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Cytokine gene expression following RSV-A infection

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1. ABSTRACT

The present study determines the cytokine gene expression in chickens following RSV-A infection, using RT-qPCR. In susceptible chickens tumors progressed to fulminating metastatic tumors while it regressed in regressors chickens and some resistant non-responder chickens did not respond to RSV-A infection and thus did not develop tumors at all. The *in vivo* expression of pro-inflammatory cytokines, Th1 cytokines and Th2 cytokines was determined at the primary site of infection, as well as in different organs of progressor, regressor and non-responder chicks at different time intervals. Our results indicated a significant upregulation of the pro-inflammatory cytokines, IL-6 and IL-8, in all the organs of progressor chicks, while they were significantly lower in regressor and non-responder chicks. The expression of the Th1 cytokines IFN-γ and TNF-α was low in all of the organs of the progressor group, except that in spleen. In contrast, regressor and non-responder groups showed high expression of IFN-γ and TNF-α. Further, there was an early upregulation of the expression of the Th2 cytokine, IL-10, TGF-β and GM-CSF, in all of the organs of progressors as compared to uninfected control.

2. INTRODUCTION

The Rous sarcoma virus (RSV), a member of avian leucosis virus (ALV) group, was the first oncogenic retrovirus to be identified as a cancer inducer (1). In susceptible chickens injected with RSV, there is a rapid neoplastic transformation and tumor development within a few days or weeks (2). The RSV has a wide host range, causing tumors in chickens, pheasants, guinea fowl, ducks, pigeons, Japanese quails, turkeys, and rockpartridges (*Alectoris graeca*) and also in mammals, including rats (3) and monkeys (4). Its replication strategy has evolved to produce a long-term or persistent infection which results from its spread both vertically from parent cells to daughter cells via the provirus, as well as horizontally from cell to cell via virions (5, 6). Distal tumors are frequently seen in various tissues and organs induced either by viral inoculation or by actual metastasis from primary sarcomas (7, 8). The enhancement of the disease by RSV is most likely the consequence of immunosuppression, but the mechanism of RSV-induced immunosuppression remains to be elucidated.

All chicken genotypes are susceptible to RSV infection and lymphoma development (9-11). However, there is a difference in disease resistance and immune response between individuals. The diversity of the major histocompatibility (MHC) proteins, T cell receptor proteins, immunoglobulins and cytokines, constitutes the major immunological basis for the variations (12-14). Chicken MHC-B complex genes encode cell surface proteins critical to the function of the immune system (15, 16). Specific MHC genotypes can alter the RSV growth pattern, either progressively or regressively (17, 18). Additional work by numerous investigators supports the hypothesis that MHC genes can regulate RSV tumor growth (19, 20).

In contrast to the information on MHC mechanisms, there is a paucity of knowledge on the role of other immune modulators in RSV infection, pathogenesis and immunity. As an important immunosuppressive disease in chickens, it is likely that the Rous sarcoma virus has complex interactions with the immune system. In avian species, adaptive immunity involves both humoral and cell-mediated immune (CMI) responses (21). While humoral or antibody - mediated immune responses are used to control extracellular microorganisms, CMI responses are important for eradicating intracellular bacteria and tumor cells and eliminating viral infections (21, 22). The functional effectors that mediate CMI include immune cells, such as cytotoxic T cells (CTLs), macrophages, and natural killer (NK) cells, which are regulated, in part, by cytokines (23). Cytokines are small proteins (5-20 kDa) that are secreted by immune and tumor cells (24, 25). They play a pivotal role in the function and regulation of the innate immune system (26). They have autocrine and paracrine functions, and thus function locally or at a distance to enhance or suppress immunity (27, 28). Depending on the tumor microenvironment and the balance of pro-inflammatory and anti-inflammatory cytokines, their relative concentrations and receptor expression, cytokines can modulate the antitumoral responses (29). Thus, it is imperative to understand the interactions between RSV and cytokines to further elucidate the oncogenesis of RSV.

Therefore, in this study, we determined the expression of cytokines in various organs of chickens

following RSV infection based on their response to RSV (i.e. progressors, regressors or non-responder).

3. MATERIALS AND METHODS

3.1. Chicks and management practices

All the procedures have been conducted in accordance with the guidelines of the Institutional Animal Ethical Committee of Indian Veterinary Research Institute. Permission for the sacrifice of the experimental birds by cervical dislocation was obtained from the Animal Ethics Committee of Indian Veterinary Research Institute, Government of India.

One - day old chicks, belonging to white-plumaged Synthetic Broiler Dam Line (SDL), were obtained from the Experimental Broiler Farm, Central Avian Research Institute (CARI), Izatnagar, Uttar Pradesh (UP), India. All birds were maintained under uniform, standard management conditions, with 15 h light: 9 h dark exposure and provided with free access to feed and water. The individual chicks were wing banded for identification. The chicks were transported to challenge sheds at the Indian Veterinary Research Institute (IVRI), Izatnagar, UP, India, where they were maintained under a controlled environment.

3.2. Virus

The Bryan Standard strain of Rous sarcoma virus (Rous associated virus-1) (BS-RSV (RAV-1)), henceforth known as RSV-A, was used in this study. The virus was obtained from Virus Lab/Tumor Immunology Lab, ICAR-IVRI, Izatnagar, India. The Infectivity titer of the virus was $1x10^3$ pock forming units (pk.f.u.)/mL. The virus was handled in biosafety level 2 facilities.

3.3. Tumor induction and categorization of chicks

One week old SDL broiler chicks were subcutaneously infected with Rous Sarcoma Virus (2000 p.f.u/0.2. mL of RSV-A suspension per chick) in the left wing-web. Chicks were observed regularly for the appearance of tumors at the primary site of inoculation. Chicks that developed primary tumor within 2 to 8 days post infection (DPI) and grew progressively and metastasize to other organs were categorized as progressors. Chicks that developed tumor between 8 to 10 (DPI) and started regressing their tumor sometimes before 30 DPI were designated as regressors. The chicks that did not develop tumor at all were deemed as non-responders. A group of uninfected control chicks was simultaneously maintained in the same facility.

3.4. Harvesting of tissues

Three birds were sacrificed at regular intervals post RSV-A challenge, from progressors on 3, 6, 9, 12 and 18 DPI and organs affected by RSV pathogenesis, primary tumor tissue, lung, liver and spleen were collected. At the early stages of infection, it is difficult to determine if a bird will or will not have tumor regression. Therefore, after visualizing the trend of tumor progression/ regression, the regressor birds were sacrificed at two time points, 27 days post infection (dpi) and 45 dpi and the lungs, liver and spleen were collected from regressor chicks. Non-responder birds (i.e. absence of tumor development) can only be identified after at least 8 dpi; therefore, the mRNA expression profiles of different organs in non-responders were determined at 12 and 27 dpi and the lungs, liver and spleen were collected at respective dpis. The mRNA expression profile of uninfected controls was also determined, in lungs, liver, spleen and the muscle from wing web on day zero.

3.5. RNA Extraction and cDNA synthesis

Total RNA from each sample was extracted using 'RNAgents- Total RNA isolation system' (Promega, Madison, WI, USA), according to the manufacturer's instructions. Briefly, the organs from the different groups were collected in RNA Later and fifty mg tissue was added and incubated with 600 µl of denaturating solution. The tissue was homogenized and 60ul of sodium acetate was added. A mixture of phenol choloroform/ isoamyl alcohol (600 µl) was added, mixed vigorously for 10 seconds and chilled on ice for 15 minutes. The mixture was centrifuged at 10,000 x g for 10 minutes at 4°C and RNA was precipitated. The RNA was air dried, dissolved in diethyl pyrocarbonate (DEPC) treated water and stored at –80°C until further use.

The first strands of cDNA from the RNA sample was prepared by Revert Aid™ First strand cDNA synthesis kit (MBI Fermantas). Briefly, the reverse transcription of RNA was performed in a 20 ul final volume Total RNA (1µg), Oligo $(dt)^{18}$ primer (0.5. µg/ml) 1µl and deionized nuclease free water (6µl). The mixture was incubated at 70°C for 5 minutes and chilled on ice immediately. The following components were added: 5x reaction buffer (4µl), ribonucleases inhibitor (20U/µl), 10 mM dNTP mix and incubated at 37°C for 5 minutes. The enzyme Revert Aid™ M.MuLV Reverse transcriptase (200U) was added and the mixture was incubated at 42°C for 60 minutes. The reaction was stopped by heating the mixture to 70°C for 10 minutes. The resultant cDNA was stored at -20°C.

3.6. Quantitative RT-PCR of cytokines

The expression of cytokines (i.e. IFN-γ, IL-6, IL-8, IL-10, TGF-β, TNF-α and GM-CSF) were quantified by mRNA expression using real-time PCR (30) by using Mx3000P™ system (Stratagene). The details of the primers used for each gene are given in Table 1. The amplification was carried out in a volume

Table 1. Primers for mRNA expression

Genes	Primer Sequences (Forward)	Primer Sequences (Reverse)	Accession No.	Annealing Temperature
IL-6	5'CTG CCC AAG GTG ACG GAG GAG GAC 3'	5'GAT TGG CGA GGA GGG ATT TCT GG 3'	AJ250838	52°C
IL-8	5'CTG TCC TGG CCG TCC TCC TGG TT 3'	5'CTT GGC GTC AGC TTC ACA TCT TG 3'	NM_205498.1	52°C
IL-10	5'TGC GGG AGC TGA GGG TGA AGT TTG 3'	5'CGC GGG GCT GGG CTG AGA G 3'	AJ621614	52°C
IFN-γ	5'ACA AGT CAA AGC CGC ACA TC 3'	5'TGG ATT CTC AAG TCG TTC ATC G 3'	AY705909	50°C
TNF-α	5'TGA GTT GCC CTT CCT GT 3'	5'CAG AGC ATC AAC GCA AA 3'	A1979890	52°C
GM-CSF	5'CTG CGC CCA CCA CAA CAT ACT CCT 3'	5'ACG ATT CCG CTT TCT TCC TCT GTC 3'	NM_01007078	55°C
TGF-β2	5'TGC ACT GCT ATC TCC TGA G 3'	5'ATT TTG TAA ACT TCT TTG GCG 3'	NM_01031045	52°C
β-Actin	5'CAT CAC CAT TGG CAA TGA GAG G 3'	5'GCA AGC AGG AGT ACG ATG AAT C 3'	L08165	55°C

of 20 µl containing 1X QuantiTect SYBR Green PCR master mix (QIAGEN GmBH, Germany), 10 pM primers and 1 µl cDNA template. PCR was carried out with standardized cycling conditions as: Initial denaturation: 94°C for 5 min, 40 cycles of denaturation: 94°C for 1min, annealing: x°C for 1 min. (annealing temp. given in Table 1), extension: 72°C for 1 min and final extension: 72°C for 10 min. All PCR reactions were performed in optical 96-well reaction plates in duplicates. For each gene, negative and positive controls were included. The results were expressed in terms of the threshold cycle value (Ct), the cycle at which the change in the reporter dye (DRn) passes the significance threshold. β-actin was used as a housekeeping gene.

3.7. Data Analysis

3.7.1. Generation of standard curve and calculating cytokine mRNA expression

Standard curves were constructed for each of the genes using serially diluted plasmids from 10^{-1} to 10^{-5} containing specific various immune - related gene and the housekeeping gene beta actin separately in each plasmid. The curve was plotted between values for $\log_{10}$cDNA vs Ct. Each RT-PCR experiment contained triplicates of the test samples, one no-template control (NTC) and one no-primer control (NPC) and a $\log_{10}$ dilution series (30). Regression analysis of the standard curve was used to calculate the slopes of the gene specific to $\log_{10}$ dilution series.

The corrected cytokine mRNA per sample was calculated using the following formula:

Corrected cytokine mRNA = ((40- Mean Ct of Target gene) x Target gene slope)/ (Difference factor of sample X Slope of beta actin) (30). The mean 40 – Ct_{target} = the triplicate mean of 40 – Ct value; $Slope_{target}$ = the slope from the standard curve regression equation for the target gene; β-actin df = the triplicate mean of β-actin-specific product of a sample / overall mean for all β-actin samples; and $Slopeb_{-actin}$ = the slope from the standard curve regression equation for the β-actin gene.

3.7.2. Statistical Analysis of mRNA Expression levels (Corrected Ct values) of various genes

The effects of time and different groups on expression of each gene (corrected Ct values) were analyzed by ANOVA. The SPSS version 16.0. software was used to analyze the data. The main fixed effects as different group and time points with interaction were included in the fixed model used.

The following formula was used to calculate corrected Ct value of each gene:

$Y_{ijk} = \mu + G_i + T_j + (G\times T)_{ij} + e_{ijk}$

Y_{ijk} = Corrected Ct Value recorded on k^{th} individual under i^{th} group and j^{th} time point.

μ = Overall mean

G_i = Effect of i^{th} group (i = 1, 2, 3)

T_j = Effect of j^{th} time (j = 1, 2,…, 5)

$(G\times T)_{ij}$ = Interaction effect of i^{th} group and j^{th} time point.

e_{ijk} = Random error distributed with mean 0 and variance σ^2

For significant effects, subclass means were compared using Duncan's multiple range test (Duncan *et al.,* 1995).

4. RESULTS

4.1. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in primary tumors

4.1.1. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in primary tumors from progressor chicks

The expression of IL-6 and IL-8 was significantly upregulated after RSV-A inoculation and was maximal at 9 days post infection (dpi) in primary tumors compared to controls (day zero)

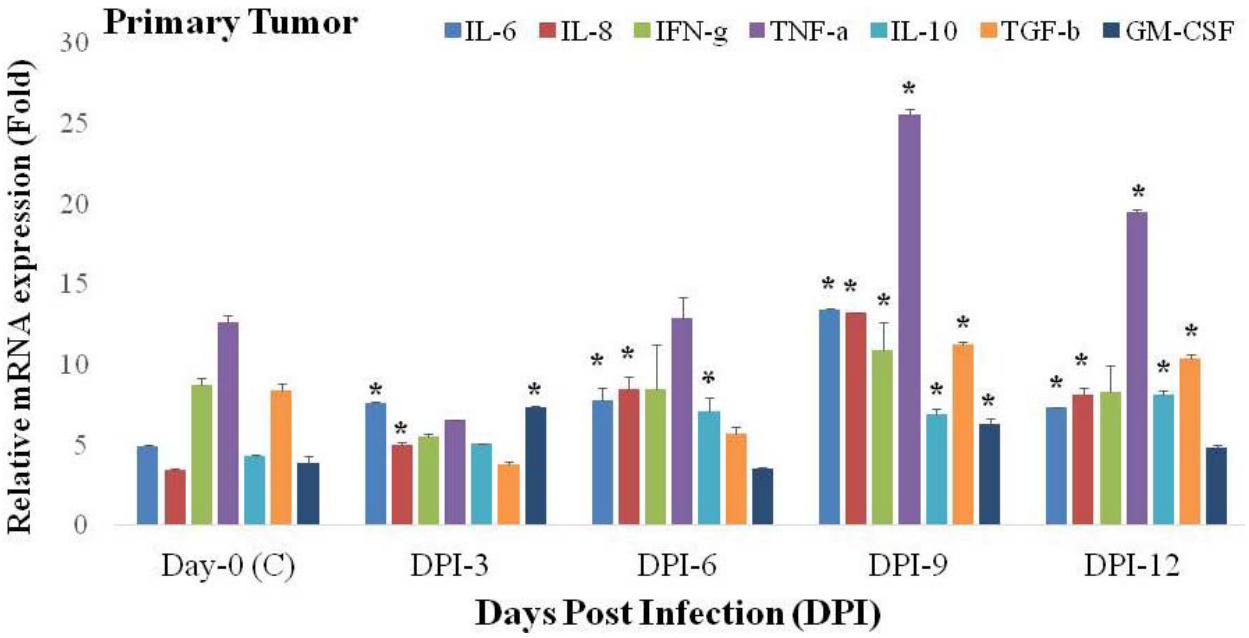

Figure 1. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines at different time points in primary tumors of Progressor chicks. Cytokine mRNA levels are expressed as relative fold - change in infected chicks compared to uninfected controls. Each value represents the mean of three samples, and the vertical bars represent the standard error of the mean. DPI, Days Post Infection; R, Regressor; NR, Non-responder. *, p-value ≤ 0.0.5.

(Figure 1 and Table 2). The expression of IFN-γ was significantly downregulated at 3 dpi as compared to controls; however, it was significantly upregulated at 9 dpi and was downregulated to control level at 12 dpi (Figure 1 and Table 2). The pattern of TNF-α expression was similar to that of IFN-γ, as TNF-α was significantly downregulated at 3 dpi, followed by significant upregulation at 9 and 12 dpi as compared to controls (Figure 1 and Table 2). The expression of IL-10 was significantly upregulated after 6 dpi until 12 dpi as compared to control (Figure 1 and Table 2). The expression of TGF-β was significantly decreased at earlier stages (3 dpi and 6 dpi) compared to controls. However, TGF-B expression was significantly increased from 9 dpi until 12 dpi compared to controls (Figure 1 and Table 2). The expression of GM-CSF was significantly upregulated at 3 dpi and 9 dpi compared to controls. In contrast, the expression of GM-CSF at 6 dpi and 12 dpi was same as that of control (Figure 1 and Table 2).

4.2. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the lungs

4.2.1. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the lungs of progressor chicks

The expression of IL-6 was significantly upregulated at 3 and 6 dpi compared to controls, but was significantly downregulated at 9, 12 and 18 dpi compared to controls (Figure 2 and Table 2). The expression of IL-8 was significantly upregulated at all time points, 3, 6, 9, 12 and 18 dpi as compared to control (Figure 2 and Table 2). The expression of IFN-γ in the lungs was significantly upregulated at 6 and 12 dpi but was not significantly different from controls at 3 and 9 dpi (Figure 2 and Table 2). Further, it was significantly downregulated at 18 dpi compared to controls (Figure 2 and Table 2). The expression of TNF-α was significantly downregulated at 3 dpi, followed by a significant up regulation at all subsequent dpi compared to controls (Figure 2 and Table 2). The expression of IL-10 in the lungs was significantly upregulated at 6 and 18 dpi compared to controls and was not significantly different from the control at all the other time points (Figure 2 and Table 2).

The expression of TGF-β was significantly upregulated at all dpi except for the 9 dpi time period compared to controls (Figure 2 and Table 2). The expression of GM-CSF was significantly upregulated across all of the dpi intervals compared to controls (Figure 2 and Table 2).

4.2.2. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the lungs of regressor chicks

The expression of IL-6 was significantly downregulated at 27 and 45 dpi compared to controls (Figure 2 and Table 2). The expression of IL-8 and

Table 2. *In vivo* expression of Pro-inflammatory, Th1 and Th2 cytokines at different time points in primary tumor and in different organs from Progressor, Regressor and Non-Responder chicks

Day	Proinflammatory cytokines		Th1 cytokines		Th2 cytokines		
PRIMARY TUMOR							
Progressor							
Day-0 (C)	$4.881^{1}\pm0.113$	$3.412^{1}\pm0.081$	$8.688^{1}\pm0.479$	$12.597^{1}\pm0.420$	$4.291^{1}\pm0.052$	$8.394^{1}\pm0.364$	$3.814^{1}\pm0.478$
DPI-3	$7.539^{2}\pm0.100$	$5.001^{2}\pm0.161$	$5.466^{2}\pm0.205$	$6.505^{2}\pm0.013$	$5.023^{1}\pm0.053$	$3.797^{2}\pm0.131$	$7.304^{2}\pm0.070$
DPI-6	$7.702^{2}\pm0.798$	$8.436^{3}\pm0.798$	$8.411^{1}\pm2.778$	$12.901^{1}\pm1.283$	$7.025^{2}\pm0.878$	$5.665^{3}\pm0.392$	$3.504^{1}\pm0.051$
DPI-9	$13.380^{3}\pm0.129$	$13.173^{4}\pm0.044$	$10.891^{3}\pm1.690$	$25.484^{4}\pm0.352$	$6.886^{2}\pm0.303$	$11.196^{4}\pm0.226$	$6.275^{2}\pm0.303$
DPI-12	$7.306^{2}\pm0.033$	$8.066^{3}\pm0.477$	$8.271^{1}\pm1.643$	$19.430^{3}\pm0.208$	$8.114^{3}\pm0.220$	$10.309^{4}\pm0.302$	$4.778^{1}\pm0.210$
LUNG							
Progressor							
Day-0 (C)	$8.162^{1}\pm0.111$	$4.934^{1}\pm0.192$	$10.056^{1}\pm0.633$	$14.689^{1}\pm0.635$	$4.636^{1}\pm0.060$	$7.436^{1}\pm0.340$	$2.658^{1}\pm0.136$
DPI-3	$10.530^{2}\pm0.123$	$8.709^{3}\pm0.079$	$9.733^{1}\pm0.442$	$10.747^{2}\pm0.040$	$4.188^{1}\pm0.083$	$9.858^{2}\pm0.038$	$4.394^{2}\pm0.087$
DPI-6	$10.247^{2}\pm0.371$	$7.706^{2}\pm0.171$	$14.082^{2}\pm0.337$	$21.386^{4}\pm0.461$	$6.936^{2}\pm0.224$	$11.954^{3}\pm0.179$	$4.940^{2}\pm0.104$
DPI-9	$7.511^{1}\pm0.169$	$7.471^{2}\pm0.044$	$10.538^{1}\pm0.950$	$17.613^{3}\pm0.071$	$3.255^{3}\pm0.070$	$6.689^{1}\pm0.027$	$4.073^{2}\pm0.052$
DPI-12	$6.172^{3}\pm0.100$	$8.117^{3}\pm0.046$	$14.594^{2}\pm0.151$	$18.065^{3}\pm0.267$	$4.567^{1}\pm0.089$	$10.165^{2}\pm0.066$	$4.612^{2}\pm0.076$
DPI-18	$6.544^{3}\pm0.149$	$8.176^{3}\pm0.048$	$8.165^{3}\pm0.825$	$19.118^{34}\pm0.159$	$5.091^{1}\pm0.063$	$10.570^{3}\pm0.037$	$4.882^{2}\pm0.037$
Regressor							
DPI-27 (R)	$4.846^{4}\pm0.141$	$6.142^{2}\pm0.021$	$17.459^{4}\pm0.112$	$13.912^{12}\pm0.099$	$5.271^{1}\pm0.011$	$4.448^{4}\pm0.106$	$2.752^{1}\pm0.056$
DPI-45 (R)	$5.478^{3}\pm0.014$	$6.809^{2}\pm0.017$	$19.487^{45}\pm1.593$	$18.482^{3}\pm0.247$	$4.765^{1}\pm0.038$	$8.607^{12}\pm0.127$	$4.917^{2}\pm0.023$
Non-Responder							
DPI-12 (NR)	$6.736^{3}\pm0.891$	$6.022^{2}\pm0.287$	$20.736^{5}\pm0.891$	$22.392^{4}\pm0.354$	$5.298^{1}\pm0.225$	$8.367^{12}\pm0.134$	$6.887^{3}\pm0.256$
DPI-27 (NR)	$3.336^{4}\pm0.285$	$6.961^{2}\pm0.020$	$18.336^{45}\pm0.285$	$19.509^{34}\pm0.222$	$3.539^{3}\pm0.040$	$7.556^{1}\pm0.042$	$5.085^{2}\pm0.207$
LIVER							
Progressor							
Day-0 (C)	$3.929^{1}\pm0.378$	$3.783^{1}\pm0.233$	$13.229^{1}\pm0.027$	$20.056^{2}\pm0.389$	$5.537^{2}\pm0.291$	$6.821^{2}\pm0.196$	$3.307^{1}\pm0.143$
DPI-3	$4.579^{1}\pm0.129$	$8.360^{4}\pm0.065$	$12.099^{1}\pm0.207$	$18.525^{1}\pm0.230$	$8.237^{4}\pm0.128$	$9.136^{3}\pm0.047$	$4.822^{2}\pm0.114$
DPI-6	$5.335^{2}\pm0.212$	$7.330^{4}\pm0.222$	$9.556^{2}\pm0.835$	$23.228^{3}\pm0.099$	$7.682^{3}\pm0.051$	$5.731^{1}\pm0.042$	$5.569^{3}\pm0.116$
DPI-9	$4.379^{1}\pm0.061$	$6.794^{3}\pm0.140$	$13.907^{1}\pm0.009$	$20.145^{2}\pm0.052$	$6.947^{3}\pm0.172$	$5.877^{1}\pm0.067$	$2.947^{1}\pm0.031$
DPI-12	$5.304^{2}\pm0.036$	$6.640^{3}\pm0.206$	$20.113^{3}\pm0.835$	$21.420^{2}\pm0.256$	$7.110^{3}\pm0.212$	$8.059^{3}\pm0.224$	$4.204^{2}\pm0.090$
DPI-18	$6.558^{3}\pm0.200$	$6.765^{3}\pm0.253$	$12.327^{1}\pm0.554$	$22.904^{3}\pm0.669$	$3.321^{1}\pm0.028$	$9.368^{3}\pm0.112$	$4.697^{2}\pm0.206$
Regressor							
DPI-27 (R)	$4.447^{1}\pm0.053$	$5.982^{3}\pm0.090$	$11.256^{1}\pm1.306$	$17.159^{1}\pm0.405$	$7.650^{3}\pm0.047$	$10.880^{4}\pm0.062$	$4.928^{2}\pm0.087$
DPI-45 (R)	$3.088^{1}\pm0.135$	$4.655^{2}\pm0.271$	$12.648^{1}\pm1.284$	$25.399^{4}\pm0.006$	$5.789^{2}\pm0.401$	$13.209^{5}\pm0.347$	$6.034^{3}\pm0.056$
Non-responder							
DPI-12 (NR)	$3.804^{1}\pm0.183$	$3.215^{1}\pm0.342$	$19.604^{3}\pm0.068$	$26.230^{4}\pm0.570$	$6.791^{3}\pm0.194$	$10.037^{4}\pm0.417$	$5.120^{2}\pm0.155$
DPI-27 (NR)	$1.881^{4}\pm0.078$	$2.866^{1}\pm0.076$	$11.023^{1}\pm1.455$	$19.011^{1}\pm0.141$	$5.997^{2}\pm0.041$	$7.833^{2}\pm0.398$	$4.634^{2}\pm0.011$
SPLEEN							
Progressor							
Day-0 (C)	$6.818^{1}\pm0.125$	$4.893^{1}\pm0.198$	$12.753^{1}\pm0.862$	$22.642^{2}\pm0.178$	$4.318^{1}\pm0.278$	$8.398^{2}\pm0.161$	$3.705^{1}\pm0.006$
DPI-3	$14.384^{4}\pm0.011$	$15.312^{4}\pm0.039$	$24.412^{5}\pm0.350$	$33.230^{5}\pm0.123$	$8.691^{5}\pm0.212$	$10.176^{3}\pm0.050$	$7.548^{3}\pm0.143$
DPI-6	$10.074^{3}\pm0.147$	$8.097^{3}\pm0.089$	$16.693^{3}\pm0.613$	$28.036^{4}\pm0.806$	$7.313^{4}\pm0.165$	$7.929^{2}\pm0.115$	$7.835^{3}\pm0.284$
DPI-9	$10.187^{3}\pm0.053$	$8.751^{3}\pm0.004$	$17.965^{3}\pm0.943$	$22.947^{2}\pm0.467$	$5.063^{3}\pm0.130$	$4.995^{1}\pm0.135$	$3.975^{1}\pm0.152$
DPI-12	$6.472^{2}\pm0.226$	$5.887^{2}\pm0.244$	$17.903^{3}\pm0.903$	$19.134^{1}\pm0.291$	$2.988^{2}\pm0.054$	$5.822^{1}\pm0.228$	$3.113^{1}\pm0.157$
DPI-18	$6.336^{2}\pm0.173$	$7.106^{3}\pm0.105$	$12.988^{1}\pm0.579$	$22.940^{2}\pm0.438$	$4.788^{1}\pm0.257$	$7.902^{2}\pm0.099$	$4.131^{1}\pm0.035$

Regressor							
DPI-27 (R)	7.761[2]±0.021	6.218[2]±0.081	10.352[1]±1.755	24.828[3]±0.400	4.500[1]±0.098	8.196[2]±0.143	6.865d±0.107
DPI-45 (R)	7.154[2]±0.299	6.312[2]±0.234	16.214[3]±0.194	25.226[3]±0.150	3.819[2]±0.004	9.839[3]±0.476	5.323[2]±0.210
Non-responder							
DPI-12 (NR)	5.544[1]±0.183	4.767[1]±0.066	19.481[4]±0.527	31.115[5]±0.159	5.827[3]±0.126	11.578[4]±0.110	7.538[3]±0.080
DPI-27 (NR)	4.063[1]±0.023	4.888[1]±0.220	14.295[2]±0.187	26.036[4]±0.379	4.402[1]±0.112	8.456[2]±0.179	5.359[2]±0.057

The means bearing different superscript differ significantly. (p≤0.05). Values represent means ± SEM

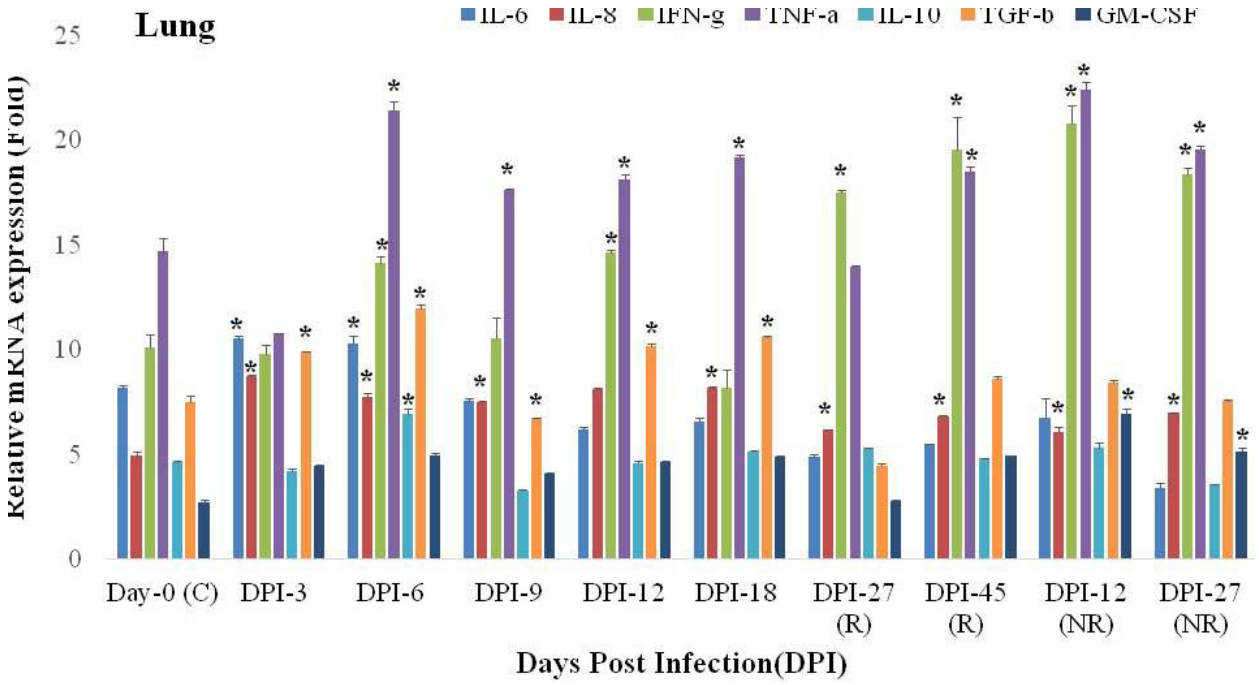

Figure 2. *In vivo* expressions of mRNA for pro-inflammatory, Th1 and Th2 cytokines at different time points in the lungs of Progressor, Regressor and Non-Responder chicks. Cytokine mRNA levels are expressed as relative fold - change in infected birds compared to uninfected controls. Each value represents the mean of three samples, and the vertical bars represent the standard error of the mean. DPI, Days Post Infection; R, Regressor; NR, Non-responder. *, p-value ≤ 0.0.5.

IFN-γ was significantly upregulated at both the time points compared to controls (Figure 2 and Table 2). The expression of TNF-α was not significantly different from controls at 27 dpi, but was significantly upregulated at 45 dpi compared to controls (Figure 2 and Table 2). IL-10 expression was significantly upregulated at 27 dpi compared to controls but decreased to the level of control by 45 dpi (Figure 2 and Table 2). TGF-β was significantly downregulated at 27 dpi but later on increased and was same as control at 45dpi (Table 2, Figure 3). GM-CSF expression was same as of control at 27 dpi but was significantly upregulated at 45 dpi (Figure 2 and Table 2).

4.2.3. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the lungs of non-responder chicks

The expression pattern of non-responder chicks was determined at two time intervals, 12 dpi and 27 dpi. The expression of IL-6 was significantly down regulated at 12 dpi and 27 dpi compared to controls (Figure 2 and Table 2). The expression of IL-8 was significantly upregulated at 12 and 27 dpi compared to controls (Figure 2 and Table 2). The expression of IFN-γ, TNF-α and GM-CSF was significantly elevated at 12 and 27 dpi compared to controls (Figure 2 and Table 2). The expression of IL-10 was significantly greater at 12 dpi than controls, whereas expression was significantly lower than controls at 27 dpi. (Figure 2 and Table 2). TGF-β expression was same as that of control at 12 and 27 dpi (Figure 2 and Table 2).

4.3. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the liver

4.3.1. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the liver of progressor chicks

The expression of IL-6 and IL-8 was upregulated from 3 dpi until the end of the experiment

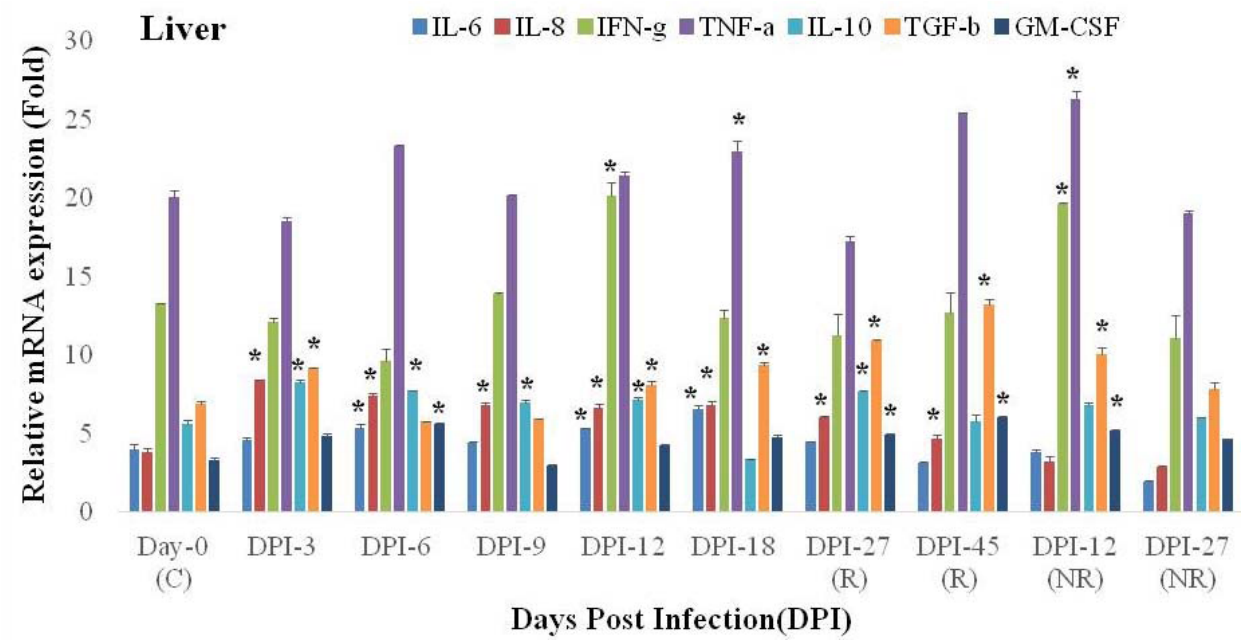

Figure 3. *In vivo* expression of mRNA pro-inflammatory, Th1 and Th2 cytokines at different time points in the liver of Progressor, Regressor and Non-Responder chicks. Cytokine mRNA levels are expressed as relative fold - change in infected birds compared to uninfected controls. Each value represents the mean of three samples, and vertical bars represent the standard error of the mean. DPI, Days Post Infection; R, Regressor; NR, Non-responder. *, p-value ≤ 0.0.5.

compared to controls (Figure 3 and Table 2). The expression of IFN-γ, compared to controls, was significantly lower at 6 dpi, but was significantly upregulated at 12 dpi (Figure 3 and Table 2). The expression of TNF-α was significantly upregulated at 6, 12 and 18 dpi compared to controls (Figure 3 and Table 2). The expression of IL-10 was significantly upregulated at 3, 6, 9 and 12 dpi compared to controls; however, expression was significantly decreased at 18 dpi compared to controls (Figure 3 and Table 2). TGF-β expression was significantly upregulated at all dpi, except at 6 and 9 dpi, where expression was significantly decreased compared to controls (Figure 3 and Table 2). The expression of GM-CSF was significantly upregulated at all time points compared to control, except at 9 dpi, which was significantly lower to controls. (Figure 3 and Table 2).

4.3.2. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the liver of regressor chicks

Compared to controls, IL-6 expression was significantly higher at 27 dpi (Figure 3 and Table 2). The expression of IL-8 was significantly greater than controls at 27 and 45 dpi (Figure 3 and Table 2). IFN-γ expression was significantly downregulated at 27 and 45 dpi compared to controls (Figure 3 and Table 2). TNF-α expression was significantly lower at 27 dpi but significantly higher at 45 dpi compared to controls (Figure 3 and Table 2). The expression of IL-10 was significantly upregulated at 27 dpi (Figure 3 and Table 2). The expression level of TGF-β and GM-CSF were significantly increased both at 27 and 45 dpi compared to controls (Figure 3 and Table 2).

4.3.3. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the liver of non-responder chicks

The expression of IL-6 was significantly downregulated at 27 dpi compared to controls, whereas the expression of IL-8 was not significantly different from the controls (Figure 3 and Table 2). Compared to controls, the expression of IFN-γ and TNF-α was upregulated at 12 dpi, but was significantly downregulated at 27 dpi (Table 2 and Figure 4). The expression of IL-10 was not significantly different from the controls (Figure 3 and Table 2). TGF-β expression was significantly upregulated at 12 dpi compared to controls and GM-CSF expression were significantly increased at 12 and 27 dpi compared to controls (Figure 3 and Table 2).

4.4. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the spleen

4.4.1. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the spleen of progressor chicks

The expression of IL-6 was significantly upregulated from 3 to 9 dpi, with the maximum at 3 dpi, compared to controls (Figure 4 and Table 2). IL-8

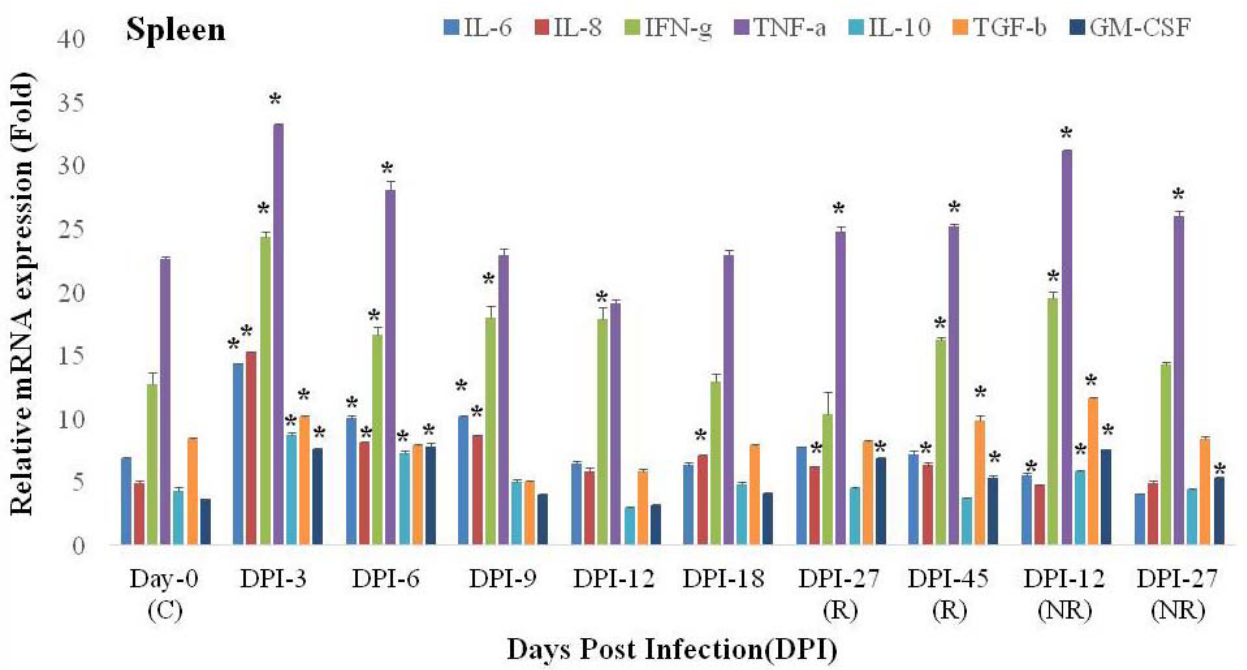

Figure 4. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines at different time points in the spleen of Progressor, Regressor and Non-Responder chicks. Cytokine mRNA levels are expressed as relative fold - change in infected birds compared to uninfected controls. Each value represents the mean of three samples, and the vertical bars represent the standard error of the mean. DPI, Days Post Infection; R, Regressor; NR, Non-responder. *, p-value ≤ 0.0.5.

expression was significantly upregulated at 3, 6, 9, 12, 18 dpi compared to controls, with the maximum increase at 3 dpi (Figure 4 and Table 2). IFN-γ and TNF-α expression were significantly increased from 3 to 9 dpi, with a significant increase at 3 dpi, compared to controls (Figure 4 and Table 2). The expression of IL-10, compared to controls, was significantly upregulated from 3 to 9 dpi, but was significantly downregulated at 12 dpi (Table 2 and Figure 5). TGF-β expression was significantly increased at 3 dpi, followed by a significant downregulation at 6, 9 and 12 dpi compared to controls (Figure 4 and Table 2). GM-CSF expression was increased at 3 and 6 dpi compared to controls. (Figure 4 and Table 2).

4.4.2. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the spleen of regressor chicks

The expression of IL-6 was same as of control at both 27 and 45 dpi (Figure 4 and Table 2). IL-8, TNF-α and GM-CSF expression were significantly upregulated at both 27 and 45 dpi compared to controls (Figure 4 and Table 2). IFN-γ and TGF-β expression at 27 dpi was not significantly different from of controls, but was significantly upregulated at 45 dpi compared to controls (Figure 4 and Table 2). The expression of IL-10 was not significantly different from controls at 27 dpi, but was significantly downregulated at 45 dpi compared to controls (Figure 4 and Table 2).

4.4.3. *In vivo* expression of mRNA for pro-inflammatory, Th1 and Th2 cytokines in the spleen of non-responder chicks

The expression of IL-6 was significantly downregulated at both 12 and 27 dpi compared to controls (Figure 4 and Table 2). IL-8 expression was not significantly different from controls at 12 and 27 dpi (Figure 4 and Table 2). The expression of IFN-γ, TNF-α and GM-CSF were significantly upregulated at 12 and 27 dpi compared to controls (Figure 4 and Table 2). IL-10 and TGF-β showed upregulated expression at 12 dpi compared to control (Figure 4 and Table 2).

5. DISCUSSION

The cytokines released from specific immune cells can produce a rapid response against viruses as well as other pathogens (31, 32). Pro-inflammatory cytokines and the balance of Th1 vs Th2 cytokines produced during infection play a pivotal role in susceptibility or resistance to various infectious diseases (33-36).

Pro-inflammatory cytokines, and the chemokines, such as IL-6 and IL-8, are released by the activation of the innate immune response in mammals (37, 38). Upon activation by bacterial or viral particles or injury, pro-inflammatory cytokines play a key role in mediating inflammation (39, 40). Th1 cytokines (e.g. IFN-γ, TNF-α) are involved in the induction of cell-

mediated immunity (41). In contrast, Th2 or Th2 - related cytokines, such as IL-10, TGF-β and GM-CSF (42, 43), are involved in the activation of humoral immunity (44, 45). In the present study, the inoculation of susceptible chickens with RSV resulted in RSV- infected responders that included progressors and regressors and RSV infected non-responders, compared to RSV uninfected control chicks. We evaluated the cytokine response to RSV infection in SDL broiler chicks in different organs of progressors, regressors and non-responders at different times post-inoculation.

Generally as mRNA level increases, protein activity also increases. However, this might not always be true and the gene expression may not project the full image for genes and their functions, as increased expression of genes might be related to increase synthesis of m-RNA or decreased degradation or suppression of siRNAs. Also, the genes might not be translated efficiently or might escape such translation. Therefore, protein quantification is important; however, if very little is known about when and why a gene will be expressed, expression profiling under different conditions is the best method to determine a cell's function from a single experiment. Hence, by determining mRNA expression we determined which genes were expressed in an organ at different stages of disease at the level of transcription that could give a global picture of cellular function.

Our results indicated a significantly greater expression of IL-6 mRNA expression in the early phase of infection (i.e 3 to 9 dpi) in all the organs compared to controls. The primary tumor, however, had significantly higher levels of IL-6 level throughout the length of the study. IL-6 is a proinflammatory cytokine and its elevated levels in the early phases of infection is indicative of an acute-phase response in susceptible chickens (46, 47). IL-6 inhibits apoptosis and may be involved in malignant transformation and tumor progression (48). Similar to our results, Kaiser *et al.* reported that IL-6 mRNA levels were significantly increased in spleenocytes between 3 and 5 dpi in susceptible chickens infected with Marek's disease virus (49, 50). Our results indicated that the expression of IL-6 was significantly lower in the liver compared to other organs, indicating that the liver was least affected by RSV infection. In addition, the lungs, liver and spleen from the regressor and non-responder groups had a significantly lower expression of IL-6, suggesting a protective response against RSV infection. Similarly, Abdul Careem *et al.* reported that the expression of IL-6 was significantly lower in chickens in the Herpes virus of turkeys (HVT) vaccinated group as compared to unvaccinated controls (51). However, in a study Xing and Schat did not detect IL-6 expression following MDV infection in chickens and this difference could be due to a pathogen - specific response of the host immune system (52).

The cytokine IL-8 produces inflammation and is a chemotactic factor for monocytes and lymphocytes (53-55). In the present study, IL-8 mRNA expression was significantly increased in all the organs, i.e. primary tumor, lung, liver and spleen, as the disease progressed and it was maximum at 3 dpi. This upregulation of IL-8 may have played a role in recruiting and activating neutrophils in response to the infection and help to control the RSV infection. Xing and Schat reported that IL-8 mRNA was expressed in the spleens of MDV-infected chicks 3 days post infection (52). In our study, the regressor and non-responder groups had a low level of IL-8 expression in all of the organs.

IFN-γ is a cytokine that mediates resistance to many different pathogens (56-58). In the current study, the expression of IFN-γ mRNA was initially downregulated in the lungs, liver and primary tumor of chicks in the progressor group and thereafter, levels were upregulated in the later stages of RSV infection. In the spleen, an upregulation occurred in the progressor group. The lungs, liver and spleen of regressor and non-responder chicks were upregulated at all the stages of RSV infection. The initial down regulation of IFN-γ in various organs of progressors suggested a severe immunosuppression, which would have enhanced RSV pathogenesis and diminished viral clearance, as reported by Price *et al.* (59). Thus, decreased IFN-γ levels would have resulted in tumor progression in progressor chicks as was found in present study also. The relatively low expression of IFN-γ in all of the organs of progressor chicks is indicative of immunosuppression, as well as the lack of a sufficient immune response by progressor chicks, leading to viral - induced pathogenesis, resulting in an increase in tumor volume. The most severe suppression was present in the primary tumor, i.e. the site of viral inoculation. In the spleen, upregulated IFN-γ mRNA levels suggested viral clearance and an anti-RSV response by the spleen of the host. Also, the upregulation of IFN-γ in regressor and non-responder chicks in present investigation suggested a sustained and sufficient immune response by host, thereby limiting viral pathogenesis. Consequently, this results in the regression of tumor size in regressor chicks or no tumor growth in non-responder chicks. Similar results were reported by Kaiser *et al.*, wherein IFN-γ mRNA expression was significantly repressed in most of the genetic lines infected with MDV by 21 days post - infection (50). Hong and Sevoian also reported that IFN levels were higher in resistant (K strain) than susceptible (S strain) chickens infected with Avian Leucosis Virus (ALV, JM strain) (60). Xing and Schat reported an increase in IFN-γ expression in the spleen of chickens following MDV infection between 3 to 15 dpi (52). Abdul Careem *et al.*reported an increase in IFN-γ expression in the spleens of vaccinated, unprotected chickens, suggesting that the increase in

IFN-gamma levels production was positively correlated with disease progression (51).

Tumor necrosis factor alpha (TNF-α) plays an important role in immunity, inflammation and apoptosis (61-66), helping in maintaining homeostasis by replacing injured and senescent tissue and stimulating necrosis of specific tumors (67-73). However, high levels of TNF-α promotes the growth of other types of tumor cells, increasing the risk of mortality (74-77). Moreover, the prolonged overproduction of TNF-α causes cachexia (78, 79). TNF-α has been reported to be a pro-tumor molecule in a number of cancers (80, 81). In the present study, there was a significant dowregulation of TNF-α at 3 dpi in the progressor group of chickens. This was followed by a significant upregulation during the later stages, in all the organs, except for the spleen. It is likely that the prolonged upregulation of TNF-α in primary tumors, lungs and liver increased the likelihood of immunosuppression, leading to tumor progression. Furthermore, if TNF-α levels remain elevated for an extended period of time, its anti-tumor efficacy decreases, thereby leading to disease progression (82, 83). However, after an initial upregulation of TNF-α in the spleen of progressors, there was a trend toward a decrease in expression, which may have been useful in controlling viral pathogenesis. The increase in TNF-α expression may have produced an anti-tumor effect in the regressor and non-responder groups. Initially, the lungs and liver of regressors expressed a low level of TNF-α, but at a later stage, TNF-α was upregulated, thereby potentially increasing tumor necrosis, although this remains to be verified. It is important to note that TNF-α is an acute phase protein that initiates the synthesis of numerous cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to the site of infection, increasing tumor regression (84-87). Similarly, the lungs, liver and spleen of non-responders expressed high levels of TNF-α, thereby increasing the probability of an anti tumor effect.

IL-10 is produced by activated macrophages or cytotoxic T-cells (88-90) and is involved in cell-mediated immunity (91, 92). IL-10 decreases the levels of Th1 cytokines (93, 94) and inhibits the actions of NK cells during the immune response to viral infection (95). In the present study, an early upregulation of IL-10 in the lungs, liver, spleen and primary tumor would have increased viral pathogenesis. IL-10 suppresses immune and inflammatory responses by inhibiting the tumoricidal capacity of macrophages by modulating cytotoxicity and cytokine production of tumor-specific T-cells and blocking the presentation of antigens by antigen-presenting cells (96, 97). In primary tumors, the expression of IL-10 continuously increased, which would have produced immunosuppression and RSV pathogenesis, leading to an increase in the size of the tumors. IL-10 also inhibits the expression of IFN-γ in chickens (93), which has antiviral efficacy (98, 99). Thus, high level of IL-10 and low level of IFN-γ could have significantly increased the severity of the tumors in the progressor group. The lungs, liver and spleen in the regressor and non-responder groups had a higher expression of IL-10 during the early stages of RSV infection, whereas in the later stages, IL-10 expression decreased to basal levels, thereby inhibiting tumor growth. Hagenbaugh *et al.* reported that IL-10 transgenic mice injected with Lewis lung carcinoma cells developed larger tumors than control mice, suggesting that the production of IL-10 prevents the development of an effective immune response against the tumor cells (100). In Marek's disease, infected chickens had significantly higher IL-10 expression compared to vaccinated chickens (51). Studies have reported that the upregulation of IL-10 may have contributed to the severity of -Newcastle diseases virus (NDV) pathogenesis (101).

TGF-β can induce apoptosis in chicken lymphocytes, resulting in viral pathogenesis (102, 103). In the present study, there was a significant upregulation of TGF-β in the lungs and liver, which could produce immunosuppression. In the primary tumor, the downregulation of TGF- β in the early stages could have sustained viral spread. In contrast, the upregulation of TGF-β in the later stages of RSV infection could have increased viral pathogenesis. However, the decreased expression of TGF-β in the spleen of the progressor group at the later stages could have increased the immunity of the host against RSV infection. The lungs and spleen of the regressor and non-responder groups had a slightly higher expression of TGF-β compared to controls, but this could have increased the mean survival time by inducing tumor regression. However, the liver of the regressor and non-responder groups would not have sustained viral pathogenesis as indicated by an increase in TGF-β expression.

GM-CSF is produced and secreted by activated T cells, macrophages, endothelial cells, and fibroblasts (104, 105). It has been reported that GM-CSF inhibits tumor growth and metastasis (106), although GM-CSF can enhance tumor progression (107). Furthermore, GM-CSF can produce significant anti-proliferative (108-110) or anti-apoptotic effects (111-113), depending on the tumor type and the stage of development (114). In the present study, an early up-regulation of GM-CSF occurred in all the organs of the progressor group of chickens may have induced over expression of IL-6, hence favoring viral pathogenesis. In this study, the GM-CSF expression level in the regressor and non-responder group of chickens was also increased compared to controls, but was not significantly different from GM-CSF expression in the progressor group. Overall, in this study, it is unlikely that GM-CSF did not significantly affect the induction, progression, or regression of primary tumors.

6. CONCLUSION

The development of molecular-based immunotherapeutic strategies for controlling neoplastic transformation in poultry requires a better understanding of the cytokine network. In humans, cytokines not only modulate tumor growth and the immune response, but they may also play a role in producing cancer-related symptoms and chronic debilitation (115). The results presented herein characterized the dynamics of specific pro-inflammatory, Th1 and Th2 cytokines. In progressor chicks, the pro-inflammatory and Th2 cytokines (IL-6, IL-8, IL-10) were significantly upregulated, whereas the Th1 cytokine particulary IFN- γ was downregulated, producing severe immunosuppression in the host and leading to RSV induced - pathogenesis. The expression pattern of the various cytokines in regressor and non-responder chicks produced some magnitude of resistance to RSV infection. We hypothesize that the expression pattern of IL-6, IL-8, IL-10 and IFN-γ be considered when developing immunotherapeutic drugs or vaccines against cancer. However, further elucidation of the interactions between tumor cells and cytokines would be important for the development of more efficacious, novel treatments.

7. ACKNOWLEDGMENT

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