

# Effects of Reticuloendotheliosis Virus Infection on Cytokine Production in SPF Chickens

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## Abstract

Infection with reticuloendotheliosis virus (REV), a gammaretrovirus in the *Retroviridae* family, can result in immunosuppression and subsequent increased susceptibility to secondary infections. The effects of REV infection on expression of mRNA for cytokine genes in chickens have not been completely elucidated. In this study, using multiplex branched DNA (bDNA) technology, we identified molecular mediators that participated in the regulation of the immune response during REV infection in chickens. Cytokine and chemokine mRNA expression levels were evaluated in the peripheral blood mononuclear cells (PBMCs). Expression levels of interleukin (IL)-4, IL-10, IL-13 and tumor necrosis factor (TNF)- $\alpha$  were significantly up-regulated while interferon (IFN)- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-3, IL-15, IL-17F, IL-18 and colony-stimulating factor (CSF)-1 were markedly decreased in PBMCs at all stages of infection. Compared with controls, REV infected chickens showed greater expression levels of IL-8 in PBMCs 21 and 28 days post infection. In addition, REV regulates host immunity as a suppressor of T cell proliferative responses. The results in this study will help us to understand the host immune response to virus pathogens.

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## Introduction

Reticuloendotheliosis viruses (REVs) are a group of viruses in the family *Retroviridae*, specifically gammaretroviruses in the same genus as mammalian C-type retroviruses [1]. The REV group includes defective REV-T [2,3], non-defective REV-A [4,5], chick syncytial virus [6], duck infectious anemia virus [7] and spleen necrosis virus (SNV) [8]. The non-defective REV-A virus has a 8.7-kb genome consisting of a group-specific antigen (gag), polymerase (pol) and envelope (env) genes flanked by long-terminal repeats (LTRs) [9].

REVs cause immunosuppression, runt disease, and lymphoma in a variety of avian hosts that include chickens, turkeys, ducks, geese, pheasants, peafowl, and some other bird species [10]. Some studies have shown that REVs are important cofactors for a number of avian diseases [11–13]. In addition, REV infection has also been associated with poor immune responses to chicken vaccines [14]. The enhancement of these diseases by concomitant REV infection is the most likely consequence of immunosuppression, but the mechanism of REV-induced immunosuppression has not been completely characterized.

Cytokines play a key role in the innate immune system [15]. Most cytokines have pleiotropic or redundant functions, and the level of one cytokine is tightly regulated by other cytokines. For example, an increase in Th2 cytokines (e.g. IL-4 and IL-10) can result a decrease in Th1 cytokines (e.g. IFN- $\gamma$  and IL-2) [16]. Therefore, it is important to examine multiple cytokines in response to REV infection to understand the roles of cytokines in viral pathogenicity. To date, several studies have focused on the effects of REV on only a few pro-inflammatory cytokines [17,18]. Other important pro-inflammatory cytokines, anti-inflammatory cytokines, and chemokines that have been associated with other retrovirus infections and pathogenicity have not been studied [19,20].

The bDNA assay, a sandwich nucleic acid hybridization platform in which targets are captured through cooperative hybridization of multiple probes, detects RNA directly, without either a reverse transcription step or polymerase chain reaction process. This assay provides a powerful method to obtain reliable measurements of multiple-gene expressions and ensures high assay specificity [21].

The main aims of this study were: 1) to determine the effect of REV-A infection on expression of mRNA for Th1-related

cytokines (IFN- $\gamma$ , IL-2, IL-15 and IL-18), Th2-related cytokines (IL-4, IL-10 and IL-13), other cytokines (IL-1 $\beta$ , IL-3, IL-17F, IFN- $\alpha$ , IFN- $\beta$ , TNF- $\alpha$ , and CSF-1) and chemokine IL-8, in specific pathogen free (SPF) White Leghorn chickens; 2) to determine the effect of REV-A infection on T cell proliferation and the balance of CD4<sup>+</sup>/CD8<sup>+</sup>.

## Materials and Methods

### Ethics Statement

Care of laboratory animals and animal experimentation were conducted following “the Australian National Health and Medical Research Council’s Australian Code of Practice for the Care and Use of Animals for Scientific Purposes” guidelines for housing and care of laboratory animals. All animal studies were approved by the Animal Ethics Committee of Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (SYXK (Hei) 2011022).

### Experimental animals and infection virus strain

All the chickens used in this experiment were one-day-old SPF White Leghorn chickens obtained from Harbin Veterinary Research Institute, The Chinese Academy of Agricultural Sciences. Chickens were kept in isolators at Harbin Veterinary Research Institute throughout the experiment.

Chickens were infected with the HLJ071 strain of REV-A (GenBank accession no. GQ375848) that was isolated from Heilongjiang Province of China in 2007. REV was propagated in chicken embryo fibroblast (CEF) as previously described [22].

### Experimental design

Forty one-day-old SPF chickens were randomly divided into two groups and were housed in the isolators. One group of chickens (n = 20) was inoculated intra-abdominally with 10<sup>4.6</sup> tissue culture infective doses 50% (TCID<sub>50</sub>) of the REV-A HLJ071 strain on day 3 of age. The rest (n = 20) were kept as uninfected controls. Infected and uninfected control chickens were kept in separate isolators with similar environmental conditions. On 7, 14, 21 and 28 days post infection (dpi), representing different stages of REV pathogenesis, five chickens were randomly selected from each group. Chicken peripheral blood mononuclear cells (PBMCs) were isolated from whole blood over a discontinuous density gradient of Ficoll-Histopaque (density = 1.077 g/ml), washed twice in PBS, and the number of viable cells was determined by an automatic cell counter (NucleoCounter, NC-100, Chemometech, Denmark). At the end of the experiment, chickens were anesthetized by CO<sub>2</sub> inhalation and euthanized by cervical dislocation.

### Qualitative RT-PCR assay

The viral RNA copy numbers in the PBMCs were determined by quantitative real-time RT-PCR. RNA was extracted from PBMCs using TRIzol<sup>®</sup> (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions and samples were subsequently subjected to DNase treatment (Invitrogen,

Carlsbad, CA, USA). Total RNA in each sample was measured using a Spectrophotometer (NanoVue, GE Healthcare, Uppsala, Sweden). cDNA was synthesized in a final volume of 30  $\mu$ L containing 6  $\mu$ L of 5 $\times$ AMV buffer, 0.5  $\mu$ L of avian myeloblastosis virus (AMV) reverse transcriptase (Takara, Shiga, Japan), 2  $\mu$ L of oligo (dT), 2  $\mu$ L of 10 mmol/L deoxynucleotide triphosphate mix, 1  $\mu$ L of Cloned Ribonuclease Inhibitor, 3.5  $\mu$ L of RNase-free water, and 5  $\mu$ L of total RNA. The reaction was done at 25°C for 10 min and 42°C for 60 min. The synthesized cDNA was stored at -20°C until used in the real-time PCR. The absolute REV genome load in the REV-infected chicken’s PBMCs was quantified using primers specific for REV-gag gene. The primers used were: forward primer (5' AGACTCGCATTGTCGATGCTTTG 3') and reverse primer (5' CAAATCTTTGCCAATCAA TATCAG 3'). Linear regression analysis of the standard curve was used to estimate the number of viral genomic RNA copies. The standard RNA curve was linear in the range between 10<sup>2</sup> molecules at the lower limit and 10<sup>9</sup> molecules at the upper limit. A real time-PCR assay was performed in a total volume of 20  $\mu$ L containing 10  $\mu$ L of SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> (2 $\times$ ; Takara, Shiga, Japan), 100 ng of cDNA, 10 pmol of forward primer, and 10 pmol of reverse primer using a LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche Diagnostics). The PCR protocol consisted of an initial denaturation step at 95°C for 120 s and 40 cycles of denaturation (95°C for 15 s), annealing (61°C for 30 s) and extension (72°C for 15 s). For each step, the temperature transition rate was 20°C/s. Experiments on each sample were performed in triplicate with the above primers. The formula used to quantify the relative amount of gene expression was 2<sup>- $\Delta$ CT</sup>. The absolute numbers of REV genome per 10<sup>6</sup> cells were calculated based on the standard curve.

### Measurement of cytokine and chemokine expression by bDNA assay

In this study, specific oligonucleotide probe sets for target genes (Table 1) for use in QuantiGene Plex 2.0 Reagent System (Affymetrix Inc., Santa Clara, California, USA) were designed by standard probe design software. bDNA analysis was performed using the reagents provided by the manufacturer (Affymetrix Inc., Santa Clara, California, USA) in a three-step procedure, which included specimen preparation, hybridization, and detection. Briefly, PBMCs from treated (infected with REV-A HLJ071 strain) or untreated chickens in microfuge tubes were mixed with 80  $\mu$ L of lysis mixture provided by QuantiGene Sample Processing Kit (Affymetrix Inc., Santa Clara, California, USA) and incubated at 50°C for 1 h to release mRNA. Aliquots of 80  $\mu$ L of lysate were transferred to capture plates, which contained 20  $\mu$ L of pooled specific probes, and were incubated for hybridization with the probes for 20 h at 54°C. The hybridization mixtures were removed, and microspheres in the capture plates were washed three times with wash buffer to remove unbound material. For signal amplification and hybridization, a volume of 100  $\mu$ L of 2.0 Pre-Amplifier Working Reagent (Affymetrix Inc., Santa Clara, California, USA) was added to each well, and the plates were incubated at 50°C for 1 h. After

**Table 1.** Regions of target genes that were used to design bDNA probe sets for the amplification of the mRNAs of 15 cytokines/chemokines and the internal control.

	Names	Sequence	Probe set	GenBank Accession
		length	region	Number
Cytokines	IFN- $\alpha$	767	19-431	NM_205427
	IFN- $\beta$	612	67-528	NM_001024836
	IFN- $\gamma$	1330	193-856	NM_205149
	IL-1 $\beta$	1107	351-709	NM_204524
	IL-2	798	75-705	NM_204153
	IL-15	857	77-718	NM_204571
	IL-17F	935	2-522	NM_204460
	IL-18	724	2-590	NM_204608
	IL-3	417	3-388	NM_001007083
	Csf1	813	17-534	NM_001193295
	IL-4	411	11-362	NM_001007079
	IL-10	528	39-394	NM_001004414
	IL-13	417	10-370	NM_001007085
	TNF- $\alpha$	740	37-479	NM_204267
Chemokine	IL-8	1182	25-650	NM_204608
Internal control	GAPDH	1288	269-503	NM_204305

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washing three times with wash buffer, a volume of 100  $\mu$ L of Amplifier Working Reagent (Affymetrix Inc., Santa Clara, California, USA) was added to each well, and the plates were incubated at 50°C for 1 h. Plates were then washed three times as described above and incubated with 100  $\mu$ L of Label Probe Working Reagent (Affymetrix Inc., Santa Clara, California, USA) at 50°C for 1 h. These plates were washed again three times, and 100  $\mu$ L of the fluorescent reagent SAPE Working Reagent (Affymetrix Inc., Santa Clara, California, USA) was added to each well. The fluorescence intensity of SAPE was proportional to the amount of mRNA transcript captured by the microspheres in each well. The amount of multiple-target mRNAs in each sample was simultaneously determined by measuring the wavelengths of color-coded microspheres and the intensities of the luminescent emission of SAPE using a Luminex 200 (Molecular Devices).

All data were analyzed using Luminex IS 2.3 program. An acquisition gate between 5000 and 20,000 was set to exclude any doublet events and ensure that only single microspheres were measured. One hundred events per region were collected. For all samples in the bDNA assay, the background signal was determined in the absence of target mRNAs and was subtracted from the signal obtained in the presence of target mRNAs. The expression levels of the cytokines and chemokines were normalized to GAPDH. Three independent experiments were performed for each treatment.

### Cell proliferation assay

PBMCs were isolated from heparinized peripheral blood of REV-A infected chickens or controls using Ficoll-Histopaque gradient centrifugation method and labeled with 2.5  $\mu$ M CFSE

(5-carboxyfluorescein diacetate succinimidyl ester, Invitrogen, Carlsbad, CA). Briefly, PBMCs ( $1 \times 10^7$  cells/ml) were incubated at 37°C for 10 min with 2.5  $\mu$ M CFSE and 5 ml of culture medium (10% FBS) was added to stop CFSE cell staining. Cells were washed three times in PBS and resuspended at a concentration of  $1 \times 10^7$  cells/ml in culture medium. Stained cells were cultured in 96-well flat bottom plate ( $4 \times 10^5$  cells/well). The temperature, time and pH of the incubations were optimized. PBMCs stimulated with 1  $\mu$ g/well of Concanavalin A (ConA; Sigma, St. Louis, MO) were used as the positive control. Cells cultured in media containing PBS were used as the negative control. After incubating at 37°C with 5% CO<sub>2</sub> for 4 days, CFSE-labeled PBMCs were incubated with a mouse monoclonal antibody, R-PE-anti-chicken CD3 (Southern Biotech, Birmingham, AL) at room temperature for 30 min. After removal of the antibody and washing three times, cells from each treatment group were fixed with 4% paraformaldehyde and analyzed using a Beckman FC500 flow cytometer.

### Flow cytometric analysis

The PBMCs were collected to detect the diversity of CD4+ CD8+. Briefly,  $1 \times 10^6$  PBMCs were incubated with mouse anti-chicken CD3-SPRD, anti-chicken CD4-FITC, and anti-chicken CD8-RPE (Southern Biotech, Birmingham, AL) at 4°C for 30 min. The cells were washed three times with PBS containing 1% fetal bovine serum. The cells were then suspended with PBS and analyzed by the FACS Calibur and CellQuest software (Becton Dickinson, Franklin Lakes, NJ). The viable lymphocytes were calculated on the basis of forward and sideward scatter characteristics, and 20,000 events were analyzed for positive staining with SPRD, FITC, and RPE antibodies.

### Statistical Analysis

Statistical analyses of significance were performed using Student's t-test. The results were considered statistically significant at the level of  $P < 0.05$  or  $P < 0.01$ .

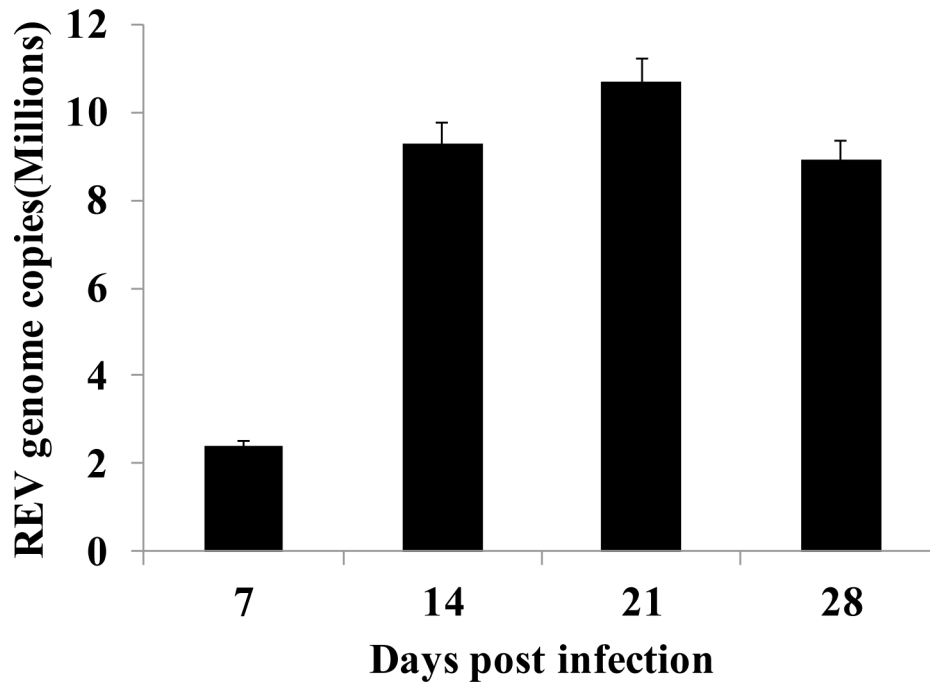
## Results

### Replication of the virus in PBMCs

To assess the replication ability of the REV HLJ071 strain in the PBMCs, the RNA genome copy numbers for the virus were detected using real-time RT-PCR at 7, 14, 21, and 28 days after infection. As shown in Figure 1, REV genome was first detected at 7 dpi, increasing rapidly from day 7 and peaked at day 21. Thereafter, REV genome copy number steadily declined until the termination of the experiment at 28 dpi.

### Kinetics of cytokine and chemokine mRNA expression after infection with the REV-A HLJ071 strain

The reproducibility of the bDNA assay was evaluated by analyzing the expression levels of 14 cytokines (IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-10, IL-13, IL-15, IL-17F, IL-18, IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , CSF, and TNF- $\alpha$ ) and one chemokine (IL-8) in duplicate in uninfected PBMCs. Three independent experiments were performed using PBMCs. The mRNA level of GAPDH was



**Figure 1. REV genome load in infected PBMCs.** Chickens were infected with the HLJ071 strain of REV and sampled at 7, 14, 21 and 28 days post-infection. Gag copy numbers in  $10^6$  PBMCs were quantitated using real-time RT-PCR. At least three samples were analyzed in duplicate at each sampling time point. The error bars represent standard error of the mean.

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measured as an internal control. The average values of duplicate wells were used to calculate the coefficient of variation (CV) of the background expression level of these 15 cytokines and chemokine in three independent experiments. As shown in Table 2, the CV values of all of the cytokine and chemokine mRNA levels with or without normalization to GAPDH were less than 5%, which indicated a high reproducibility of the measurements. These results demonstrated that the bDNA assay was a reliable method to simultaneously evaluate the expression levels of multiple cytokines and chemokines.

To determine the effects of REV infection on cytokine and chemokine mRNA expression, transcript levels of 14 cytokines (IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-10, IL-13, IL-15, IL-17F, IL-18, IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , CSF, and TNF- $\alpha$ ) and one chemokine (IL-8) were examined. At 7, 14, 21, and 28 days after infection with REV-A HLJ071 strain, PBMCs were isolated and detected by bDNA assay. As shown in Figure 2, the expression of pro-inflammatory cytokines IFN- $\alpha$ , IL-1 $\beta$  and IL-17F were down-regulated in chickens after REV infection compared to uninfected chickens. Analysis of Th1 and its regulatory cytokines IFN- $\gamma$ , IL-2, IL-15 and IL-18 revealed that the levels of these cytokines were decreased following REV infection. After REV infection, the expression levels of Th2 and regulatory cytokines (IL-4, IL-10 and IL-13) were drastically increased after 7 dpi, and moderately increased at 14 days and then up-regulated after 21 and 28 days following infection. The production of TNF- $\alpha$  was markedly up-regulated after 7 and 14

dpi. The levels of IFN- $\beta$ , IL-3 and CSF were significantly decreased at 7 and 14 days after infection and then moderately increased at 21 and 28 dpi. We analyzed the IL-8 expression and no significant changes were observed at 7 and 14 days post infection, while the expression levels of IL-8 were gradually up-regulated after 21 and 28 dpi.

#### Inhibition of T cell functions by REV infection

To explore the functions of REV infection in chicken immune response, we assessed the effect of REV-A on PBMC proliferation. As shown in Figure 3, the rate of PBMC proliferation in chickens infected with REV-A was significantly decreased compared to that of uninfected chickens ( $p < 0.05$ ).

CD4+/CD8+ ratios were calculated from the number of cells labeled with the fluorescent monoclonal antibodies of anti-CD4 or anti-CD8 analyzed using a flow cytometer. As shown in Figure 4, the ratios of CD4+/CD8+ in chickens infected with REV-A were lower than those of uninfected chickens.

#### Discussion

In this study the kinetic responses of immune-related cytokines and chemokines in PBMCs were examined following experimental infections of chickens with REV-A. Cytokines and chemokines play important roles in regulating innate immune responses and acquired immunity [15]. Analysis of cytokine and chemokine profiles will provide more information on the mechanism of immunosuppression caused by REV. In

**Table 2.** Reproducibility of the bDNA multiplex assay and the effects of GAPDH normalization.

	Unnormalized mRNA levels			mRNA levels normalized with GAPDH		
	Mean <sup>a</sup>	SD <sup>b</sup>	CV <sup>c</sup> (%)	Mean	SD	CV (%)
<b>Cytokines</b>						
IFN- $\alpha$	503.86	10.72	2	0.118	0.0041	3
IFN- $\beta$	597.70	11.81	2	0.139	0.0057	4
IFN- $\gamma$	9505.47	83.24	1	1.901	0.0401	2
IL-1 $\beta$	1964.67	12.43	1	0.43	0.0122	3
IL-2	217.78	10.91	5	0.053	0.0025	5
IL-15	710.48	28.93	4	0.16	0.0046	3
IL-17F	1827.52	56.23	3	0.389	0.0132	3
IL-18	9299.72	110.45	1	1.927	0.0419	2
IL-3	3060.02	61.23	2	0.702	0.0164	2
Csf1	3043.08	40.45	1	0.642	0.0132	2
IL-4	8100.37	90.76	1	1.718	0.0389	2
IL-10	5605.61	76.54	1	1.144	0.0343	3
IL-13	5317.56	85.63	2	1.103	0.0389	4
TNF- $\alpha$	469.17	23.57	5	0.09	0.0032	4
<b>Chemokine</b>						
IL-8	10515.62	139.98	1	2.391	0.0517	2

<sup>a</sup> The average values of three independent experiments using PBMCs prepared from the same chicken. Each value was calculated from the expression levels of duplicate wells. The mRNA level of GAPDH was measured as an internal control of baseline expression level.

<sup>b</sup> Standard deviation of values from three independent experiments.

<sup>c</sup> The coefficient of variation (CV) was calculated by the formula:  $CV = SD / \text{mean} \times 100\%$ .

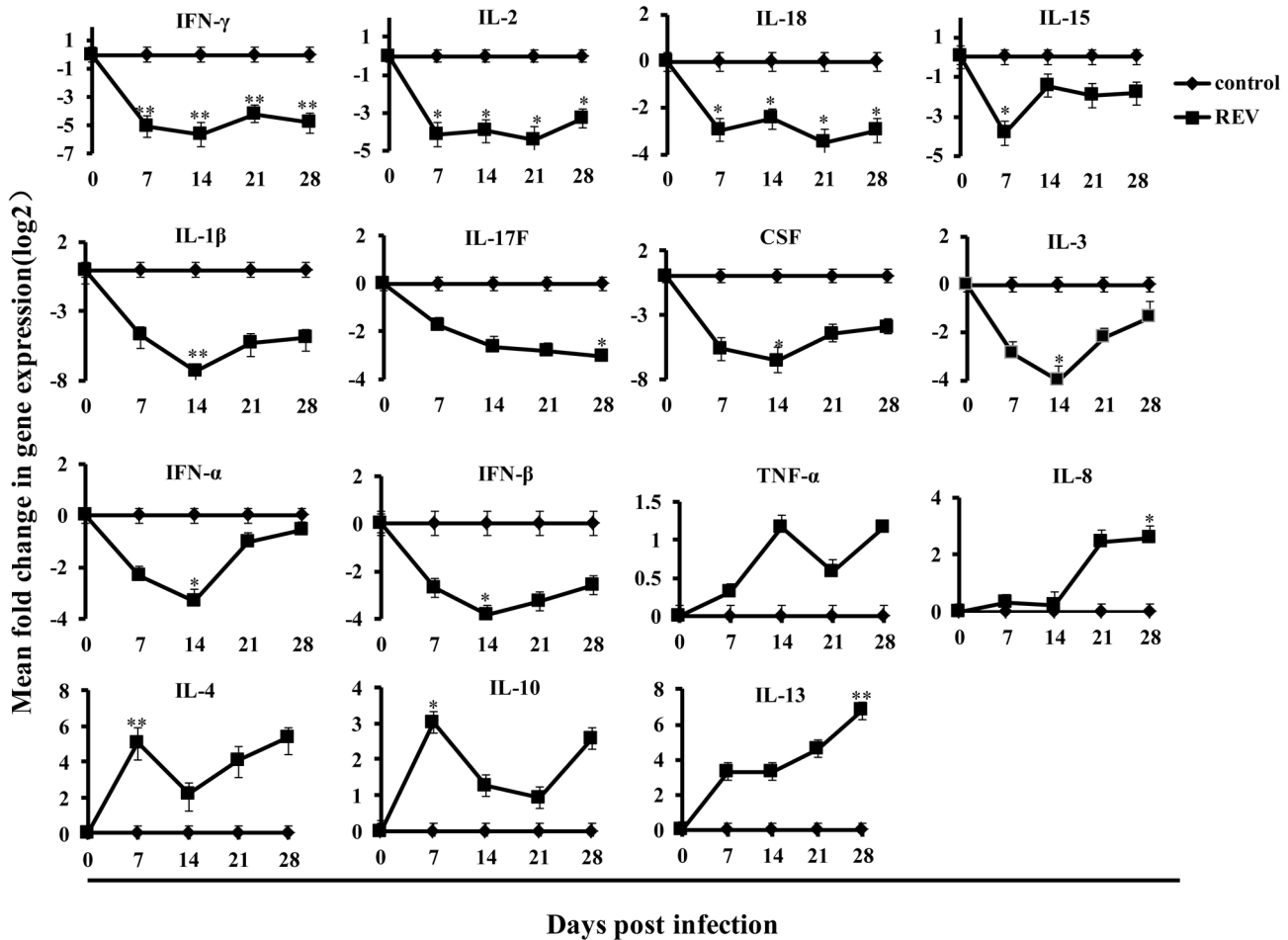
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comparison to the cases with humans or mice, the detection of avian cytokines and chemokines has been hampered by the lack of specific antibodies and reliable bioassays [23]. Up to now, there are no comprehensive studies on the role of cytokines or chemokines during *in vivo* REV-A infections of the chickens. In this study, expression levels of IL-4, IL-10, IL-13 and TNF- $\alpha$  were significantly up-regulated while the expression levels of IL-1 $\beta$ , IL-18, IFN- $\gamma$ , IL-2, IL-15, IL-17F, IFN- $\alpha$ , IFN- $\beta$ , IL-3 and CSF-1 were markedly decreased in PBMCs at all stages of infection. Compared with controls, REV infected chickens showed greater expression levels of IL-8 in PBMCs 21 and 28 dpi. The expression of Th1-related cytokines IFN- $\gamma$ , IL-2, IL-15 and IL-18 were down-regulated, while the production of Th2-related cytokines IL-4, IL-10 and IL-13 were drastically increased, which showed a trend for Th1 to Th2 conversion. PBMC proliferation assay showed that REV inhibited proliferative responses in chicken lymphocytes stimulated with T helper mitogen ConA. Furthermore, the chickens infected with REV induced a lower ratio of CD4+/CD8+. These data suggested that REVs have immunosuppressive effects in chickens.

One set of molecules involved in the immune response is Th2 cytokines. In mammals, it has been known for some time that the balance between Th1/Th2 lymphocyte subsets determines susceptibility to some disease states [24]. Thus, an unusually dominant Th1 response is often associated with

autoimmunity, while improper development of Th2 immunity can lead to allergic diseases [25]. Th2 cells are necessary for inducing the humoral response to combat virus infection [26]. As in mammals, the chicken genome contains a cluster of Th2 cytokine genes including IL-10, IL-4 and IL-13, all of which are expressed in lymphoid tissues [27]. IL-10 plays an important role in regulating immune responses and inhibiting the synthesis of inflammatory and pro-inflammatory cytokines (such as IL-1 $\beta$ ), thus down-regulating inflammatory Th1 responses [28]. IL-10 is highly regarded as an immunosuppressive cytokine and plays a role as the mediator of tumor regression [29]. It has been reported that IL-10 can be secreted by tumor cells [30] and high IL-10 levels were observed in areas of spontaneous regression of primary melanoma [31]. The predominant expression of immunosuppressive cytokines in cancer patients induces an immunosuppressive state in the immunological microenvironment of the tumor [29] and enables tumor to escape from immune recognition [32,33]. Since low levels of IL-10 increase resistance and high levels increase susceptibility [34], the high expression of IL-10 may help us to understand the increase in susceptibility to concurrent or secondary bacterial or viral infections post REV-A infection. IL-4 plays an important role in the differentiation of naive T cells toward a Th2 phenotype. IL-13 is structurally similar to IL-4 and is produced by Th2 CD4+ T cells as well as epithelial cells. IL-10, IL-4 and IL-13 are markedly increased in earlier days and later days of REV-A infection. It is associated with down-regulating inflammatory (Th1) response and results in driving Th2 cell development. In HIV, it has been reported that viral infection induced Fc $\epsilon$ RI+ hematopoietic cells to produce IL-4, which inactivated the host adaptive immune response [35]. In addition, the conversion from a Th1 response to a Th2 response during viral infection favors infection and the spread of HIV-1 [36].

Another set of molecules involved in the immune response is Th1 cytokines. IL-2 and IL-15 are structurally homologous to Th1 or Th1 related cytokines produced by mononuclear phagocytes and other cell types in response to viral infection [37,38]. Both cytokines stimulate proliferation of chicken T lymphocytes and NK cells [38]. Interferons were first described in chickens [39] and have been shown to have various immunomodulating effects on a wide variety of tissues and combat the replication of the viruses in the host cells. The decreased levels of IL-2, IL-15, IFN $\alpha$ , IFN $\beta$  and IFN- $\gamma$  indicated that infection of REV-A suppressed Th1-type immune response. It has been reported that the expression level of immunosuppressive cytokines, such as IL-10, was significantly higher than that of immunostimulatory cytokines, such as IL-2 and IFN- $\gamma$ , in various cancer patients [40,41]. IFN- $\gamma$  production during REV infection has been examined by quantitative RT-PCR [17] and by antigen capture ELISA [18]. Infection with REV-CS resulted in a 10-fold increase in IFN- $\gamma$  mRNA levels in 9- to 10- or 30-day-old birds [17]. Chickens infected with SNV strain of REV at 5 days of age showed 3 to 5 fold increased level of IFN- $\gamma$  between 7 and 28 dpi as measured by Ag capture ELISA [18]. It remains to be determined if the difference between the cytokine responses



**Figure 2. The relative cytokine and chemokine mRNA levels in PBMCs of chickens infected with REV-A strain HLJ071 or uninfected controls for 7, 14, 21 and 28 days.** Values were normalized to the endogenous GAPDH control and were presented as the log<sub>2</sub> mean fold-change in mRNA expression (relative to the uninfected control). Data are the means of three independent experiments. \* indicates P < 0.05 and \*\* indicates P < 0.01 when the REV-infected group was compared with the control group.

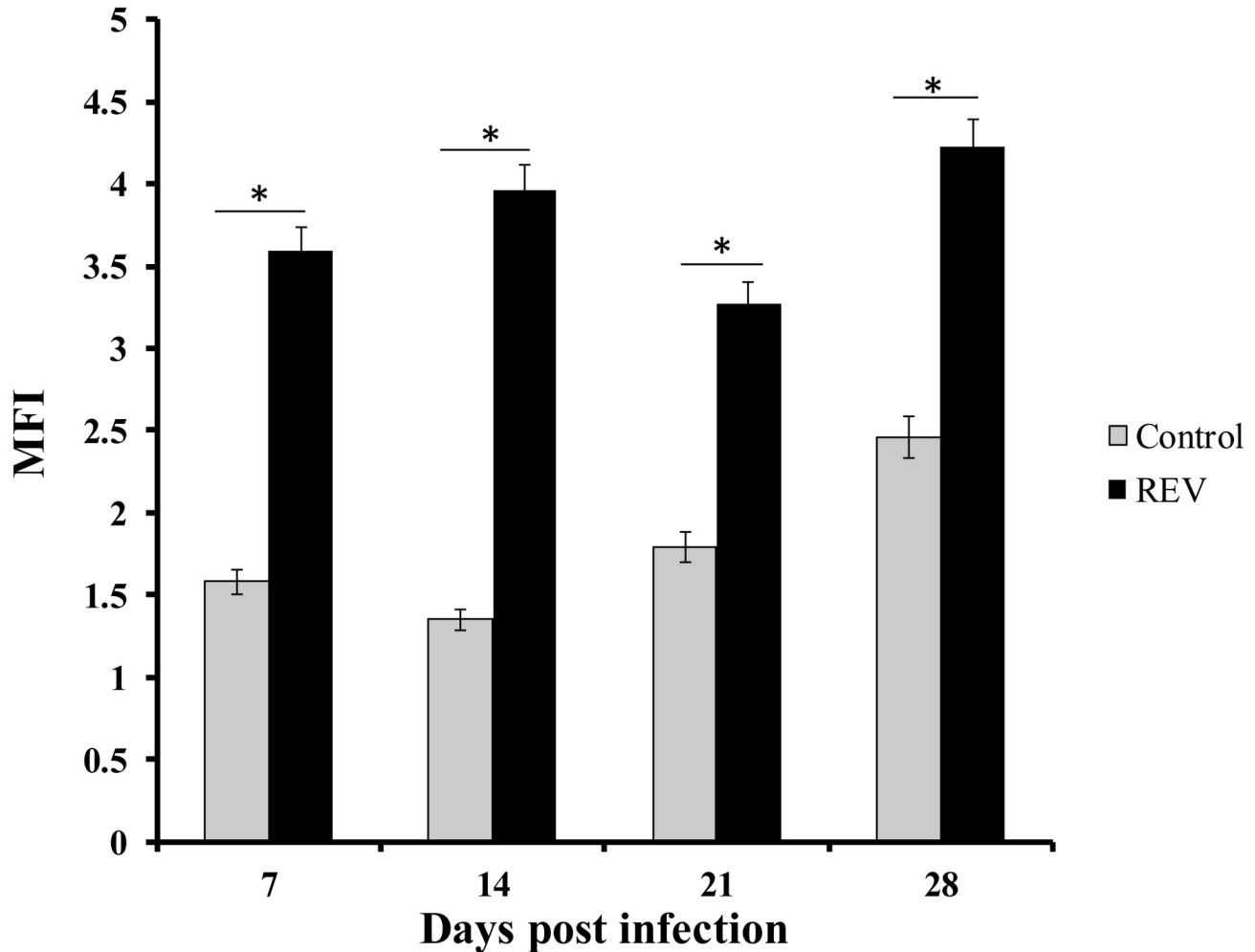
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induced by these two REV strains is related to their pathogenicity, antigenicity, and/or other unknown factors.

IL-1 $\beta$  is a powerful pro-inflammatory cytokine secreted by many different types of cells, with stimulated macrophages being the major producer. In this study IL-1 $\beta$  was down-regulated after REV infection when bDNA technology was utilized for testing. No significant changes in the level of IL-1 $\beta$  transcripts was observed at either 7 or 14 days post REV-CS strain infection [17], which was different from the results in this study. In another study, infection with MDV JM-16 strain could not induce IL-1 $\beta$  expression in the spleen but RK-1 strain led to induction of IL-1 $\beta$  at 4 dpi in both N2a and P2a chicken lines [42]. IL-18 is structurally homologous to IL-1 $\beta$  and plays important roles in initiating inflammation. In mammals, IL-18 is an inducer of cell-mediated immunity, especially in combination with IL-12, and is primarily associated in Th1 responses to intracellular pathogen infections [43]. IL-18 transcription was down-regulated between 7 and 28 dpi post REV-A infection.

IL-17 is produced by activated memory T cells and, like IL-1 $\beta$ , induces the production of other pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ . The results in this study demonstrated that IL-17F transcript was gradually down-regulated following REV-A infection. Thus, the decreased levels of transcripts for several chicken pro-inflammatory cytokines in this study suggest that these molecules may be relevant to the tumor and immune-suppression caused by REV infection.

The CSFs are a family of polypeptide growth factors critical to the development of haematopoietic cells, including those of the monocyte/macrophage lineage [44]. CSF-1 is an important mediator of inflammation, and also represents a key element of a possible haematopoietic growth factor network active at sites of inflammation [45]. For example, in the inflamed joint CSF-1 may drive the differentiation and activation of monocytes/macrophages, causing the release of inflammatory mediators and resulting in inflammation and local tissue damage.



**Figure 3. The proliferation of PBMCs post infection of REV.** The PBMCs ( $1 \times 10^7$  cells/ml) were isolated from heparinized peripheral blood of REV-A infected or uninfected control chickens. The mean fluorescence intensity (MFI) was statistically analyzed. \* indicates  $P < 0.05$  when the REV-infected group was compared with the control group.

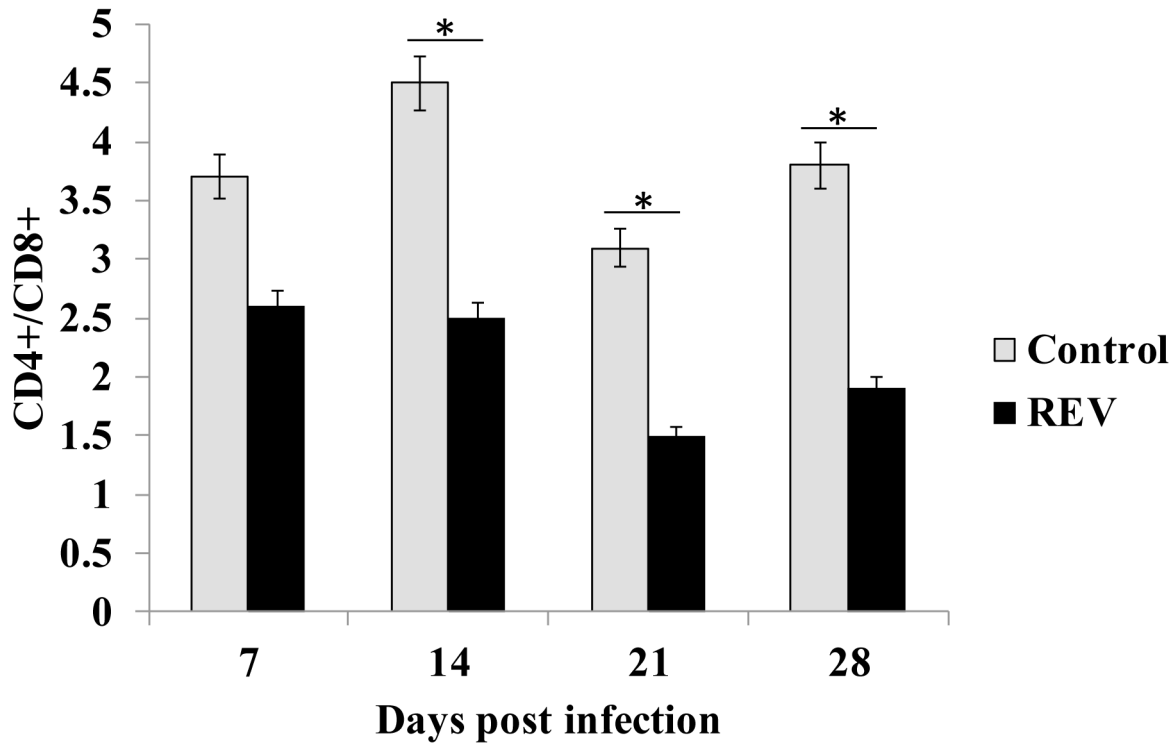
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Compared with mammalian IL-3, chicken IL-3 was reported to be expressed at higher levels in all tissues [27], which concurs with our mRNA expression data. In this study, CSF-1 and IL-3 were significantly down-regulated at 7 and 14 dpi, and gradually increased at 21 and 28 dpi. The down-regulated expression of CSF-1 and IL-3 were consistent with other pro-inflammatory cytokines and demonstrated the immunosuppressive state after REV-A infection.

TNF- $\alpha$  is a potent immunomodulator and proinflammatory cytokine that has been implicated in the pathogenesis of autoimmune and infectious diseases. It has been reported that TNF- $\alpha$  system was activated during HIV-1 infection and the raised levels increased with disease progression and degree of immunodeficiency [46]. Since TNF- $\alpha$  has a strong antitumoral action [47], the up-regulated expression of TNF- $\alpha$  post REV-A infection may correlate with tumor caused by REV.

Chemokines are another group of regulators of immunity that has important roles in disease etiopathology and the immune response after viral infection [48]. IL-8 is a member of the chemokines, which is known as an important mediator of inflammation that recruits and activates leukocytes to sites of infection [49,50]. In addition, the potential role of IL-8 in viral infections of chickens was also indicated. For examples, large increases in IL-8 mRNA were seen in the brains after Marek's disease virus infection [42] or in the chicken macrophages exposed to infectious bursal disease virus [51]. Moreover, human and murine tumors also frequently secrete IL-8 [52]. IL-8 was up-regulated in the late stage of infection in this study, and the over-expression of IL-8 would induce excessive accumulation of lymphocytes and mononuclear cells in infected tissues and cause cytokine imbalances.

There are a number of possible explanations as to why IL-1 $\beta$ , IL-2 and IFN- $\gamma$  expression levels presented in this study



**Figure 4. The subpopulation ratios of CD4+/CD8+ in the PBMCs of chickens infected with REV detected by flow cytometry.** The PBMCs were isolated from the heparinized peripheral blood and stained with mouse monoclonal antibodies against chicken CD3, CD4, and CD8. CD4+/CD8+ ratios were calculated from the number of cells labeled with the fluorescent monoclonal antibodies of anti-CD4 or anti-CD8 analyzed using a flow cytometer. All data were expressed as mean  $\pm$  standard error. \* indicates  $P < 0.05$  when the ratio of the REV-infected group was compared with that of the control group.

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were different from the previous study [17]. Firstly, the virus strains and chicken lines used in both studies were different. Secondly, the age of infection differed in the two studies, with chickens being infected at 9-30 days of age in the report by Schat et al. [17] and chickens being infected at 3 days of age in this study. Thirdly, the total amount of RNA used in the test assays might be different in each report. In this study,  $10^6$  PBMC RNA was used in each reaction while the actual amount of input RNA used in the reaction by Schat et al. [17] was unknown. Thus, if less RNA is added to each reaction, a sample may be negative for a specific cytokine at that level of sensitivity, but may be positive if more input RNA is added. Finally, the methodology used to test cytokines was different in these two studies. bDNA assay was used in this study compared to qualitative RT-PCR assay used in the previous report [17]. The bDNA assay is a sandwich nucleic acid hybridization platform in which target-specific RNA molecules are captured through cooperative hybridization of multiple probes. It has been demonstrated that this assay enables the reliable detection and quantitation of multiple-gene expressions simultaneously [53].

Our data demonstrated that T cell proliferative responses were decreased and the ratio of CD4+/CD8+ was lower in REV infected chickens. The inhibition of T-cell proliferation and the

lower ratio of CD4+/CD8+ induced by REV would enable the virus to downregulate the host immune response, thereby compromising the ability of the host to develop effective protective immunity to other pathogens.

To the best of our knowledge, this is the first comprehensive study of differential cytokine and chemokine expression in PBMCs infected with REV-A strains using bDNA multiple measurement technology. Based on the results in this study, REV infection causes disruption of cytokine networks, inhibits chicken lymphocyte proliferation, enhances the immunosuppressive effect, and thus increases susceptibility to concurrent or secondary bacterial or viral infections and results in poor immune responses to chicken vaccines. Further investigations are required to evaluate the effect of differential expression of these cytokines and chemokines on the tumors and immune response of viral infection.

#### Author Contributions

Conceived and designed the experiments: YW MX XS. Performed the experiments: MX. Analyzed the data: MX XS SH XC. Contributed reagents/materials/analysis tools: YZ HC. Wrote the manuscript: YW MX XC.



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