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Cytokines Evaluation of Different Marek's Disease Virus Vaccines in Broiler Chickens

Leila K. Abdelhamid, Helmy A. Torky, Samy A. Khalil
Department of Microbiology, Faculty of veterinary medicine, Alexandria University, Egypt.

Key words:

Marek's disease vaccines, Pro-inflammatory Cytokines, RT-PCR, Chicken

Correspondence to:
Leila K. Abdelhamid
Email:
leila.kotp@alexu.edu.eg

ABSTRACT

Marek's disease is an immune suppressive disease that threatens poultry industry. It is controlled by vaccination against MDV. However, the post vaccination immune mechanisms remain vague. Therefore, the objective of this study was to examine the transcripts of the cellular immune responses of two commercial vaccines (CVI988 and HVT strains) compared to control non-vaccinated chickens through the analyses of several cytokine responses. The mRNA was extracted from the bursa of Fabricius and thymus, sampled at 7, 14 and 21 days post-immunization (PI) of both vaccinated and control groups. Several pro-inflammatory and anti-inflammatory cytokine gene expressions were analyzed using RT-PCR. Early significant up-regulation of IFN-γ, a pro-inflammatory cytokine was observed. Also, upregulation of other cytokines like IL-1β and IL-6 was observed at different time points along with minimal non-significant expression of inducible nitric oxide synthase (iNOS) in both of vaccinated groups. Meanwhile, a late upregulation of the anti-inflammatory cytokine, IL-10 was only noticed by 21 day PI. These findings show that both vaccinal strains mimic the natural infection by inducing early cell-mediated inflammatory responses, while devoid the long-term immune suppressive effect of natural infection. Additionally, vaccination triggers anti-tumor cell-mediated responses as indicated by the late increase of IL-10 expression. Furthermore, the observed cytokine responses following CV1988 strain supports its previously reported efficacy over HVT strain, making the Rispens vaccine more suitable candidate for Egyptian field conditions. Additionally, the early observed up-regulation of IFN-γ cytokine nominates its administration as an adjuvant with the Rispens vaccine; this may provide synergistic immunomodulatory capacities to the available vaccines and better levels of protection.

1. INTRODUCTION

Marek's disease (MD) is an economically destructive lymphoproliferative disease of chicken, caused by an alphaherpes virus called Marek's disease virus (MDV). The disease is characterized by development of various tumors in infected chickens. These tumors are usually associated with appearance of the disease clinical symptoms such as blindness, paralysis and death(Calnek, 2001). Additionally, this disease has a sever immune suppressive nature which may be due to cell cytolysis and lymphomas development(Calnek, 2001). Therefore, MD infection is associated with great economic losses that are related to the cost of vaccination, morbidities, mortalities and the increased susceptibility of the infected birds to other infections as a result of MD induced immunosuppression (Islam et al. 2002).

MDV is highly cell associated and usually targets both B and T lymphocytes during the cytolytic and latent phases of infection respectively. MDV primary targets the bursa of Fabricius during the early stage of infection resulting in B cells cytolysis (Schat et al. 1981), then targets the thymocytes resulting in thymic atrophy (Morimura et al. 1996). So, the T cell mediated responses are reported to have a more significant role than antibody mediated responses in controlling MDV infection. Precisely, cytotoxic T–lymphocytes (CTLs) (Omar & Schat, 1996) and their secreted product.

Cytokines as the secreted products of CTLs are proposed to play key roles against MDV infection, shaping the viral pathogenesis (Xing & Schat, 2000) and may possess protective responses following

vaccination (Djeraba et al. 2000; Kano et al. 2009).In spite of, the fact that MDV was the first oncogenic virus to which vaccines were available (Baigent et al. 2006), the virus is continually evolving toward greater virulence impacting those vaccines efficacy. Even with the intensive application of variable MD vaccines worldwide, the problems of MD outbreaks still arise. This situation indicates the inability of the available vaccines to counteract the viral transmission among the vaccinated birds. Additionally, the precise mechanisms of post vaccines immune responses are still vague. That warrants further evaluation of the commercially available vaccines on the Egyptian level such as CVI988 (Rispens) and Herpes virus of turkey (HVT) vaccines. Examining the transcripts of the cellular immune responses of these two vaccines through cytokine profile analysis, may provide better understanding of vaccinations induced immunity. This may help in developing of better controlling strategies against MDV infection.

Therefore, the objective of this study was comparing the cytokine responses of two commercially available vaccines on the Egyptian level; CVI988 (Rispens) and Herpes virus of turkey (HVT) vaccines in broiler chickens by evaluation of the mRNA gene expression pattern of a number of pro-inflammatory and anti-inflammatory cytokines (including INF gamma, iNOS, IL-1β, IL6 and IL-10). This was to determine the role of bursal and thymic cytokines in vaccines-induced immunity.

2. MATERIAL AND METHODS

2.1. Chickens.

One day old Cobb broiler chicks were obtained from healthy flocks and were free from hatchery vaccination. They were maintained in a disease controlled environment as approved by committee ethics of faculty of veterinary medicine, Alexandria University, Egypt. Feed and water were supplied ad-libitum.

2.2. Vaccines strains.

Two commercially available vaccines were used to immunize chicks. Rispens (CVI 988 attenuated live strain) and turkey herpesvirus (HVT) vaccines were purchased from Ceva and Vaxitec - IFT Animal Health Companies respectively.

2. 3. Study design and tissue sampling.

One hundred twenty Cobb broiler chicks were randomly assigned into three groups; one kept as untreated control and the other two were vaccinated with CVI988 or HVT vaccination. Vaccines were administered subcutaneously at first day of hatch as has been recommended by the manufacturer, while the control group was mock vaccinated, received only the vaccine diluent. Chicken bursa of Fabricius and thymus were sampled at 7, 14 and 21 days post-vaccination. In each time point 6 to 9 chicks per group were euthanized for sample collection. Samples were snap frozen in liquid nitrogen and stored at -80 C until RNA extraction.

2. 4. RNA extraction and reverse transcription

Total RNA extraction was carried out from control and vaccinated groups using Trizol reagent (Life Technologies, Ambion) following the manufacture procedures. The quality of the RNA yield was assessed by agrose gel electrophoresis. DNase treatment and the reverse transcription into single stranded cDNA was done following the manufacturer's protocols using RQ1 RNase-Free DNase (Promega) and High Capacity cDNA Reverse Transcription kit (Applied Biosystems) respectively.

2. 5. Primers

The previously published oligonucleotide primers for broiler chickens cytokines were used for relative quantification of cytokine gene expression and GADPH was used for normalization as shown in table (1) (Hong et al. 2006). All the selected primers were BLAST searched to ensure their specificity to broiler cytokines.

2. 6. Relative cytokines analysis by RT-PCR.

The relative cytokines genes expression using cDNA templates was conducted using Real-time Polymerase Chain Reaction with Power SYBR®Green PCR Master Mix (Life Technologies) in Mx3005P Real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). On each time point, samples were evaluated as biological triplicates and the mean cycle threshold (CT) values obtained were used to determine the fold changes in gene expression using the GADPH gene as internal control for normalization. The Variations in the cytokines transcripts expression among different groups (Rispens or HVT vaccinated) were expressed as the fold change compared to the control (unvaccinated group) using the Livak; $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Table (1) Sequence of the Primers used in RT-PCR for cytokines analysis.

Target cytokine	Primer sequence, Nucleotide sequence (5'→ 3') Forward	Reverse	Accession number
IFN-γ	AGCTGACGGTGGACCTATTATT	GGCTTTGCGCTGGATTC	Y07922
IL-1 B	TGGGCATCAAGGGCTACA	TCGGGTTGGTTGGTGATG	Y15006
IL -6	CAAGGTGACGGAGGAGGAC	TGGCGAGGAGGGATTTCT	AJ309540
IL-10	CGGGAGCTGAGGGTGAA	GTGAAGAAGCGGTGACAGC	AJ621614
iNOS	TGGGTGGAAGCCGAAATA	GTACCAGCCGTTGAAAGGAC	U46504
GADPH	GGTGGTGCTAAGCGTGTTAT	ACCTCTGTCATCTCTCCACA	K01458

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2.7. Statistical analysis.

The differences in gene transcripts between groups were analyzed using by analysis of variance followed by Tukey test using graphpad prism7 software, and comparisons were considered significant at $P \leq 0.05$.

3. RESULTS

After assessing the quality of the extracted RNA, using Gel electrophoresis, the CDNA product of the extracted mRNA from both bursa of Fabricius and thymus was used for cytokines genes expression analysis following CVI988 and HVT vaccination.

3.1. Cytokines and iNOS genes expression in bursa of Fabricuis of different Vaccinated and control chickens.

Up-regulation of the pro inflammatory and anti-inflammatory cytokines; IFN-γ, IL-1β, IL-6 and IL-10, with maintained expression of the iNOS was observed in chicken bursal tissue at different experimental time-points following both Rispens and HVT vaccination compared to the unvaccinated control group. Table (2) shows the mean ΔCT ± SEM values of relative mRNA expression of the tested cytokines to the different treatment groups at 7, 14 and 21 days PI. Fig.1 shows the fold changes for different cytokines genes expression over control group.

An early statistically significant up-regulation of IFN-γ was observed by day 7 PI without a statistical difference in its expression between the two vaccinated groups as demonstrated in Fig.1.A. Further up-regulation of IFN-γ was continued at 14 and 21 days PI without a significant difference than its expression in mock-vaccinated chickens.

In contrast, the increased expression of IL-1β was statistically significant following Rispens vaccine at each time point, while this significant up-regulation -following HVT vaccination was only observed during the first 2 weeks of immunization. Additionally, a statistical significant difference was observed in IL-1β expression between Rispens and HVT vaccinated chickens at day21 PI.as shown in Fig.1.B. In parallel, a significant expression of IL-6 transcript was observed in both of Rispens and HVT vaccinated groups at the different time points with significant difference in its expression between the vaccinated groups only at day 7 PI as revealed in Fig.1.C.

On the other hand, the expression of the anti-inflammatory cytokine IL10 showed a little non-significant expression during the first two weeks PI followed by a significant up-regulation only observed at day 21 PI with a significant difference in its expression following HVT vaccination compared to CVI988 immunization (Fig.1.D).

Meanwhile, examination of the signaling molecule iNOS gene, revealed a maintained expression without a statistical significance when expressed as fold change relative to control at all examination times as represented in Fig.1. E.

3.2. Cytokines and iNOS genes expressions in the thymus of vaccinated chickens.

In the thymus, the expression of the tested cytokines (IFN-γ, IL-1β, IL-6 and IL-10) mRNA and the signaling molecule iNOS gene followed closer patterns however, higher expression than those at bursal tissue. Table (3) shows the mean ΔCT ± SEM values of relative mRNA expression

for different cytokines to each experimental group at 7, 14 and 21 days PI. Fig. 2 demonstrated the fold changes in cytokines genes expression in the thymus of Vaccinated chicken over its control expression.

IFN-γ transcript expression was statistically significant following both vaccines at days 7 compared to the unvaccinated group. Then the fold changes in its expression followed a trend reduction toward the day 21, with a reduced but statistically significant expression following Rispens immunization re-observed at day 21 PI with a noticed significant difference between the two vaccinated groups as shown in Fig.2.A. Similarly, IL-6 transcript showed a significantly increased expression in both immunized groups at day 7, followed by reduced expressions at the subsequent examination points. However, by the day 21 a significant difference in IL-6 expression between the vaccinated groups with a significant up-regulation in Respins immunized chicken were re-observed as demonstrated in Fig.2.B. Meanwhile, IL-1 IL-1β cytokine showed significant up-regulation of its transcript at only days 7 PI for both vaccinated groups with a non-significant expression during subsequent examination times as in Fig.2.C.

The mRNA transcripts were normalized relative to that of GADPH, housekeeping gene. The analyzed data represented as fold change over control. Data shows significant upregulation of the pro-inflammatory cytokines especially early after vaccination with late IL-10 upregulation. The difference in cytokine expression between groups was determined by analysis of variance followed by Tukey test and comparisons were considered significant at $P \leq 0.05$. * for each vaccinated group over control and the little star over the inverted bracket for significance between the 2 vaccinated groups .

Table (2) Mean ΔCT ± SEM values of relative mRNA expression for different cytokine genes in bursa tissue.

Cytokine	Groups	Day7	Day14	Day21
IFN-γ bursa	Control	14.391 ± 0.1817	14.54 ±0.050	15.64± 0.4102
	Rispen	15.98833 ± 0.194959	13.12± 1.03	15.01± 0.0907
	HVT	15.715 ± 1.3233	14.36± 0.1981	15.18± 0.14
IL-1 β bursa	Control	14.683± 0.058973	14.856± 0.1995	15.8866±0.199
	Rispen	11.29± 0.355	13.04±0.113	14.62±0.07234
	HVT	12.0333± 0.297228	13.7133±0.2142	15.52667±0.147234
IL-6 bursa	Control	14.93667±0.145	15.3456± 0.125	15.9966±0.149
	Rispen	10.37± 0.1401	13.21±0.226053	14.98667±0.16676
	HVT	11.56± 0.34775	13.9333±0.2579	15.24667±0.108372
IL-10 bursa	Control	12.4667± 0.65484	12.52± 0.1852	13.62±0.545924
	Rispen	12.1333±0.37373	12.6233±0.26193	11.9433±0.337754
	HVT	12.9667±0.24065	13.53±0.645084	10.28667±0.108372
iNOS bursa	Control	16.655± 0.015	16.66± 0.45	17.34667±0.374002
	Rispen	17.256667±0.24251	18.15±0.865	17.5667±0.410744581
	HVT	17.12±0.520395149	17.50333±0.247004	17.47333±0.269093

Table (3). Mean ΔCT ± SEM values of relative mRNA expression for different cytokine genes in thymes tissue.

Cytokine	Groups	Day7	Day14	Day21
INF-γ thymus	Control	12.41667 ± 0.1217	8.60667 ±0.27083	13.953± 0.191514
	Rispen	7.15667 ± 0.1935	6.793± 0.72996	13.0233± 0.22318
	HVT	7.68 ± 0.10263	7.77667± 0.6293	14.2733± 0.1433
IL-1β thymus	Control	13.7533± 0.16013	8.86± 0.441173	14.14±0.460326
	Rispen	11.76333±0.029627	8.766667±0.806687	14.92±0.235018
	HVT	11.78± 0.4	9.12±0.300056	15.18667±0.23607
IL-6 thymus	Control	15.26±0.19	9.396667±0.286609	13.9533±0.2903637
	Rispen	9.51± 0.172143	7.01±0.997714	12.8±0.170098
	HVT	9.82333± 0.258994	17.46±0.20951	14.366±0.03179797
IL-10 thymus	Control	12.49± 0.13769	7.5633± 0.2896	11.723±0.2862
	Rispen	12.5±0.15631	6.62±0.95459	7.987±0.82585
	HVT	12.53672±0.44423	7.5166±0.2577	8.44333±0.38299
iNOS thymus	Control	16.655± 0.015	16.66± 0.45	17.34667±0.374002
	Rispen	17.256667±0.24251	18.15±0.865	17.5667±0.410744581
	HVT	17.12±0.520395149	17.50333±0.247004	17.47333±0.269093

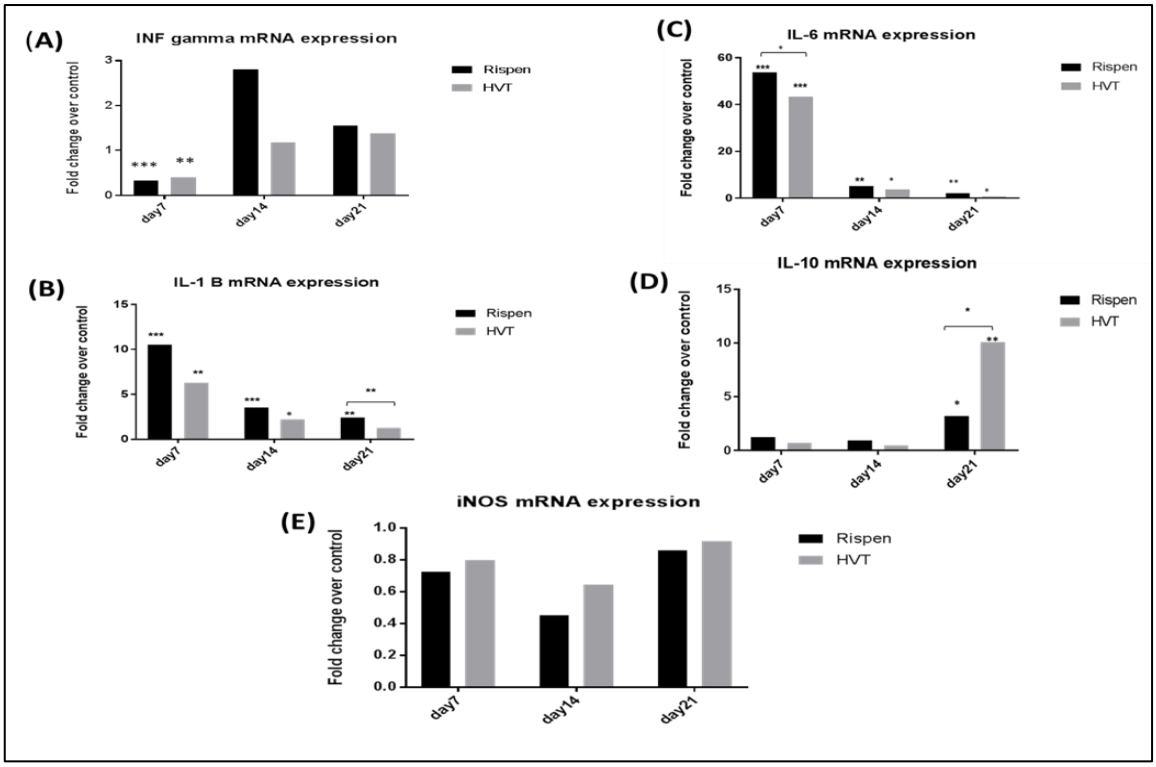

Fig.1. Relative mRNA transcript expression levels of (A) IFN –γ, (B) IL-1β,(c) IL-6 ,(d) IL-10 and (E) iNOS genes in bursa of fabricuis for Rispens and HVT immunized chicken groups.

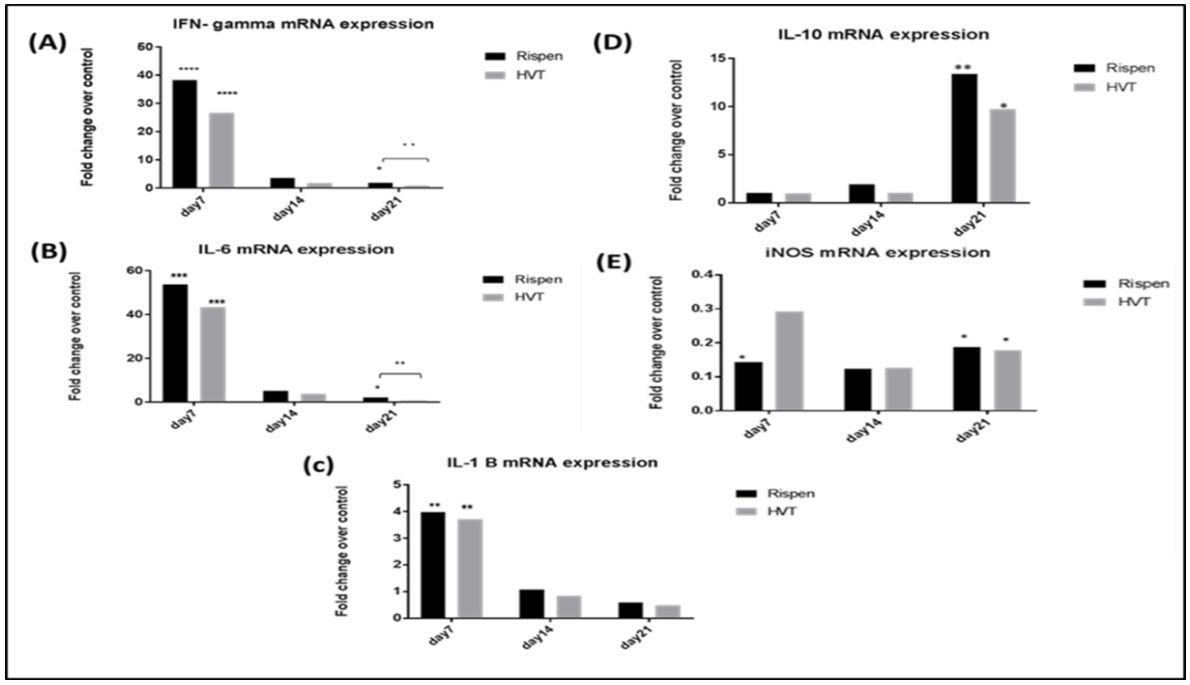

Fig.2. Relative mRNA transcript expression levels of (A) IFN –γ, (B) IL-6,(c) IL-1 β, (d) IL-10 and (E) iNOS genes in the thymus for Rispens and HVT immunized chicken groups.

The mRNA transcripts were normalized relative to that of GADPH, housekeeping gene. The analyzed data represented as fold change over control. Results show significant up regulation of the pro-inflammatory cytokine especially early after vaccination with late IL-10 upregulation as well as minimal while significant iNOS expression in the thymic tissue. The difference in cytokine expression between groups was determined by analysis of variance followed by Tukey test and comparisons were considered significant at P≤0.05 with (*) for each vaccinated group over control and the little star

over the inverted bracket for significance between the 2 vaccinated gr

On contrary, the anti-inflammatory; IL-10 transcript expression kept as minimal and not statistically significant during the first 2 weeks post vaccination with a significant up-regulation following both vaccines at 21 day PI, without a major difference between the two vaccinated groups (FIG.2.D).

The iNOS mRNA transcript, in the thymus, showed minimal but statistically significant up-regulations observed at different examination points as shown in Fig.2.E.

4. DISCUSSION

The early observed significant expression of the major Th1 pro inflammatory cytokine; IFN-γ in chicken bursa and thymus following immunizations against MDV is in agreement with previous studies that recorded similar IFN-γ expression following vaccination and/or MDV infection in other tissues. For example, Djeraba and co-workers reported an increased IFN- γ mRNA expression in the splenocytes of vaccinated bird with CVI-988 strain and challenged with very virulent MDV, RB-1B strain (A Djeraba et al., 2000). In parallel, Abdulcareem et al., (2008) have reported an increased expression of IFN- γ gene in feather tips of CVI988 and HVT vaccinated chickens by 10 days PI (M F Abdul-Careem et al., 2008). Similarly, up-regulation of IFN-γ in Peripheral blood mononuclear cells (PBMCs) and splenocytes following Rispens vaccination has been observed (Kano et al., 2009). Additionally, Heidari et al., (2014), reported an up regulation of IFN-γ in the cecal tonsils of genetically susceptible and resistant chickens, during the early cytolytic phase of infection with bacterial artificial chromosome (BAC) cloned very virulent (vv) strain of MDV; rMd5-BAC.

As suggested by Abdul-Careem et al., (2008) , the up-regulation of IFN-γ following vaccination could be related to this cytokine antiviral activity mediated by macrophage activation. IFN-γ has been reported to inhibit MDV replication by activating macrophages to initiate cell mediated cytotoxicity for MDV-infected cells (Lee, 1979; Djeraba et al., 2000; Djeraba et al., 2002). Furthermore, IFN-γ directly activates CD8+ CTLs to kill the virally–infected cells (Whitmire et al., 2005). Additionally, in vitro investigations revealed that IFNs are essential for vaccine viral replication of both CVI988 and HVT strains in vitro (Levy et al., 1999). So, the recorded up-regulation of IFN-γ mRNA expression in the current study reveals the importance of this pro-inflammatory cytokine in immune response to MDV vaccinal CV1988 and HVT strains in a common agreement with previous findings and that supports the notion to use IFN- γ as an adjuvant for more potent vaccine formulations.

Furthermore, the significant expression following Rispens immunization that was re-observed again at day 21 PI in thymus, with the noticed difference between its expressions in the two vaccinated groups may be linked with the CVI988 viral strain replication in chicken lymphoid tissue. Gimeno et al., (2011) showed that the CVI988 strain replicates in the bursa and thymus and its viral DNA can be detected in those organs by the day 6 post s/c inoculation, then this replication decline toward the day 14 followed by a second replication wave as recorded in their following examination time point at 26 days. Their results also revealed that CVI-988 viral DNA load in thymus was higher than in bursa. So the reduced however significant IFN-γ by 21 days in thymus of Rispens immunized group may be associated with this second replication wave.

Meanwhile, up regulation of other pro-inflammatory cytokines; IL-1β and IL-6 was also observed, following vaccination with both CVI988 and HVT vaccines. Those findings are in contrast to what has been observed during MD infection and in agreement with others findings following vaccination. For instance, Kano et al., (2009), found a decreased expression of those cytokines during the early cytolytic phase of infection with virulent MDV, while they were upregulated in CV1998 vaccinated groups.

Despite, up-regulation of IL-1β and IL-6 has observed in our findings, they may not directly contribute in the vaccines induced immunity. Kano et al., 2009, reported no differences in those cytokine responses among vaccinated challenged and non-challenged birds following CVI988 vaccination. Similarly, Abdul-Careem et al., 2007, reported similar findings following bivalent vaccination with HVT+SB-1 strain.

Generally, for all the pro-inflammatory cytokines, the current study showed that fold changes in IFN-γ, IL-1β and IL-6 transcripts expression due to Rispens vaccination were higher than that up-regulation due to HVT strain immunization throughout the experiment. These findings are in agreement with previous studies that showed that Rispens strain give more potent responses that are much closer to classical type MDV infection (Witter, 2001).

For the cellular signaling molecule; iNOS, a sustained non-significant expression of has observed following Rispens and HVT vaccination

in chicken bursa. This is in contrast to previous findings following infection. For example, kano et al., 2009, has reported a transient increase in iNOS mRNA expression following infection with a virulent RB1B strain. Similarly, Dejarba et al., (2002) reported a strong expression of iNOS followed by NO production in the spleen and serum of histocompatible B21/B21 chickens following RB-1B MDV infection. However, in agreement with our study, they did not notice similar responses with the HVT vaccine strain. Meanwhile, they reported that vaccination with HVT before RB-1B infection resulted in a strong expression of iNOS transcripts as well as NO production in the spleen (Djeraba et al. 2002). This response may be interpreted as, following infection iNOS catalyzes the NO produced by the activated macrophages, which in turn induces the immune suppression correlated to infection by inhibiting lymphocytes proliferation (Bingisser et al., 1998). While, the observed maintained expression of iNOS along with up regulation of the pro-inflammatory cytokines following vaccination in the bursa of Fabricius, may provide an indication that the vaccines strains are capable of inducing early inflammatory cell mediated response similar to the natural infection, without eliciting the immune suppressive effect of the wild virus. On the other hand, the minimal however significant expression of iNOS in thymus may be interpreted by the inherent ability of thymocytes to produce iNOS to help in the process of negative selection by deletion of the autoreactive thymocytes to ensure the self-tolerance. Tai et al., showed that murine fetal thymus expressed modest but significant levels of iNOS without external stimuli and adult thymus can produce higher levels following T cell receptors (TCR) stimulation (Tai et al., 1997).

Finally, the anti- inflammatory cytokine, IL-10 is proposed to be a regulatory cytokine in chickens (Rothwell et al., 2004). In the present study there was up-regulation of IL-10 following both vaccinations, observed at 21 day PI. In bursa a significant difference between HVT and Rispens groups was observed while both vaccines resulted in IL-10 upregulation in thymus without a significant difference between both vaccinated groups. This late increased IL-10 expression is in agreement with Kano et al., (2009) results. They reported that the expression of IL-10 was more prominent in splenocytes and PBMCs of Rispens vaccinated chicken than those of RB1B infected unvaccinated birds. However, in the current study there was statistically significant difference in the expression of IL-10 following HVT which resulted in higher expression than Rispens vaccination in bursa, this might be explained by the non-oncogenic nature of the Serotype 3 HVT strain. IL-10 is reported to possess anti-tumor activities and can control primary tumor growth and inhibit its metastatic activity (Berman et al., 1996; Fujii, 2001). Therefore, the non-oncogenic HVT may provoke higher IL-10 expression. Meanwhile, the proposed higher replication of Rispens strain over the HVT one in the thymus tissue as suggested by Gimeno et al., (2011) may provide an explanation for the non-significant difference in IL-10 expression in the thymus.

The noticed IL-10 responses might support Payne and his colleagues, 1978, proposal that the second step in vaccinal immunity against MDV is anti-tumor cell mediated responses (Payne et al., 1978). Similarly, this observation supports previous studies that MDV greatly reduce cell transformation and tumor development in vaccinated birds (Baaten, Butter, & Davison, 2004; Baigent, S.J. et al., 2006).

Additionally, the increased expression of the IL-10 was associated with reduced expression of the pro-inflammatory cytokines. This may be explained as counter-regulatory properties of IL-10 to suppress TH1 cytokines for maintaining a balanced immune response following vaccination. IL- 10 is a primary Th2 cytokine and is suggested to maintain a balanced immune response by regulating Th1 activity (Rothwell et al., 2004).

Collectively, for different cytokines transcripts, their expression as fold changes over their expression in the non-vaccinated group in thymus was higher than in bursa. This may be linked with starting of examination at day7 PI and onward. Vaccines strains are reported to induce similar pathogenesis as wild type MDV. Vaccines virus induce a persistent infection that reduce viremia due to further pathogenic infection (Baigent et al., 2006). Initially, MDV became cell associated with the B- cells in the bursa of Fabricius (two to seven day), and then at seven days the virus infects the T-cells in thymus during the latency phase (Shek et al. 1983). Furthermore, Gimeno et al., 2011, provided further support to this explanation; by the increased viral strains DNA load in the thymus tissues over bursal one as described earlier in this discussion. Their studies findings may explain the higher responses in thymus.

In conclusion, both vaccines strains induce early inflammatory responses, and devoid the immune suppressive effect associated with MDV infection. Meanwhile, the upregulation of IL-10 by 21 day PI

indicates the importance of IL-10 for maintaining balanced immune responses following MDV vaccinations. Additionally, Rispens vaccine elicits better cytokine responses than these of HVT vaccine. So, the current findings nominate the Rispens vaccine as a more potent one for Egyptian broiler farming conditions especially if combined with the pro-inflammatory cytokine; INF-γ.

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