Veterinary Microbiology 235 (2019) 248–256

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Influence of Marek's disease virus vaccines on chicken melanoma differentiation-associated gene 5-dependent-type I interferon signal transduction pathway with a highlight on their secondary impact on the immune responses post Newcastle disease virus vaccination

Ola Hassanin[a,*], Fatma Abdallah[b], Mahmoud H.A. Mohamed[a,c], Doaa M. Abdel Fattah[d]

[a] Department of Avian and Rabbit Medicine, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, Egypt
[b] Department of Virology, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, Egypt
[c] Department of Clinical Studies, Collage of Veterinary, King Faisal University, Saudi Arabia
[d] Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, Egypt

ARTICLE INFO

Keywords:
Marek's disease virus
Melanoma differentiation-associated gene 5 "MDA5" dependent
Newcastle disease virus
Chicken interferon-alpha
Vaccine and chicken

ABSTRACT

Marek's disease virus (MDV) leads to a lytic infection of B-lymphocytes in chickens, and also latently infects T-lymphocytes. Although Marek's disease vaccines have been widely in use, little is known about the innate immune response of this important livestock vaccine. In this study, we tested the effect of different commercially applied Marek's disease vaccines on the expression pattern of selected genes related to chicken interferon-alpha (chIFN-α) (melanoma differentiation associated gene 5 "MDA5" dependent) signal transduction pathway. Both MDV serotype I (Rispens) and serotype III (Herpesvirus of turkey "HVT") vaccines could stimulate MDA5 dependent-type I interferon response as early as three days post vaccination in a dose-dependent manner. The stimulation continued up to 10 days in the instance of HVT vaccine and declined in the case of Rispens. Surprisingly, increasing the doses of the two vaccines led to dose-dependent down-regulation in the expression pattern of the investigated pathway, five and ten days post vaccination. Additionally, to shed the light on the consequent effect on the immune responses of the other viral vaccine, another experimental model based on Newcastle disease virus (NDV) vaccines was designed using HVT, HVT-VP2 and Rispens MDV vaccines. The three MDV vaccines were found to reduce chicken humoral immune response post NDV vaccination. However, only Rispens and HVT-VP2 had suppressive effects on the expression of MDA5-dependent-chIFN-α related cytokines. Consistent with this finding, the protection rate and NDV- humoral immune response post challenge with virulent NDV strain was lower in case of Rispens and HVT-VP2 vaccines.

1. Introduction

Despite the presence of traditional vaccination programs, Marek's disease virus (MDV) remains a main concern for the poultry industry due to the emergence of further virulent MDV strains. In addition to its importance in livestock, MDV is considered an excellent model for studying herpesvirus-induced oncogenicity and immunosuppression. The cellular and innate immune responses are two distinguishable but interrelated mechanisms to confront MDV invasion. Specifically, the innate immunity plays a critical role in priming and guiding the consequent adaptive immune responses, including humoral and cellular immune responses to be customized more specifically against MDV (Parvizi et al., 2010). On the other hand, the virus life cycle in birds is associated with early transient immunosuppression phase during the early cytolytic infection (2–7) days post infection (dpi) and late permanent immunosuppressive phase during virus reactivation in CD4 + T cells 18 dpi (Boodhoo et al., 2016). Recently, it has been demonstrated that oncogenic MDV strains can modulate the function of immune cells via the COX-2/PGE2 pathway which can be involved in the immunosuppression process (Gurung et al., 2017). However, until now, little has been known about the exact role of MDV in modulating the chicken innate immune response, and particularly the interferon response. Different types of MDV vaccines, such as serotype I Rispens and serotype III HVT vaccines, are widely used to prevent the appearance of the later MDV symptoms. These vaccines considered as good candidates to understand the molecular mechanisms of MDV pathobiology and

* Corresponding author.
E-mail address: olahassanin@zu.edu.eg (O. Hassanin).

https://doi.org/10.1016/j.vetmic.2019.07.008
Received 7 May 2019; Received in revised form 4 July 2019; Accepted 7 July 2019

molecular immunology.

Interferons (IFNS) are mediators of the innate immune defense which are produced at an early stage of virus infection in order to rapidly provide the host with an antiviral state. The interplay between avian IFNS and viruses in the term of interferons induction and their signaling effectors has been reviewed in details (Santhakumar et al., 2017b). Upon secretion of IFN-α/β, activation of a signal transduction pathway that triggers the transcription of a diverse set of genes that inaugurate an antiviral response in target cells. These genes are termed as IFN-inducible genes or IFN-stimulated genes (ISGs) (Randall and Goodbourn, 2008). Interferon-stimulated genes act on multilayered manner via a direct effect on the viral replication cycle and re-recruiting IFN induction through an amplification loop. One example, chicken Melanoma differentiation-associated gene 5 (MDA5) (Karpala et al., 2011; Lee et al., 2012) that has an essential role in the IFNs production upon cell stimulation with dsRNA. Previous studies demonstrated that chicken MDA5 implicated in the activation of IFN-β and mitochondrial antiviral signaling genes induced by Infectious bronchitis virus (IBV), Avian influenza virus (AIV) and Infectious bursal disease virus (IBDV) (Karpala et al., 2011; Lee et al., 2014; Yu et al., 2017). Another example, the Myxovirus-resistance (Mx) proteins are interferon-induced antiviral gene. When it was initially recognized in avian species, it is thought that avian Mx proteins lack of antiviral activity (Bazzigher et al., 1993; Bernasconi et al., 1995). Succeeding studies reported the polymorphism of the Mx gene from different breeds (Ko et al., 2002) and the antiviral specificity of the chicken Mx protein linked to a specific amino acid mutation at position 631 (Ser to Asn) (Fulton et al., 2014; Ko et al., 2002, 2004b). Furthermore, In vivo studies elucidated the significant up-regulation of the Mx1 in the chicken spleen cells as early as 48 h post AIV vaccination, indicating it is one of the innate immune response components against AIV vaccine (Abdallah and Hassanin, 2015; Hamad et al., 2018). The avian double-stranded RNA-dependent protein kinase (PKR) has had been cloned and sequenced from chicken cDNA of various chicken breeds (Ko et al., 2004a). However, following highly pathogenic Avian influenza virus (HPAIV) infection, up-regulation of PKR was not sufficient to counteract the HPAIV (H5N1) and protect the chicken from mortality (Daviet et al., 2009). In contrast, overexpression of goose PKR protein significantly controlled the replication of Newcastle disease virus (NDV) in goose embryo fibroblasts (Liu et al., 2018).

In a previous study, stimulation raised two typical ISGs, Mx and 2′-5′-oligoadenylate synthetase-like protein (OASL), in MDV infected chicken embryo fibroblasts CEFs. In the same study, no significant change in IFN-β expression was detected during MDV infection suggesting that MDV inhibits IFN-β production (Zou et al., 2017). Recent studies have suggested that MDV infection results in the down-regulation of IFN-α level in infected birds. Moreover, MDV is able to block the interferon response of the NDV vaccine, a potent IFN-α inducer (Quere et al., 2005). This effect on IFN-α is not correlated with the virulence of the MDV strain since the down-regulation of IFN-α mRNA was detected in genetically resistant chickens from 1 to 7 dpi with either RB-1B MDV or HVT (Quere et al., 2005). Down-regulation or even absence of IFN-α transcripts was mainly detected in the spleens of MDV infected birds, particularly in the latent stage (Heidari et al., 2008; Xing and Schat, 2000).

Here we report on the ability of different MDV vaccine strains that are routinely used in the field to counteract the MDA5-dependent-interferon signaling machinery and its antiviral regulators. Additionally, it was essential to investigate whether these vaccine strains, similar to the virulent strains, have a late immune suppression profile with or not secondary impact on NDV vaccine immune responses.

2. Material and methods

2.1. Birds

One hundred and five, one-day old SASSO baby chicks (for study I) and Seventy five, one-day old SASSO baby chicks (for study II) were housed separately in animal house research facility at the Faculty of Veterinary Medicine, Zagazig University. All birds had ad libitum access to feed and water throughout the experiment. The birds were subjected to regular clinical examination and sampling procedures in search of alterations that could indicate the presence of any infections. All the procedures were performed in strict accordance with the recommendations in the guidelines of the Institutional Animal Care and Use Committee (ZU-IACUC) under approval number ZU-IACUC/2/F/79/2019, Zagazig University.

2.2. Vaccines

1 VAXXITEK HVT + IBD (MERIAL, inc), Bursal disease-Marek's disease vaccine serotype III, live vaccine based on the use of a recombinant turkey Herpesvirus (HVT) expressing the VP2 gene of IBDV The vaccine was titrated in 10x replicates according to (Thornton, 1985) and the dose was adjusted to contain 1000 PFU/0.2 ml administrated subcutaneously (Batch No. 1022-4725-00) serial RI906.
2 BIO-MAREK HVT (FATRO), live freeze-dried vaccine, serotype III. The vaccine was titrated in 10x replicates according to (Thornton, 1985) and diluted to specific diluent to contain 1000 PFU/0.2 ml administrated subcutaneously (Batch No. 701033).
3 MD RISPENS (CEVAC), LIVE MDV vaccine serotype I. The vaccine was titrated in 10x replicates according to (Thornton, 1985) and the dose was adjusted to contain 1000 PFU/0.2 ml administrated subcutaneously (Serial No. 344-062).
4 Volvac® ND LaSota (Boehringer Ingelheim), live freeze-dried vaccine, contains at least $10^{6.5}$ ELD_{50} of live NDV (LaSota strain). The vaccine was administrated via occulonasal route.
5 Volvac® ND KV (Boehringer Ingelheim), oil emulsion killed vaccine, contains LaSota NDV. The vaccine was injected at a dose of 0.5 ml per bird, subcutaneously in the middle third part of the back of the neck.

2.3. Virus

Virulent Newcastle disease virus sub-genotype VIId strain (NDV/chicken/Egypt/1/2015) that published in GenBank under the accession numbers KX231852 was used a challenge virus. It was chosen because it is antigenically and phylogenetically representative for NDV circulating strains in the Middle East. The virus was propagated and titrated in 9^{th} days old embryonated chicken eggs (ECEs) and adjusted to contain $10^{6.3}$ embryo infective dose 50 (EID_{50})/ 0.1 ml approximately.

2.4. Serology

Hemagglutination inhibition (HI) assay was done using 8 HAU of LaSota NDV vaccine strain and 1% chicken red blood cells. Serum was initially diluted into 1/5 and two fold dilution series of each tested serum sample was carried out according to (OIE, 2012). Titres were expressed as $\log_2$ geometric mean titres (GMT).

2.5. Quantitative real-time PCR for the relative expression of the innate immune-related antiviral cytokines

RNA isolation was performed from 30 mg of spleen tissue using the RNeasy® Mini Kit (Qiagen) according to the manufacturer's instructions. The purified RNA from each sample was reversed transcribed to produce cDNA using the M-MLV Reverse Transcriptase (enzynomics) and

Table 1
Gene expression fold change in chicken spleens after MDV vaccination at different time points.

Vaccine/dose	Gene	[a]D3 Fold Change	[b]D5 Fold Change	[c]D10 Fold Change	Vaccine/dose	Gene	[a]D3 Fold Change	[b]D5 Fold Change	[c]D10 Fold Change
HVT/1000PFU	**IRF7**	5.40	71.55	38.57	**Rispens/1000PFU**	**IRF7**	24.40	180.01	28.34
	Mx1	2.76	38.76	43.36		**Mx1**	3.60	64.43	23.74
	PKR	1.42	13.89	89.33		**PKR**	6.60	19.97	85.93
	MDA5	2.76	23.06	26.45		**MDA5**	3.61	61.20	9.48
	chIFN-α	2.02	20.98	46.45		**chIFN-α**	1.11	111.41	34.51

[a] D3; Day three post vaccination.
[b] D5; Day five post vaccination.
[c] D10; Day ten post vaccination.

oligo dT primer. The cycling parameters consisted of a reverse transcription step at 42 °C for 60 min and an inactivation step at 95 °C for 5 min. Quantitative PCR for MDA5, interferon regulatory factor 7 (IRF7), Mx1, PKR, IFN-α and GAPDH was carried out for the created cDNA using previously designed primers (Hassanin et al., 2014; Santhakumar et al., 2017a) purchased from Eurofins, Genomics. The quantitative Real-time PCR was performed using a Bio-Rad real-time thermal cycler CFX96™ and TOPreal™ qPCR 2X preMIX (SYBR Green with low ROX), Enzynomics according to the manufacturer's instructions. The thermo profile was; 95 °C for 10 min hold, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The relative gene expression levels of the innate immune response related genes were quantified using GAPDH as the endogenous control and were normalized to the non-MDV vaccinated control samples using ΔΔCt method. Melting curve was carried out to detect whether there was any non-specific amplification at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s. A standard curve assay was performed for each primer pair in order to verify the high linearity of the reaction ($R^2 \geq 0.99$).

2.6. Animal experiment

Study I: One hundred and five, one-day old SASSO baby chicks were divided into 7 treatment groups of fifteen birds each. Six groups were vaccinated subcutaneously with different doses of MDV vaccines; HVT1000 PFU, HVT2000 PFU, HVT3000 PFU, Rispens1000 PFU, Rispens2000 PFU, Rispens3000 PFU. One group was sham-vaccinated and used as control (n = 15). To assess the effect of MDV non-oncogenic strains on MDA5-dependent-IFN-α signaling transduction pathway, five Chicks from each group were humanely euthanized at 3, 5 and 10 days post-vaccination (PV). The spleen tissues, which were collected and immediately processed for RNA extraction and cytokine measurements.

Study II: to assess the impact of one day old MDV vaccination on NDV immunization, seventy five, one-day old SASSO baby chicks were divided into five treatment groups (n = 15). Birds from three groups were received 1000PFU/0.2 ml subcutaneous dose of either HVT, HVT-VP2 or Rispens vaccine at day one. Seven days later, birds from the three MDV vaccinated groups received 0.5 ml of inactivated NDV vaccine subcutaneously and boosted after 7 days with $10^{6.5}$ EID_{50} of LaSota vaccine via eye drop. One control group (n = 15) received only NDV vaccination at 7 and 14 days old and other control group (n = 15) was left without neither MDV nor NDV vaccination. Twenty-one days after NDV first immunization, all birds were challenged by the intramuscular route (IM) with $10^{6.3}$ EID_{50} of virulent NDV (NDV/chicken/Egypt/1/2015) in a volume of 0.1 ml. The inoculum titer was verified later by back titration of the inoculated virus. All the birds were monitored daily for clinical signs and mortality. Serum samples were collected weekly at 14, 21, 28 and 35 days of age for monitoring of NDV HI antibody titers. Spleen samples were taken from five birds after being humanely euthanized immediately before challenge for the purpose of gene expression assay.

2.7. Statistical analysis

The logarithm2 (log_2) mean titer of the NDV HI test was statistically analysed and graphed with One-way ANOVA with Tukey's at $P < 0.05$ using GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The results were expressed as scatter plots ± S.E. (standard error). The gene expression levels were compared between groups via One-way ANOVA with Tukey's (for study I) and Welch's *t*-test (for study II).

3. Results

3.1. Marek's disease virus vaccines interact with MDA5-dependent-chIFNα signal transduction pathway in chicken spleens

We first analyzed the expression of different antiviral cytokines involved in chIFNα responses at three, five and ten days post application of different doses from MDV vaccines, using real-time qRT-PCR.

As shown in Table 1, vaccination with serotype III (HVT) vaccine at 1000 PFU led to rapid increasing of IRF7, Mx1, PKR, MDA5, and chIFNα mRNA(s) in the chicken spleens as early as three days and lasting up to ten days PV ($P < 0.05$). In the instance of serotype I (Rispens) vaccine, after peaking at 5 days PV, IRF7, Mx1, MDA5 and chIFNα transcripts showed fold change significant decline at ($P < 0.05$) 10 days PV (Table 1).

A dose-dependent up-regulation in the expression of IRF7, Mx1, PKR, MDA5, and chIFNα mRNA(s) in the chicken spleens as early as three days PV (Fig. 1a–e) when the chickens were vaccinated with different doses (1000, 2000 & 3000 PFU) of either HVT or Rispens strains at one day of age. Rispens strain induced more evident and statistically significant ($P < 0.05$) increases in the transcriptional levels of IRF7, Mx1 and MDA5 mRNA, particularly when increasing the doses up to 2000 and 3000 PFU, compared with HVT strain. Both strains induced comparable levels of chIFNα mRNA with different applied doses.

At five and ten days PV, increase the vaccine dose to 2000 and 3000 PFU led to a dose-dependent down-regulations in all the measured antiviral cytokines compared to 1000 PFU of both vaccines. The most evident suppression was in the case of vaccination with Rispens 3000 PFU at both five and ten days PV. The statistical comparison with 1000 PFU dosage of HVT revealed that vaccination with either 2000 or 3000 PFU dosage led to statistically significant down-regulation of IRF7, Mx1 and PKR mRNA levels at both five and 10 ten days PV (Figs. 2 and 3a–c). The down-regulations of MDA5 and IFN-α mRNA levels were detected only at ten days PV (Fig. 3d & e). Furthermore, vaccination with either 2000 or 3000 PFU dosage of Rispens strain led to statistically significant downregulation of all the antiviral cytokines compared with 1000 PFU dosage at five days PV (Fig. 2a–e). Similar results were obtained at ten days PV at both doses of Rispens strain with the exception of MDA5 and Mx1 mRNA levels at 2000 PFU dosage (Fig. 3a–e).

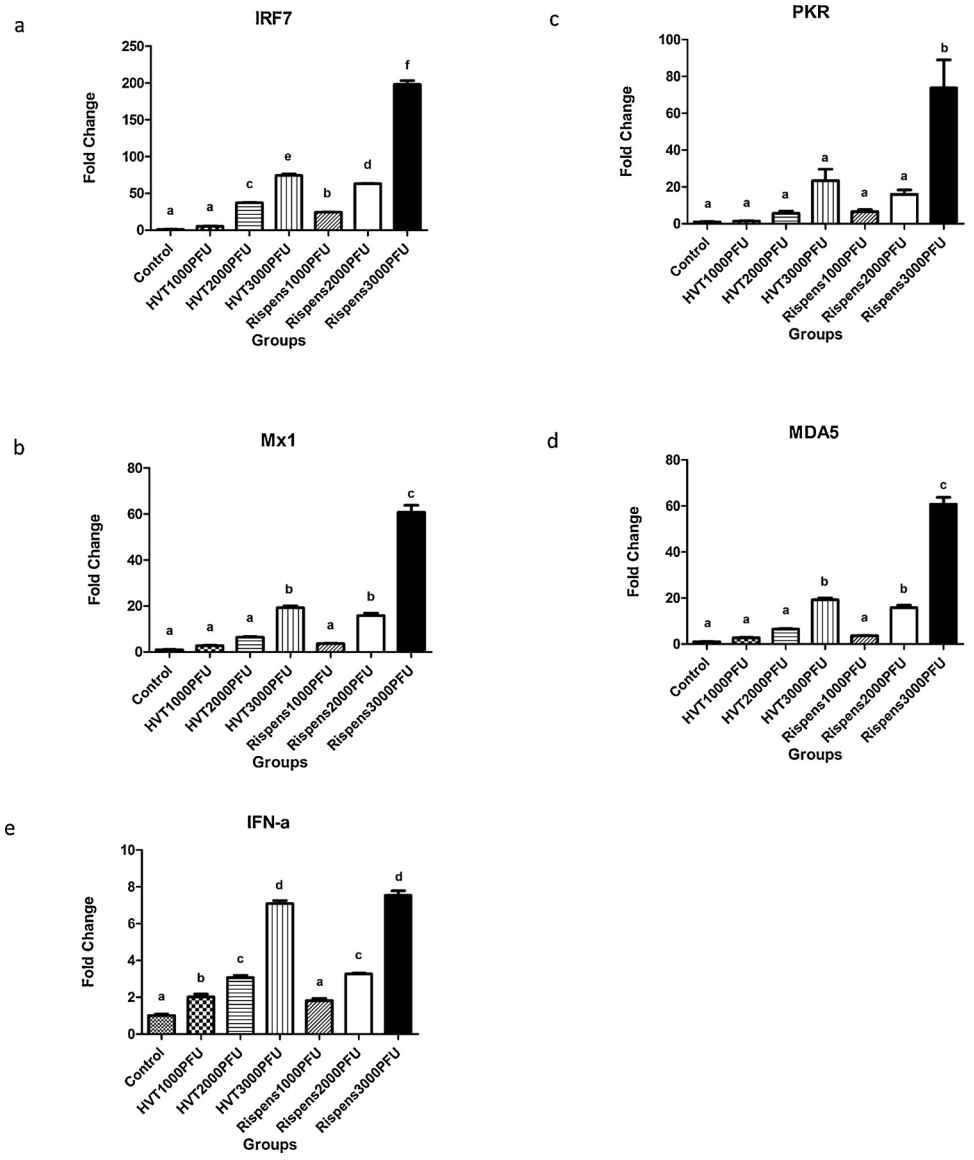

Fig. 1. MDV vaccines dose-dependent up-regulation of MDA5-dependent-chIFNα signal transduction pathway in bird spleens during early cytolytic stage. Birds were vaccinated with either HVT or Rispens strain at 1000, 2000 and 3000 PFU. Birds were humanely euthanized at 3rd days post-vaccination and their spleen tissues were collected. RNA was extracted and an IRF7 (a), Mx1 (b), PKR (c), MDA5 (d) or chIFN-α (e) specific quantitative real-time PCR was performed. All data were normalized to GAPDH as an internal control and all the data were expressed as mean of fold difference (Bar) relative to non-MDV vaccinated group, which is given an arbitrary value of 1. Error bars represent the standard errors of means. The statistical analysis was done by One-way ANOVA with Tukey's at $P < 0.05$ using GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Different letters refer to statistical significant differences.

3.2. Different commercially applied MDV vaccines interfere with NDV humoral immune responses

In order to screen whether early MDV vaccination has a late impact on NDV humoral immune response, day-old birds were immunized with either HVT, HVT-VP2 or Rispens MDV vaccine followed by vaccination with inactivated and live NDV LaSota vaccines at 7 and 14 days, respectively.

In general, a statistical significant down-regulation ($P < 0.05$) of NDV-HI Ab titers were detected in all MDV vaccinated groups from 2 to 4 weeks of age compared to non-MDV vaccinated group. As shown in Fig. 4a, the HVT-VP2 vaccinated group had the lowest elevation in the NDV-HI Ab titer at 14 days old with a geometric mean 1.4 $\log_2$. Both HVT and Rispens strains vaccinated groups had geometric mean 2.2 and 2.7 $\log_2$, respectively which were also lower than non-MDV vaccinated group (3.6 $\log_2$). The three MDV (HVT, HVT-VP2&Rispens) vaccinated groups, had geometric mean titers of 3.6, 3.3 & 3.3 $\log_2$ at 21 days old and 4.7, 4.4 & 4.4 $\log_2$ at 28 days old (Fig. 4b & c), respectively which were statistically significant ($P < 0.05$) lower than non-MDV vaccinated group (4.9 $\log_2$ at 21 days old and 5.9 $\log_2$ at 28 days old).

3.3. Different MDV vaccine strains interact differentially with NDV induced innate immune responses in chicken spleens

Twenty one days post NDV first vaccination, birds vaccinated with day old HVT vaccine showed no down regulatory effect on the expression of different chIFNα regulated genes in their spleens compared with the non-MDV vaccinated (Fig. 5). However, the insertion of IBD-VP2 gene led to statistical significant decrease in the IRF7 (90%), Mx1 (50%), MDA5 (90%), PKR (50%) and chIFNα (94%) mRNA levels compared with the non-MDV vaccinated birds. Similar to HVT-VP2, Rispens vaccination also led to down-regulation in the IRF7 (50%), Mx1 (60%), MDA5 (70%), PKR (50%) and chIFNα (84%) mRNA levels compared with the non-MDV vaccinated group (Fig. 5).

3.4. Rispens and HVT-VP2 strains have negative impact on the bird protection and humoral immune responses against virulent NDV challenge

As shown in Fig. 6, the sham non-vaccinated group challenged with a virulent genotype VIId NDV strain (NDV/chicken/Egypt/1/2015) had 80% mortality between days 4th to 7th post challenge (PC). Groups of birds receiving either NDV vaccines only or plus HVT vaccine demonstrated two cases of mortality on days 4th and 5th PC, with 80% of birds surviving challenge. Birds immunized with Rispens and two NDV

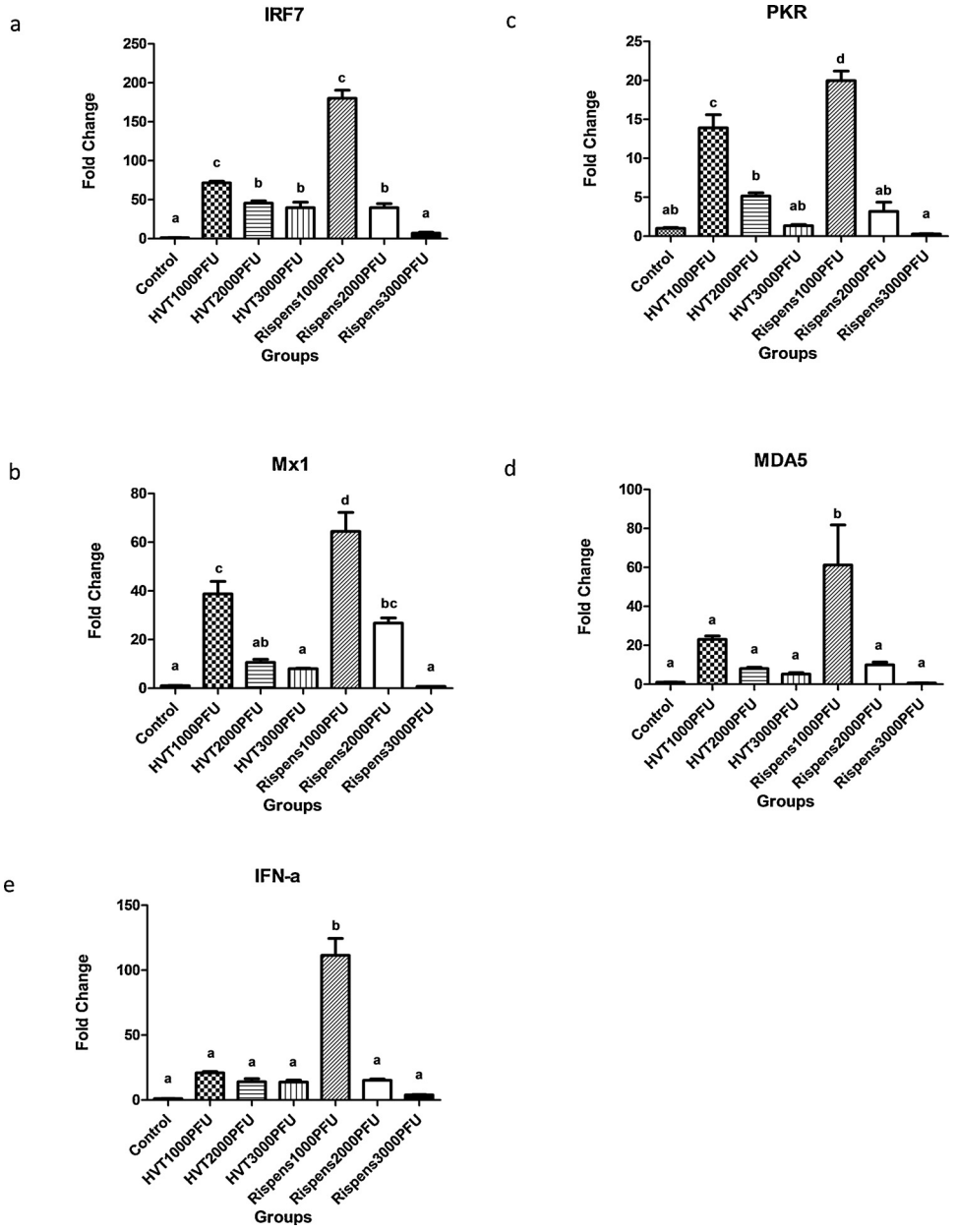

Fig. 2. MDV vaccines counteract MDA5-dependent-chIFNα signal transduction pathway in bird spleens in a dose-dependent manner during late cytolytic stage. Birds were vaccinated with either HVT or Rispens strain at 1000, 2000 and 3000 PFU. Birds were humanely euthanized at 5th days post-vaccination and their spleen tissues were collected. RNA was extracted and an IRF7 (a), Mx1 (b), PKR (c), MDA5 (d) or chIFN-α (e) specific quantitative real-time PCR was performed. All data were normalized to GAPDH as an internal control and all the data were expressed as mean of fold difference (Bar) relative to non-MDV vaccinated group, which is given an arbitrary value of 1. Error bars represent the standard errors of means. The statistical analysis was done by One-way ANOVA with Tukey's at $P < 0.05$ using GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Different letters refer to statistical significant differences.

vaccines demonstrated 70% protection with mortality observed between days 4th and 6th PC. The highest mortality 60% was observed in birds immunized with HVT-VP2 and two NDV vaccines between days 4th to 7th PC.

Consistent with protection results, the NDV-HI Ab titer 7 days post-challenge were the lowest and most statistically significant ($P < 0.05$) with a mean titer of 6.5 $\log_2$ in the case of HVT-VP2 vaccination followed by Rispens vaccination with a titer of mean titer of 7.7 $\log_2$. No statistical significant difference was existed between non-MDV vaccinated group and HVT vaccinated group with mean titers of 8.3 and 8 log2, respectively (Fig. 4d).

4. Discussion

Herpesviruses develop several mechanisms to weaken the innate immunity (Su et al., 2016). Productive replication of herpesviruses leads to the accumulation of higher order RNA structures. These RNAs are sensed in the cytoplasm by MDA5, but not retinoic-acid inducible gene I (RIG-I) (Melchjorsen et al., 2010; Paludan et al., 2011) which does not exist in Gallus Gallus species. Several herpesviruses encode proteins that counteract with MDA5 in order to decrease type I interferon transduction and its associated antiviral cytokines in the host cells. Subsequently, decreased the production of several downstream molecules including transcriptional regulators and antiviral cytokines.

Marek's disease virus is a member of *herpesviridae* family, in the subfamily *alphaherpesvirinae* and it is the first tumor prevented by vaccination. Infection with oncogenic MDV causes down-regulation of innate immune defense at both in vitro and in vivo levels (Hunt et al., 2001). Insights into the roles of MDV vaccines along with their variable immune pathways in the chicken immune system will give rise to safer and more effective vaccines. Therefore, the study scope was to analyze whether the early phase of MDV-vaccination incorporated with mechanisms of either stimulation or knocking-down IFN-α signaling response that involved in limitation of herpesvirus replication (Zoller et al., 1992). Furthermore, it was of interesting to investigate whither the different applied MDV vaccines have negative impact on other viral vaccines or not.

Genome of Rispens strains was observed in chicken spleens as early

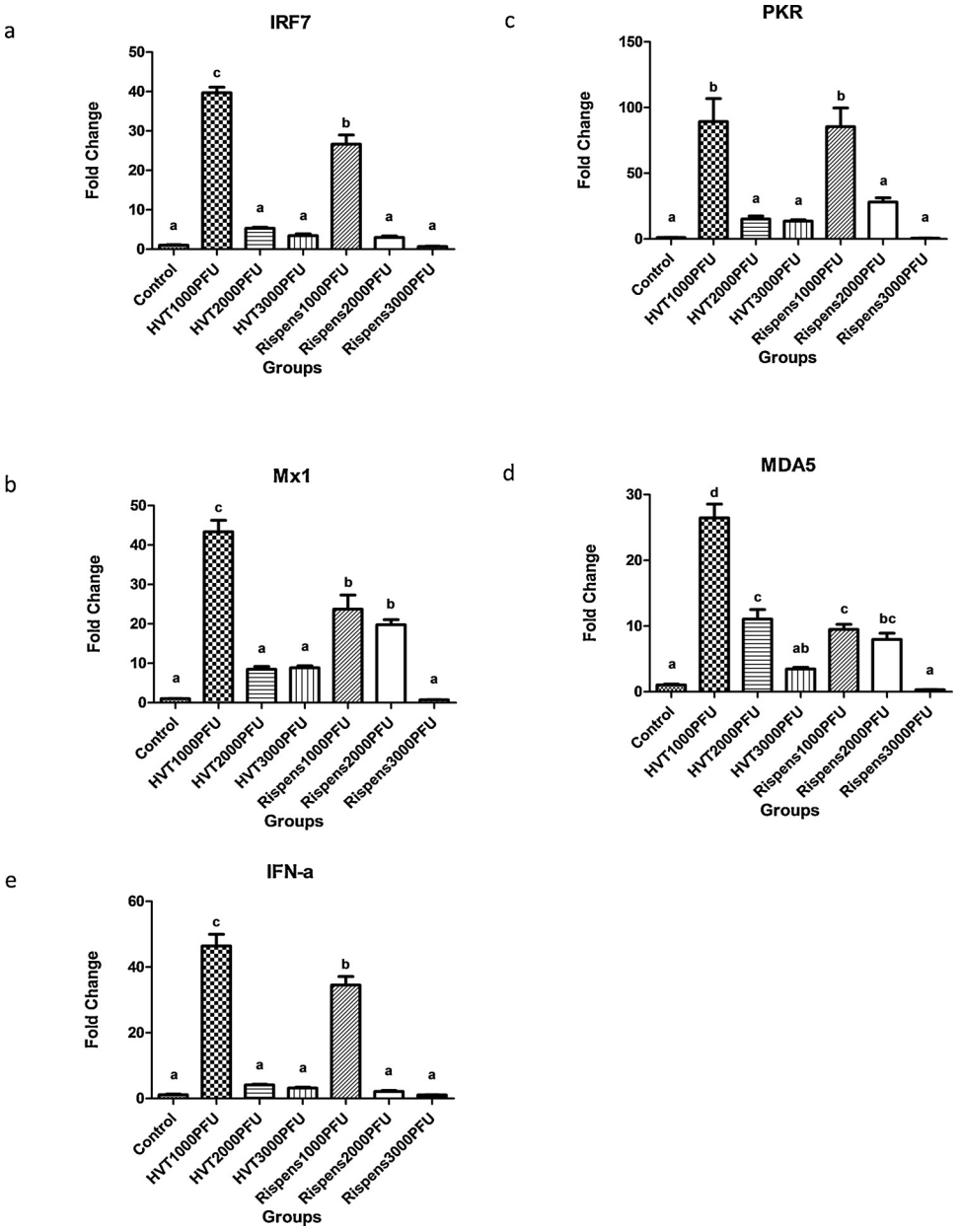

Fig. 3. MDV vaccines counteract MDA5-dependent-chIFNα signal transduction pathway in bird spleens in a dose-dependent manner during late stage. Birds were vaccinated with either HVT or Rispens strain at 1000, 2000 and 3000 PFU. Birds were humanely euthanized at 10th days post-vaccination and their spleen tissues were collected. RNA was extracted and an IRF7 (a), Mx1 (b), PKR (c), MDA5 (d) or chIFN-α (e) specific quantitative real-time PCR was performed. All data were normalized to GAPDH as an internal control and all the data were expressed as mean of fold difference (Bar) relative to non-MDV vaccinated group, which is given an arbitrary value of 1. Error bars represent the standard errors of means. The statistical analysis was done by One-way ANOVA with Tukey's at $P < 0.05$ using GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Different letters refer to statistical significant differences.

as 2 days PV (Baigent et al., 2005). Furthermore, the replication kinetics and virus load revealed that MDV genome copies increasing rapidly from day 4 to a peak at day 6, cytolytic phase of infection, and then steadily declined (Baigent et al., 2005). In our study, the genome of different MDV vaccines were detected in the spleens of vaccinated chickens three days PV (data not shown). As a result of MDV replication in the spleen cells, up-regulations of pattern recognition receptors (MDA5), IFN-α, transcriptional regulator (IRF7) and downstream antiviral elements (Mx1 and PKR) were established as early as 3 days post MDV vaccination in a virus-dose-dependent manner. This activation was continued up to 10 days post HVT vaccination at 1000 PFU dose only. In coordination with MDV serotype I replication kinetics, alteration of the Mx1, IRF7, MDA5 and chIFNα transcripts fold changes was detected in the case of vaccination with 1000 PFU of Rispens strain at 10 days PV which coordinate with MDV serotype I replication Kinetics. Earlier study showed Rispens strain enters latency in lymphoid tissues at 7 days PV which is probably associated with shutting down of the host's immune responses to switch from cytolytic stage (Baigent et al., 2005). Increasing the dose of the two applied vaccines led to virus-dose-dependent down-regulation of chicken MDA5-dependent-IFN-α signaling markers at both 5 and 10 days PV. These data support that the non-oncogenic MDV strains of low virulence, member of herpesviridae, have a mechanism to delay the interferon response in chicken spleens during the late phase of cytolytic infection. Consistent with our data, infection of chicken cells with the HVT or SB-1 strains interferes with expression of the major histocompatibility complex (MHC or B complex) class I (BF) glycoproteins during active but not latent stage of infection (Hunt et al., 2001). Interestingly, similar to mammals, chicken Interferon consensus motif contained in a sequence from −174 to −194 of the BF-IV MHC gene acts as a strong interferon-response element (Zoller et al., 1992). Further, it was proofed earlier that chicken infection with the non-oncogenic HVT and oncogenic RB1B led to up-regulation in genes involved in the immune response at one and seven dpi included genes involved in interferon response [e.g. the signaling molecule (IRF1, IRF7, and IRF10) and IFN-α] (Hu et al., 2015). Additionally, infection with either of the two viruses decreased IFN-α transcripts in MDV-genetically resistant B21/B21 chickens, indicating the role of IFN-α and their associated antiviral cytokines in MDV

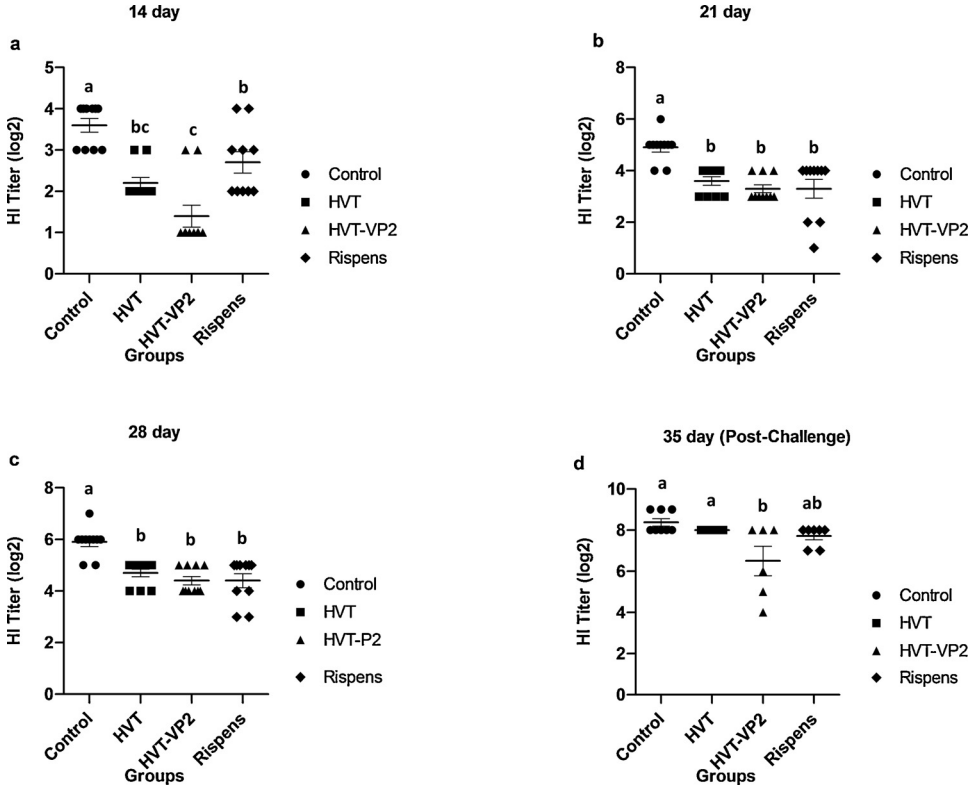

Fig. 4. Different MDV vaccines possess negative impact on NDV humoral HI Ab titres. Scatter plots of different vaccinated groups at 14th (a), 21th (b), 28th (c), and 35th (d) days of age. Control (received only NDV vaccines at 7th and 14th days old), HVT (received HVT vaccine at one day old and NDV vaccines at 7th and 14th days old), HVT-VP2 (received HVT-VP2 vaccine at one day old and NDV vaccines at 7th and 14th days old) and Rispens (received Rispens vaccine at one day old and NDV vaccines at 7th and 14th days old). Titres are expressed as $\log_2 \pm$ SE and the statistical analysis was done by One-way ANOVA with Tukey's at $P < 0.05$ using GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Different letters refer to statistical significant differences.

resistance.

The suppressive effect was more evident in case of MDV serotype I vaccine Rispens rather than that MDV serotype III vaccine HVT. This interesting observation suggesting that MDV vaccine serotype I Rispens, encodes protein(s) that not existed in HVT strain and involved in enhancing the immunosuppression process via the alteration of type I interferon signaling machinery in the host cells. Knocking out of both copies of Meq, basic leucine zipper protein, from a very virulent + MDV 686 strain reduce the late-MDV induced immune suppression (Faiz et al., 2018). Rispens strain possesses longer isoform of Meq termed as LMeq, in which a 60 AA sequence is inserted in the proline rich region. Previous results indicated that both Meq as well as its long isoform LMeq downregulate Mx1 on the transcription level in DF1 cell line, indicating that the inhibition of chIFN-α signaling through Meq does not contribute to the difference in pathogenicity in these strains (Hassanin, 2010). Additionally, it is worth to mention, a previous in vivo study showed that HVT vaccination alone caused lower effects on the circulating B-cell and T-cell numbers but failed to affect the primary lymphoid organ weights and humoral antibody responses, suggesting the very mild effect (Islam et al., 2002).

MDV infection also led to late immune suppression, one example early infection with very virulent + MDV strain led to total abrogation of protection induced by Infectious laryngotracheitis (ILT) vaccine against ILT challenge (Faiz et al., 2016). Consistently, here we provide similar late MDV immune suppression model induced by the non-oncogenic MDV commercially applied MDV vaccines. The three used MDV vaccines were able to significantly reduce the humoral Ab responses to NDV vaccines at different time points. On disagreement with these data,

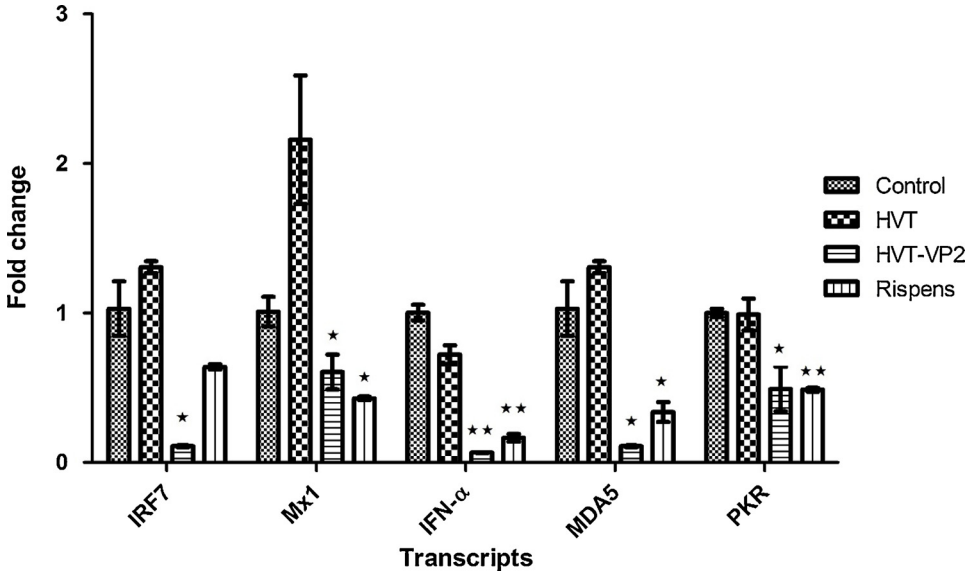

Fig. 5. MDV vaccine strains interact differentially with NDV induced innate immune responses in chicken spleens. Birds were vaccinated with either HVT, HVT-VP2 or Rispens strain at one day old. After 7th and 14th days old birds were immunized with inactivated and live NDV, respectively. Birds were humanely euthanized at 28th days post-vaccination and their spleen tissues were collected. RNA was extracted and an IRF7, Mx1, PKR, MDA5 or chIFN-α-specific quantitative real-time PCR was performed. All data were normalized to GAPDH as an internal control and all the data were expressed as fold difference relative to control group(received only NDV vaccines at 7th and 14th days old), which is given and arbitrary value of 1. The statistical analysis was done using Welch's *t*-test at $P < 0.05$ using GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

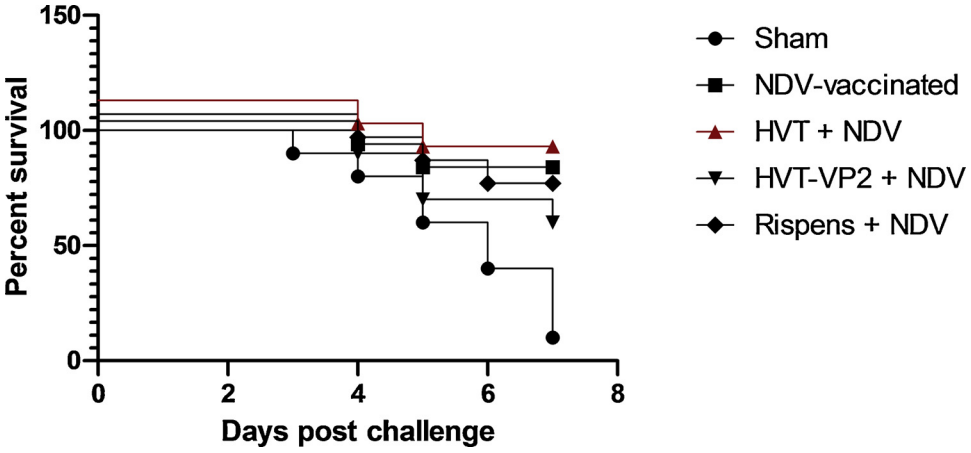

Fig. 6. Rispens and HVT-VP2 strains have negative impact on NDV vaccines induced protection against challenge with virulent NDV (genotype VIId). Survival plot of chickens from Sham (neither MDV nor NDV vaccinated), NDV-vaccinated (received NDV vaccines only at 7th and 14th day old), HVT + NDV (received HVT vaccine at one day old and NDV vaccines at 7th and 14th days old), HVT-VP2 + NDV (received HVT-VP2 vaccine at one day old and NDV vaccines at 7th and 14th days old) and Rispens + NDV (received Rispens vaccine at one day old and NDV vaccines at 7th and 14th days old), was graphed using GraphPad Prism version 8.

administration of high-tittered MD vaccines either in ovo or at hatch did not affect the efficacy of an IB vaccination (strains Ark and Mass) given by eye drop at hatch (Avakian et al., 2000). However, in the current study, the survival data proofed that Rispens and HVT-VP2 vaccination showed more suppressive effect than HVT vaccines. Similarly, no down-regulation in type I interferon signaling components in the spleens of the HVT vaccinated group. On the other hand, insertion VP2 open reading frame into HVT vector reduced the efficacy of the type I signaling machinery post NDV vaccination. It is worth to mention here, IBDV VP2 of serotype 1&2 induced apoptosis in a chicken B-lymphocyte cell line, DT40 (Rodriguez-Lecompte et al., 2005), and it is apoptotic inducer during the course of IBDV infection (Fernandez-Arias et al., 1997; Qin et al., 2017). This cellular apoptotic effect could explain the significant down-regulation in the levels of all the tested cytokines. Additionally, serotype I vaccine Rispens reduce the mRNA levels of all the tested cytokines compared with the HVT vaccine. Oncogenic serotype I MDV was able to block the response to inactivated NDV, a potent inducer of IFN, in resistant B21/B21 chickens and had a more suppressive effect than HVT on IFN gene transcription (Quere et al., 2005).

In general, this study conclude that MDV non-oncogenic strains elicit up-regulation of type I interferon signal-transduction pathway (MDA5 dependent) in chicken spleens as early as three days post vaccination. The exerted up-regulation was knocked down via a higher doses of MDV vaccines at 5 and 10 days post vaccination especially in the instance of serotype I strain. This negative regulatory consequence can hinder the humoral as well as the innate immune responses of other avian vaccines such as NDV vaccine, suggesting incrimination of MDV vaccines in the MDV induced late immune suppression phenomena.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

References

Abdallah, F., Hassanin, O., 2015. Positive regulation of humoral and innate immune responses induced by inactivated Avian Influenza Virus vaccine in broiler chickens. Vet. Res. Commun. 39, 211–216.
Avakian, A.P., Wakenell, P.S., Grosse, D., Whitfill, C.E., Link, D., 2000. Protective immunity to infectious bronchitis in broilers vaccinated against Marek's disease either in ovo or at hatch and against infectious bronchitis at hatch. Avian Dis. 44, 536–544.
Baigent, S.J., Smith, L.P., Currie, R.J., Nair, V.K., 2005. Replication kinetics of Marek's disease vaccine virus in feathers and lymphoid tissues using PCR and virus isolation. J. Gen. Virol. 86, 2989–2998.
Bazzigher, L., Schwarz, A., Staeheli, P., 1993. No enhanced influenza virus resistance of murine and avian cells expressing cloned duck Mx protein. Virology 195, 100–112.
Bernasconi, D., Schultz, U., Staeheli, P., 1995. The interferon-induced Mx protein of chickens lacks antiviral activity. J. Interferon Cytokine Res. 15, 47–53.
Boodhoo, N., Gurung, A., Sharif, S., Behboudi, S., 2016. Marek's disease in chickens: a review with focus on immunology. Vet. Res. 47, 119.
Daviet, S., Van Borm, S., Habyarimana, A., Ahanda, M.L., Morin, V., Oudin, A., Van Den Berg, T., Zoorob, R., 2009. Induction of Mx and PKR failed to protect chickens from H5N1 infection. Viral Immunol. 22, 467–472.
Faiz, N.M., Cortes, A.L., Guy, J.S., Fletcher, O.J., West, M., Montiel, E., Gimeno, I.M., 2016. Early infection with Marek's disease virus can jeopardize protection conferred by laryngotracheitis vaccines: a method to study MDV-induced immunosuppression. Avian Pathol. 45, 606–615.
Faiz, N.M., Cortes, A.L., Guy, J.S., Reddy, S.M., Gimeno, I.M., 2018. Differential attenuation of Marek's disease virus-induced tumours and late-Marek's disease virus-induced immunosuppression. J. Gen. Virol. 99 (7), 927–936.
Fernandez-Arias, A., Martinez, S., Rodriguez, J.F., 1997. The major antigenic protein of infectious bursal disease virus, VP2, is an apoptotic inducer. J. Virol. 71, 8014–8018.
Fulton, J.E., Arango, J., Ali, R.A., Bohorquez, E.B., Lund, A.R., Ashwell, C.M., Settar, P., O'Sullivan, N.P., Koci, M.D., 2014. Genetic variation within the Mx gene of commercially selected chicken lines reveals multiple haplotypes, recombination and a protein under selection pressure. PLoS One 9, e108054.
Gurung, A., Kamble, N., Kaufer, B.B., Pathan, A., Behboudi, S., 2017. Association of Marek's Disease induced immunosuppression with activation of a novel regulatory T cells in chickens. PLoS Pathog. 13, e1006745.
Hamad, M., Amen, O., Mahmoud, M., Hassanin, O., Saif-Edin, M., 2018. Effectiveness of different avian influenza (H5) vaccination regimens in layer chickens on the humoral immune response and interferon-alpha signalling immune marker. Vet. Res. Commun. 42, 145–152.
Hassanin, O., 2010. Genome-wide Analysis of Marek's Disease Virus Proteins and their Role in Modulating the Innate Immune Response in Chickens. University of Edinburgh.
Hassanin, O., Abdallah, F., Awad, A., 2014. Effects of florfenicol on the immune responses and the interferon-inducible genes in broiler chickens under the impact of E. coli infection. Vet. Res. Commun. 38, 51–58.
Heidari, M., Zhang, H.M., Sharif, S., 2008. Marek's disease virus induces Th-2 activity during cytolytic infection. Viral Immunol. 21, 203–214.
Hu, X., Qin, A., Xu, W., Wu, G., Li, D., Qian, K., Shao, H., Ye, J., 2015. Transcriptional analysis of host responses to Marek's disease virus infection in chicken thymus. Intervirology 58, 95–105.
Hunt, H.D., Lupiani, B., Miller, M.M., Gimeno, I., Lee, L.F., Parcells, M.S., 2001. Marek's disease virus down-regulates surface expression of MHC (B Complex) Class I (BF) glycoproteins during active but not latent infection of chicken cells. Virology 282, 198–205.
Islam, A.F., Wong, C.W., Walkden-Brown, S.W., Colditz, I.G., Arzey, K.E., Groves, P.J., 2002. Immunosuppressive effects of Marek's disease virus (MDV) and herpesvirus of turkeys (HVT) in broiler chickens and the protective effect of HVT vaccination against MDV challenge. Avian Pathol. 31, 449–461.
Karpala, A.J., Stewart, C., McKay, J., Lowenthal, J.W., Bean, A.G., 2011. Characterization of chicken Mda5 activity: regulation of IFN-beta in the absence of RIG-I functionality. J. Immunol. 186, 5397–5405.
Ko, J.H., Asano, A., Kon, Y., Watanabe, T., Agui, T., 2004a. Characterization of the chicken PKR: polymorphism of the gene and antiviral activity against vesicular stomatitis virus. Jpn. J. Vet. Res. 51, 123–133.
Ko, J.H., Jin, H.K., Asano, A., Takada, A., Ninomiya, A., Kida, H., Hokiyama, H., Ohara, M., Tsuzuki, M., Nishibori, M., Mizutani, M., Watanabe, T., 2002. Polymorphisms and the differential antiviral activity of the chicken Mx gene. Genome Res. 12, 595–601.
Ko, J.H., Takada, A., Mitsuhashi, T., Agui, T., Watanabe, T., 2004b. Native antiviral specificity of chicken Mx protein depends on amino acid variation at position 631. Anim. Genet. 35, 119–122.
Lee, C.C., Wu, C.C., Lin, T.L., 2012. Characterization of chicken melanoma differentiation-associated gene 5 (MDA5) from alternative translation initiation. Comp. Immunol. Microbiol. Infect. Dis. 35, 335–343.
Lee, C.C., Wu, C.C., Lin, T.L., 2014. Chicken melanoma differentiation-associated gene 5 (MDA5) recognizes infectious bursal disease virus infection and triggers MDA5-related innate immunity. Arch. Virol. 159, 1671–1686.
Liu, W.J., Yang, Y.T., Huang, Y.M., Zhou, D.R., Xu, D.N., Cao, N., Jiang, D.L., Pan, J.Q., Tian, Y.B., 2018. Identification of goose PKR gene: structure, expression profiling,

and antiviral activity against newcastle disease virus. J. Interferon Cytokine Res. 38, 333–340.
Melchjorsen, J., Rintahaka, J., Soby, S., Horan, K.A., Poltajainen, A., Ostergaard, L., Paludan, S.R., Matikainen, S., 2010. Early innate recognition of herpes simplex virus in human primary macrophages is mediated via the MDA5/MAVS-dependent and MDA5/MAVS/RNA polymerase III-independent pathways. J. Virol. 84, 11350–11358.
OIE, 2012. Manual of Standards for Diagnostic Tests and Vaccines for Terrestrial Animals.
Paludan, S.R., Bowie, A.G., Horan, K.A., Fitzgerald, K.A., 2011. Recognition of herpesviruses by the innate immune system. Nature reviews. Immunology 11, 143–154.
Parvizi, P., Abdul-Careem, M.F., Haq, K., Thanthrige-Don, N., Schat, K.A., Sharif, S., 2010. Immune responses against Marek's disease virus. Anim. Health Res. Rev. 11, 123–134.
Qin, Y., Xu, Z., Wang, Y., Li, X., Cao, H., Zheng, S.J., 2017. VP2 of infectious bursal disease virus induces apoptosis via triggering oral Cancer Overexpressed 1 (ORAOV1) protein degradation. Front. Microbiol. 8 1351-1351.
Quere, P., Rivas, C., Ester, K., Novak, R., Ragland, W.L., 2005. Abundance of IFN-alpha and IFN-gamma mRNA in blood of resistant and susceptible chickens infected with Marek's disease virus (MDV) or vaccinated with turkey herpesvirus; and MDV inhibition of subsequent induction of IFN gene transcription. Arch. Virol. 150, 507–519.
Randall, R.E., Goodbourn, S., 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol. 89, 1–47.
Rodriguez-Lecompte, J.C., Nino-Fong, R., Lopez, A., Frederick Markham, R.J., Kibenge, F.S., 2005. Infectious bursal disease virus (IBDV) induces apoptosis in chicken B cells. Comp. Immunol. Microbiol. Infect. Dis. 28, 321–337.
Santhakumar, D., Iqbal, M., Nair, V., Munir, M., 2017a. Chicken IFN kappa: a novel cytokine with antiviral activities. Sci. Rep. 7, 2719.
Santhakumar, D., Rubbenstroth, D., Martinez-Sobrido, L., Munir, M., 2017b. Avian interferons and their antiviral effectors. Front. Immunol. 8, 49.
Su, C., Zhan, G., Zheng, C., 2016. Evasion of host antiviral innate immunity by HSV-1, an update. Virol. J. 13, 38.
Thornton, D.H., 1985. Quality control and standardization of vaccines. In: Payne, L.N. (Ed.), Marek's Disease (Scientific Basis and Methods of Control). Martinus Nijhoff Publishing, Boston.
Xing, Z., Schat, K.A., 2000. Expression of cytokine genes in Marek's disease virus-infected chickens and chicken embryo fibroblast cultures. Immunology 100, 70–76.
Yu, L., Zhang, X., Wu, T., Su, J., Wang, Y., Wang, Y., Ruan, B., Niu, X., Wu, Y., 2017. Avian infectious bronchitis virus disrupts the melanoma differentiation associated gene 5 (MDA5) signaling pathway by cleavage of the adaptor protein MAVS. BMC Vet. Res. 13, 332.
Zoller, B., Ozato, K., Kroemer, G., Auffray, C., Jungwirth, C., 1992. Interferon induction of chicken MHC class I gene expression: phylogenetic conservation of the interferon-responsive element. Virology 191, 141–149.
Zou, H., Su, R., Ruan, J., Shao, H., Qian, K., Ye, J., Qin, A., 2017. Toll-like receptor 3 pathway restricts Marek's disease virus infection. Oncotarget 8, 70847–70853.