, 1979), NDV (Yeh et al., 1999), hepatitis B virus (HBV) (Suri et al., 2001) and DHBV (Long et al., 2005; Schultz and Chisari, 1999). Importantly, we have shown here that the recombinant chicken IFN-γ expressed by plasmid DNA stimulates NO production from chicken macrophages. If indeed the plasmid-derived IFN-γ had exerted anti-viral activities in our study, this must have occurred in early stages of virus replication because by day 21, there was no significant association between IFN-γ expression and genome load. In addition to antiviral activities, NO also has anti-tumor activities (Bogdan, 2001; Blanchette et al., 2003), which in the present study might have reduced the incidence of tumors or delayed the onset of tumor formation in MDV-infected birds. The inhibitory effects of IFN-γ on viral replication and maintenance of latency (Steed et al., 2006) have been confirmed by the results demonstrating that mice deficient in IFN-γ or its receptor have an elevated number of cells harboring viruses reactivating from latency producing infectious particles and large vessel vasculitis (Weck et al., 1997). Other studies have provided evidence of CD8+ T cells employing IFN-γ in maintenance of HSV-1 latency (Decman et al., 2005), suggesting that the two mechanisms described above can be intertwined. This has led us to conclude that the administration of rChIFN-γ encoding plasmid in our study enhances antiviral immunity induced by HVT. The exact underlying mechanisms by which rChIFN-γ exerts its function in the context of the present study require further examination and these experiments are underway.

In our study, the vaccine-conferred immunity was associated with significantly lower MDV genome copy numbers in the spleen which also correlated with lower expression of MDV-*meq* and *vIL-8* genes. The above viral genes are associated with the different phases of MDV replication cycle. MDV-vIL-8 is a chemokine-like molecule that is associated with the cytolytic phase of the virus and has been shown to play a role in attracting T cells to the site of infection, in which the MDV-infected B cells are undergoing cytolysis (Cui et al., 2004; Parcells et al., 2001; Liu et al., 1999), whereas MDV-*meq* is associated with transformation and may also play a role in latency and anti-apoptotic properties of the virus (Jones et al., 1992; Liu et al., 1997). MDV reactivates from latency and causes a late cytolytic phase around 14 dpi (Calnek, 1986), which may result in replication of the virus, hence higher expression of *meq* and vIL-8, in birds with lesions, at these time points is expected. Other studies have also shown similar significant up-regulation in spleen, lung and bursa of Fabricius of birds infected with MDV-RB1B (Abdul-Careem et al., 2009).

The expression of chIFN-γ was significantly elevated in spleens of chickens with lesions. However, an elevated expression of IFN-γ was also observed in the vaccinated chickens that had no lesions, which had also received pcDNA:chIFN-γFLAG. We could not determine whether this elevated level of IFN-γ was plasmid-derived or was produced as a native molecule by host cells or was a combination of both. It is possible that the administration of the chIFN-γ encoding plasmid may have contributed to an increase in endogenous expression of IFN-γ, in a positive feedback manner. Furthermore, we were able to detect the presence of pcDNA:chIFN-γFLAG plasmid DNA up to 7 days after intra-muscular administration in skin, muscle, bursa of Fabricius and spleen of treated chickens (data not shown). By day 21, we were still able to detect the presence of plasmid DNA in muscle and, to a lesser extent, spleen of treated birds (data not shown). Overall, it is plausible that an early increase in IFN-γ in spleens of the plasmid-administered birds may be an explanation for the low tumor incidence in this group, since IFN-γ may drive the virus into an early latency, similar to the scenario seen in resistant versus susceptible birds (Kaiser et al., 2003).

IL-10 plays a significant role in regulating the host immune response and is known to counteract the effects of IFN-γ (Endharti et al., 2005). In the present study, IL-10 expression was significantly induced in the unvaccinated RB1B infected group, in agreement with other studies (Abdul-Careem et al., 2007; Parvizi et al., 2009b; Parvizi et al., 2009a). The expression of IL-10 was also up-regulated to a lesser extent in some of the vaccinated birds that had lesions. This up-regulation in cytokine expression may be indicative of a strategy used by MDV to subvert host immune responses (Buza and Burgess, 2007), similar to what has been observed in Epstein Barr virus (EBV) infection (Marshall et al., 2003). In general, the results from IL-10 expression suggest that IL-10 expression is significantly reduced in the vaccinated chickens with and without lesions, suggesting that lower expression of IL-10 is not correlated with protection against MD.

Here, we also evaluated the expression of IL-18 and IL-6, proinflammatory cytokines shown to have an important role in the modulation of immune responses. Importantly, the lower expression of these cytokines is associated with genetic resistance against MD and vaccine-induced protection (Kaiser et al., 2003; Abdul-Careem et al., 2007). In agreement with these studies, we also observed lower expression of IL-6 and IL-18 in chickens vaccinated that had no lesions compared to unvaccinated MDV-infected chickens, confirming an inverse correlation between the expression of these cytokines and vaccine-conferred protection.

In conclusion, the findings in this study support the notion that co-administration of pcDNA:rChIFN-γ and HVT vaccine reduces tumor incidence after challenge with a vvMDV. In addition, the results demonstrate that virus replication and viral load are significantly reduced in the chickens that display no gross lesions. In the absence of any significant side effects, such as weight loss or toxicity, the use of IFN-γ as an adjuvant could be considered to enhance the protective efficacy of vaccines against Marek's disease. However, prior to embarking upon any commercial vaccine formulations containing IFN-γ, a cost-benefit analysis would be required. We anticipate that expression of IFN-γ by a recombinant HVT may provide a cost-effective method for delivery of this cytokine.

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