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Research paper

# Transcriptional analysis of host responses related to immunity in chicken spleen tissues infected with reticuloendotheliosis virus strain SNV

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ABSTRACT

In avian species, the Reticuloendotheliosis virus (REV) causes severe immunosuppression and other symptoms, including avian dwarfing syndrome, and chronic tumors in lymphoid and other tissues. The pathogenesis of REV and its interaction with the host have yet to be fully elucidated with transcriptional studies on the changes in host gene expression after REV infection at the body level. In this study, the Spleen Necrosis Virus (SNV) was used to inoculate the one-day-old specific pathogen free (SPF) chicken to simulate congenital infection. We identified 1507 differentially expressed genes (DEGs) at 7, 14 and 21 dpi using Next Generation Sequencing (NGS) technology. Through the Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of these DEGs, it was found that DEGs were mainly involved in the categories of signal transduction, immune system and signaling molecules and interaction. Among them, Pattern recognition receptors (PRRs), chemokine, T cell receptor, JAK-STAT, TNF, and NF-kappa B signaling pathway, and the Hematopoietic cell lineage play an important role in the tumorigenic and immunosuppressive regulation of REV. In addition, a series of DEGs associated with inflammatory factors (CCL4, TNFRSF18, CDKN2), apoptosis (IRF1, PDCD1, WNT5A), innate immunity (TLR, MAD5, TRIM25), and adaptive immunity (LY6E, CD36, LAG3) were also discovered. We further verified 33 selected immune- relevant DEGs using quantitative RT-PCR (qRT-PCR). These findings provide new insights and research directions for revealing the pathogenesis of REV infection and the interaction between REV and the chicken immune system.

## 1. Introduction

Reticuloendotheliosis (RE), characterized by the formation of acute reticulocyte tumors, dwarf syndrome, and the formation of chronic tumors in lymphoid tissue and other tissues, thus causing severe immunosuppression, is a pathological syndrome caused by the reticuloendotheliosis virus (REV) (Amarasinghe et al., 2018; Bi et al., 2018). REV targets lymphocyte or reticular endothelial cells, causing tumor cell infiltration or atrophy in immune organs. It impairs the body's immune function, leading to significant humoral and cellular immunosuppression (Walker et al., 1983). In addition to seriously affecting the growth and immune response of chickens, REV can work with Avian leukosis virus (ALV), chicken anemia virus (CAV), and Marek's disease virus (MDV) to cause more serious mixed infectious diseases, resulting in more severe immunosuppression and more serious diseases (Li et al., 2015; Liu et al., 2009; Xue et al., 2017). This in turn

*Abbreviations:* REV, reticuloendotheliosis virus; SNV, spleen necrosis virus; SPF, specific pathogen free; NGS, next generation sequencing; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; PRR, pattern recognition receptor; RE, reticuloendotheliosis; DEG, differentially expressed gene; CAV, chicken anemia virus; MDV, Marek's disease virus; TCID, tissue culture infectious dose; dpi, day post-inoculation; gag, group-specific antigen gene; RPKM, reads per kilo bases per million reads; SRA, sequence read archive; qRT-PCR, quantitative RT-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCA, principal component analysis; TLR, toll-like receptor; NLR, NOD-like receptor; and RLR, RIG-I-like receptors; INF, infection; CON, control; ISG, interferon-stimulated genes; IBDV, infectious bursal disease virus; ALV, Avian leukosis virus; AIV, Avian influenza virus; CEF, chicken embryo fibroblast; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum

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leads to a significant increase in morbidity and mortality, brings greater difficulties to the prevention and control of the disease, and brings serious economic losses to the poultry industry (Bao et al., 2015).

With the rapid development of next-generation sequencing (NGS) technology, it is easier to screen out genes associated with viral pathogenicity from the genome (van Dijk et al., 2014). By analyzing the structure and function of genes, transcriptional analysis can reveal important information such as possible pathogenic mechanisms and biological processes involved in the virus infection, which is of great significance for disease prevention and control as well as basic research (Costa et al., 2010). The application of NGS technology in veterinary medicine enables the analysis of the transcriptome after a host if infected with a virus, providing new insights into the interaction between the host and virus.

To date, there is still a lack of transcriptional studies on the changes in host gene expression after REV infection at the body level. Moreover, the pathogenesis of REV-induced immunosuppression and tumorigenesis has not yet been fully elucidated. Therefore, to acquire transcriptome information and to study the interactions between REV and the host, SPF chicken were infected with REV-SNV. Spleen tissue was collected at 7, 14, and 21 days post-inoculation (dpi) and then subjected to transcriptome sequencing. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were used to examine differentially expressed genes (DEGs) identified during different stages of infection. Our findings may provide novel information that will increase our understanding of the immune mechanisms underlying REV infection.

## 2. Materials and methods

### 2.1. Ethics statement

All of the animal infection experiments were conducted in accordance with international, national, and institutional guidelines. Animal procedures were approved by the Animal Care and Use Committee of Shandong Agricultural University (No. SDAUA-2018-165).

### 2.2. Animal infection assay and sample collection

REV strain SNV (GenBank: DQ003591.1) was graciously provided by Professors Shuhong Sun and Zhizhong Cui from Shandong Agricultural University (Trager, 1959). The 10 day old SPF chicken embryos used for the preparation of chicken embryo fibroblasts (CEFs) and SPF White Leghorn line chickens were purchased from Jinan SAIS Poultry Co. Ltd., China. CEFs were cultured in Dulbecco's modified Eagle medium (DMEM; BI, Kibbutz, Beit Haemek, Israel) supplemented with 10% fetal bovine serum (FBS; BI, Kibbutz, Beit Haemek, Israel). The CEFs were cultured at 37 °C in a humid incubator containing 5% $CO_2$ and subsequently used for virus propagation. All chicks were kept in negative-pressure-filtered air isolators and fed as recommended. Twenty-four SPF chickens were randomly divided into two groups: the control group (group CON) and the REV-infected group (group INF). Group INF was inoculated with 200 μL containing 5000 tissue culture infectious doses ($TCID_{50}$) by intraperitoneal injection at the age of 1 day. At the same time, group CON was inoculated with 200 μL DMEM by intraperitoneal injection as the mock inoculum.

Three (group CON) or four (group INF) SPF chickens of per group were euthanized at 7, 14, or 21 days post-inoculation (dpi). The spleens of these animals were collected, then immediately frozen in liquid nitrogen and stored at −80 °C for later use.

### 2.3. Total RNA isolation and virus infection assays

Total RNA was isolated using Trizol Reagent (Invitrogen Life Technologies), after which the concentration, quality, and integrity were determined using a NanoDrop spectrophotometer (Thermo Scientific). High-quality samples (i.e., A260/280 was between 1.8 and 2.2, A260/230 was ≥2.0, and RNA integrity numbers were ≥7) were used in subsequent experiments.

Primers were designed using Oligo 7.0 software (Rychlik, 2007), and the sequences of the two primers were: gag reverse 5′-GAGACTC TATCAGGCTTATCGG-3′ and gag forward 5′-CAGTTCTTCTTCCAATGT CCC-3′, which can amplify the section of the group-specific antigen gene (gag) to verify the REV infection in chickens. A PrimeScript™ One Step RT-PCR Kit Ver.2 (Dye Plus) (Takara, Dalian, China) was used to amplify the section of gag, with the reaction conditions as follows: reverse transcription at 50 °C for 30 min, initial denaturation at 94 °C for 3 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. The PCR products were subjected to agarose gel electrophoresis and sequenced by TSINGKE Biololgical Technology (China).

### 2.4. Library construction and next-generation sequencing

Three micrograms of RNA were used as input material for the RNA sample preparations. Sequencing libraries were generated using a TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under an elevated temperature in an Illumina proprietary fragmentation buffer. First strand cDNA was synthesized using random oligonucleotides and SuperScript II. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities and the enzymes were removed. After adenylation of the 3′ ends of the DNA fragments, Illumina PE adapter oligonucleotides were ligated to prepare for hybridization. To select cDNA fragments longer than 200 bp, the library fragments were purified using an AMPure XP system (Beckman Coulter, Beverly, CA, USA). DNA fragments with ligated adaptor molecules on both ends were selectively enriched using Illumina PCR Primer Cocktail in a 15 cycle PCR reaction. Products were purified (AMPure XP system) and quantified using an Agilent high-sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent). The sequencing library was then sequenced on an Illumina HiSeq 2500 sequencer at Personal Biotechnology Co., Ltd. (Personal Bio, Shanghai, China).

### 2.5. Analysis of mRNA raw sequencing data

The raw data were filtered using Cutadapt (Version 1.2.1) to remove joint contamination at the end. In addition, data with a Sanger sequencing quality value of < 20 were eliminated at the same time. Then the quality control analysis of clean data was conducted in base mass distribution, base content distribution, GC content distribution, and sequence base quality by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The reads obtained from sequencing were mapped to the chicken genome (WASHUC2.69) in Ensembl using Bowtie/Tophat (2.0.5) (http://tophat.cbcb.umd.edu), and the reads of each gene were normalized using reads per kilo bases per million reads (RPKM). The significance was determined by normalizing the raw reads and calculating the *p*-value using DESeq (http://bioconductor.org/packages/release/bioc/html/DESeq.html). Genes with a fold change (RPKM (chGH/PBS)) > 1.5 or < 2/3 and a p-value < 0.05 were identified as DEGs (Wang et al., 2010).

### 2.6. GO and KEGG analyses

GO enrichment analysis was performed using GOslim (Ashburner et al., 2000; Consortium et al., 2000) and KEGG (Kanehisa et al., 2004) analysis was employed to discover differentially expressed genes and analyze the signaling pathways involved. The raw data were submitted

to the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra/); the SRA submission ID is SUB4782429 and the BioProject ID is PRJNA505870.

### 2.7. Quantitative RT-PCR analysis of mRNAs

The expression levels of 33 selected mRNAs were validated by real-time quantitative RT-PCR (qRT-PCR). For real-time PCR, the primers were designed using Primer Premier 6.0 software and Oligo7.0 software based on the sequences in GenBank. All primers sequences used in this study, which were synthesized by TSINGKE Biololgical Technology (China), are listed in Supplementary Table S1. qRT-PCR analysis was performed using LightCycler®96 (Roche Diagnostics GmbH, Germany) with a One-Step SYBR® PrimeScript™ RT-PCR Kit (Takara, Dalian, China) following the manufacturer's instructions. The relative expression values were normalized, with the chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene serving as an internal control. After amplification, the relative fold change of the differentially expressed genes was calculated through the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001). Triplicate qRT-PCRs were performed on each RNA sample to guarantee the reproducibility of the amplification.

### 2.8. Statistical analysis

All data are shown as the mean ± standard deviation of three replicates. We performed the Pearson's correlation analysis of Log2 (fold change) by NGS and Log2 (fold change) by qRT-PCR with the software GraphPad Prism, Version 7.0.

## 3. Results

### 3.1. REV infection in SPF chickens

The RNAs extracted from the spleens of chickens in all groups were picked for RT-PCR validation. The amplified products of PCR were subjected to agarose gel electrophoresis and the resulting bands from the testing were purified, recovered, and sequenced (Fig. 1). The results showed that specific fragments with a length of 368 bp were amplified from all samples of the infection group by RT-PCR, which was consistent with the expected findings. Meanwhile, the results of sequencing and analysis by NCBI/BLAST indicated that the extracted RNA was REV-SNV (GenBank: DQ003591.1).

### 3.2. Transcriptome sequencing data

Nineteen spleen sample libraries from six samples obtained at 7 dpi (CON1–1, CON1-2, CON1-3, INF1-1, INF1-2, and INF1-3), six samples obtained at 14 dpi (CON2-1, CON2-2, CON2-3, INF2-1, INF2-2, and INF2-3), and seven samples obtained at 21 dpi (CON3-1, CON3-2, CON3-3, INF3-1, INF3-2, INF3-3, and INF3-4) were generated using NGS. Among them, the quality of the samples INF1-4 and INF2-4 was not in accordance with the requirements for constructing mRNA libraries, so these were removed. As shown in Supplementary Table S2, the raw reads, clean reads, clean reads (%), N (%), Q20 (%), Q30 (%), and GC (%) were recorded for each library. For all libraries, the percentages of clean data, Q20, and Q30 were > 97%, > 94%, and > 87%, respectively. The Q20 and Q30 values and GC contents among the sequences indicated the high quality of the sequencing data and ensured their suitability for the next step of the analysis.

In addition, correlations between biologically replicated samples were determined by sample correlation tests. As shown in Fig. 2a, the correlation between samples for biological replication (such as between INF1-1 and INF1-2, INF1-1 and INF1-3, INF1-2 and INF1-3, etc.) was strongly correlated ($R^2$ > 0.8), indicating that all groups have good biological repeatability. As shown in Fig. 2b, in the dimension of principal component (PC) 1, the control groups (CON1, CON2, and CON3) and the infected groups (INF1, INF2, and INF3) can be clearly distinguished, indicating the sensitivity of SPF chickens to REV infection. However, in the dimension of PC2, the groups analyzed at 7/14 dpi (INF/CON1 and INF/CON2) and the groups analyzed at 21 dpi (INF/CON3) can be clearly distinguished, indicating that SPF chickens have large differences in gene expression patterns during different growth stages.

### 3.3. Analysis of differences in gene expression of chicken infected with REV

The final data were obtained through normalization and statistical analysis by DESeq (version 1.18.0) to identify DEGs associated with REV infection in SPF chickens. A total of 1507 DEGs were found to be differentially expression compared with the control group at 7, 14, and 21 dpi. Among these DEGs, 574 were identified in samples obtained at 7 dpi, 260 of which were downregulated and 314 of which were upregulated (Fig. 3a). Five hundred and forty-five DEGs were identified in samples obtained at 14 dpi, 235 of which were downregulated and 310 of which were upregulated (Fig. 3b). In addition, 854 DEGs were identified in samples obtained at 21 dpi, with 291 DEGs downregulated and 561 upregulated (Fig. 3c). A Venn diagram analysis showed that 112 genes were represented by DEGs at all three tested time points (Fig. 3d). The details of the DEGs are listed in Supplementary Table S3.

### 3.4. Gene ontology analysis

For all three time points, Gene Ontology (GO) category analysis of the genes corresponding to the 1507 DEGs was performed to explore the roles of DEGs. As shown in Fig. 4a–c, these results provide an overview of the host response to REV infection with respect to the top 10 enriched GO terms of DEGs in each category at 7, 14, and 21 dpi. The following GO terms were most commonly implicated in the biological process category: immune response, defense response, immune system process, response to cytokine, and response to interferon-gamma. The results indicate that the host develops a strong immune response during REV infection. More detailed analysis results concerning GO terms and genes enriched in GO terms are listed in Supplementary Table S4.

### 3.5. KEGG pathway analysis

To further explore the function of DEGs after REV infection in SPF

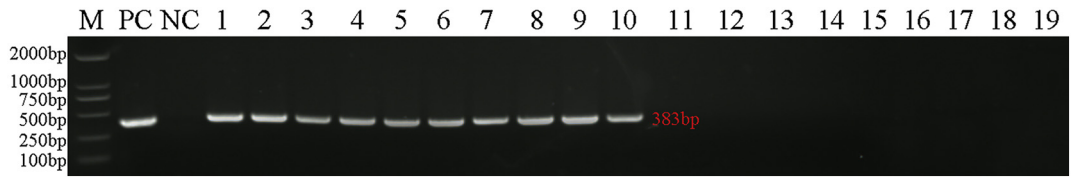



**Fig. 1.** Detection of viral RNA of REV-SNV from infected chicken spleen.
The total RNA extracted from virus stock of REV was used as the positive control (PC). The ddH2O was used as a negative control (NC). The DNA marker (M) was DL2000 (Takara, Dalian China). Lanes 1–3, 4–6, and 7–10 are the PCR product from the IFN group at 7, 14, and 21 dpi, respectively. Lanes 11–13, 14–16, and 17–19 are the PCR product from the CON group at 7, 14, and 21 dpi, respectively.

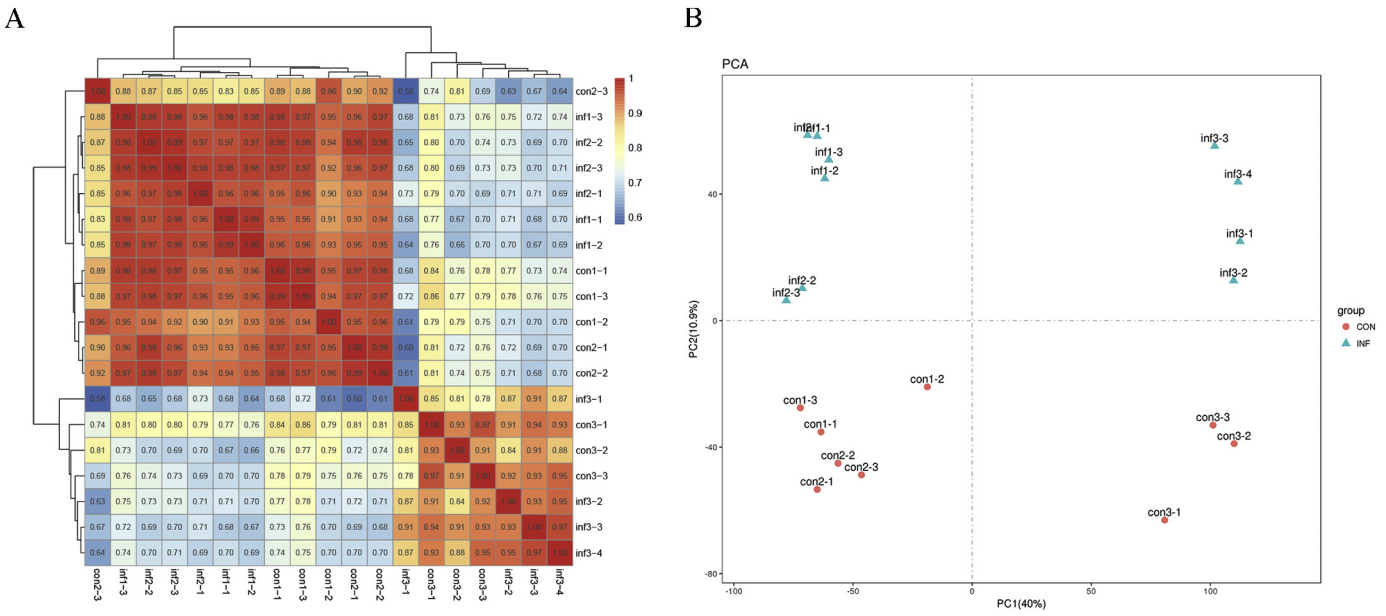



**Fig. 2.** Sample correlation tests (a) and principal component analysis (PCA) (b).
Note: (a) The abscissa and ordinate labels in the figure are sample numbers, and the values in the box are correlation coefficients. The closer the correlation coefficient is to 1, the higher the similarity. Red indicates a high correlation coefficient and blue indicates a low correlation coefficient. (b) The abscissa is the first principal component and the ordinate is the second principal component. The blue triangle icon represents the infected (INF) group and the red circle icon represents the control (CON) group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chickens, KEGG enrichment analysis was conducted. As shown in Fig. 5a–c, the KEGG functional terms contained a large number of enriched genes mainly involved in the categories of signal transduction, immune system, and signaling molecules and interaction at 7, 14, and 21 dpi. In addition, we counted the 20 most enriched KEGG pathways in which DEGs were involved at 7, 14, and 21 dpi. As shown in Fig. 5d–f, the DEGs were considerably enriched in the category of immune system, including antigen processing and presentation, hematopoietic cell lineage, chemokine signaling pathway, complement and coagulation cascades, RIG-I-like receptor signaling pathway, Toll-like receptor signaling pathway, intestinal immune network for IgA production, NOD-like receptor signaling pathway, and T cell receptor signaling pathway. These signaling pathways may play a key role in REV-induced tumorigenic and immunosuppressive mechanisms. The details of the KEGG terms are included in Supplementary Table S5. (See Fig. 6.)

### 3.6. qRT-PCR assay

To validate the NGS data, qRT-PCR was used to verify 33 DEGs, which were generated at the intersection of 7, 14, and 21 dpi. The functions of these 33 DEGs are mainly related to cell differentiation, proliferation, apoptosis, tumor development, and immune response. Among the 33 related mRNAs, 21 mRNAs were upregulated and 12 mRNAs were downregulated in infected samples compared with uninfected samples, as determined by qRT-PCR analysis (Supplementary Table S6). As shown in Fig. 6a–c, the fold changes in the expression of the 33 DEmRNAs were comparable between the three datasets, with correlation coefficients of $R^2 = 0.961$ ($p < 0.0001$), $R^2 = 0.9681$ ($p < 0.0001$) and $R^2 = 0.9467$ ($p < 0.0001$), respectively. Taken together, these results indicate that the NGS results in this study are reproducible.

## 4. Discussion

As a member of the Gammaretrovirus genus in the Retroviridae family, REV, MDV, and ALV are called three major viral tumor disease in poultry. Furthermore, REV is an important immunosuppressive disease (Wang et al., 2012). However, the harm of REV has not received sufficient attention for a long time due to its atypical clinical symptoms. In recent years, REV has spread rapidly around the world, and the increasing rate of REV in China has gradually increased (Jiang et al., 2013). The direct and indirect harm to the poultry industry has become increasingly serious. Currently, high-throughput NGS techniques represent a powerful tool that can be applied to high-throughput whole-viral-genome sequencing to better our understanding of the interaction between viral and host transcriptomes (Radford et al., 2012; Yang et al., 2017). However, to the best of our knowledge, there is still a lack of transcriptional studies on the changes of host gene expression after REV infection at the cellular level. REV exhibits pathogenic characteristics of a higher immunosuppressive effect for chickens at a young age (Sun et al., 2009). In chickens, the spleen is an important peripheral immune organ—a place for colonization as well as the immune response of mature T and B lymphocytes. The main pathogenicity of the REV strain SNV is concentrated in the spleen (Trager, 1959). For these reasons, we primarily analyzed the effect of REV invasion of the spleen on the host immune response at 7, 14, and 21 dpi. In this study, 1507 DEGs were discovered by analyzing the changes of the genome transcriptional level in the spleen of SPF chickens after infection with REV-SNV. These DEGs were significantly enriched primarily in the pathways of the immune system, signal transduction, signaling molecules and interaction, cell growth, and death categories.

The innate immune response is the first line of defense against infectious disease. Here, pattern recognition receptor (PRR) signaling pathways were identified to be involved in the response to REV infection at 7, 14, and 21 dpi, including Toll-like receptors (TLR), NOD-like receptors (NLR), and RIG-I-like receptors (RLRs). A large number of RNA fragments were produced during the process of proliferation and expression after REV invaded the host. The recognition of these fragments by TLR3 and TLR4 receptors stimulated the increase of TLR gene expression in the host (Blasius and Beutler, 2010; Gillespie et al., 2011). TLR3 recruited TAK1 or TRAF3 through the TRIF-dependent pathway, stimulating the generation of NF-κB and IRF3, respectively. Finally, inflammatory cytokines and IFN-1 were produced, so as to achieve the purpose of killing the invading virus. The relative expression of the

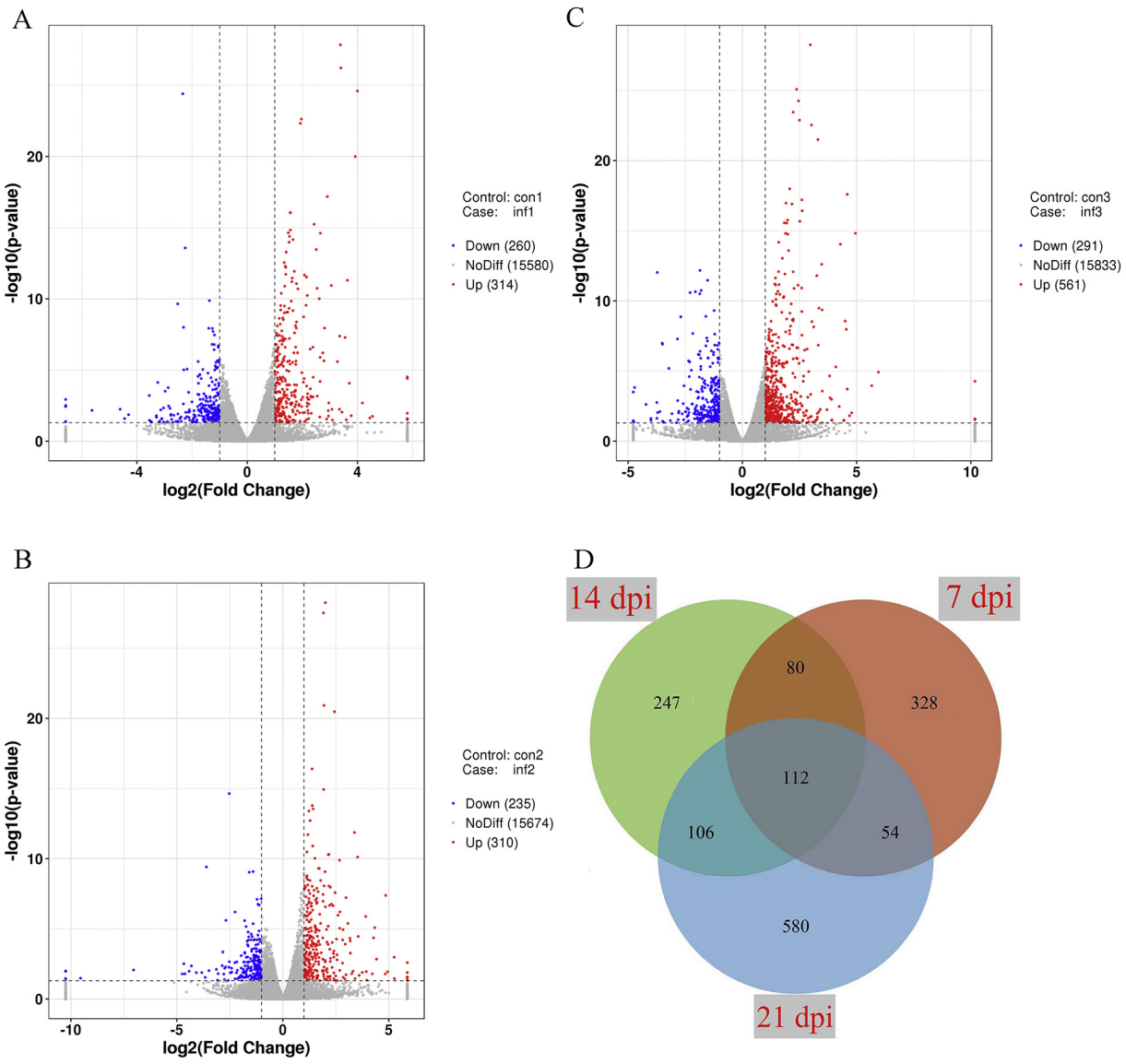



**Fig. 3.** Gene expression difference analysis (a–c) and Venn diagram (d).
Note: (a–c) Expression differential analysis of genes at 7, 14, and 21 days pot-inoculation (dpi), respectively. The abscissa is the expression difference multiple (log2 value) and the ordinate is the expression difference significance *p*-value (−log10 value). In the figure, the vertical line is the 2-fold expression difference threshold; the horizontal line is the threshold of $p = 0.05$. Red dots indicate significant differentially expressed genes and blue dots indicate non-significant differentially expressed genes. (d) The number and overlap of differentially expressed genes in spleen infected with reticuloendotheliosis virus (REV) at 7, 14, and 21 dpi. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TLR3 gene changed significantly after avian influenza virus (AIV) (Karpala et al., 2008) and infectious bursal disease virus (IBDV) (Rauf et al., 2011) infection in chickens in previous studies, and it has been reported that the expression of the TLR3 gene in the lungs increases significantly after 12 h of MDV infection (Amarasinghe et al., 2018). In this study, the relative expression of TLR3 increased at all three time points, indicating that the invasion of REV virus stimulated macrophages and other cells in the spleen to increase the expression of the TLR gene in order to resist and remove REV. In addition, it has been reported that TLR4 can be activated by LPS to induce the production of proinflammatory cytokines such as IL-1β, IL-6, and IL-18 as well as soluble mediator NO via iNOS activation (Farnell et al., 2003; He et al., 2006). The activation of TLR4 can stimulate immune responses, leading to protection against viruses. It was indeed reported that the activation of TLR4 delayed the onset of disease in RB-1B-infected chickens and reduced MDV genome copy numbers in infected spleens (Parvizi et al., 2014). In this study, the relative expression of TLR4 increased at 14 dpi, which aided the host in activating macrophages to fight off viral infection.

As a result of intracellular viral replication, non-self RNA appearing in cells is recognized by a family of cytosolic RNA helicases termed RLRs. RLR proteins, including RIG-I, MDA5, and LGP2, are expressed in both immune and nonimmune cells. Upon the recognition of viral nucleic acids, RLRs recruit specific intracellular adaptor proteins to initiate signaling pathways that lead to the synthesis of other inflammatory cytokines, which are important for eliminating viruses. TRIM25 E3 ubiquitin ligase induces the Lys 63-linked ubiquitination of RIG-I to elicit host antiviral innate immunity, which is crucial for the cytosolic RIG-I signaling pathway (Gack et al., 2007). The relative expression of the TRIM25 gene changed significantly after ALV-J infection in chickens in previous studies (Hang et al., 2014). It was also reported that the gene silencing of endogenously expressed TRIM25 inhibited release and replication upon overexpression of the retroviruses (Uchil et al., 2008). In this study, the relative expression of TRIM25 was

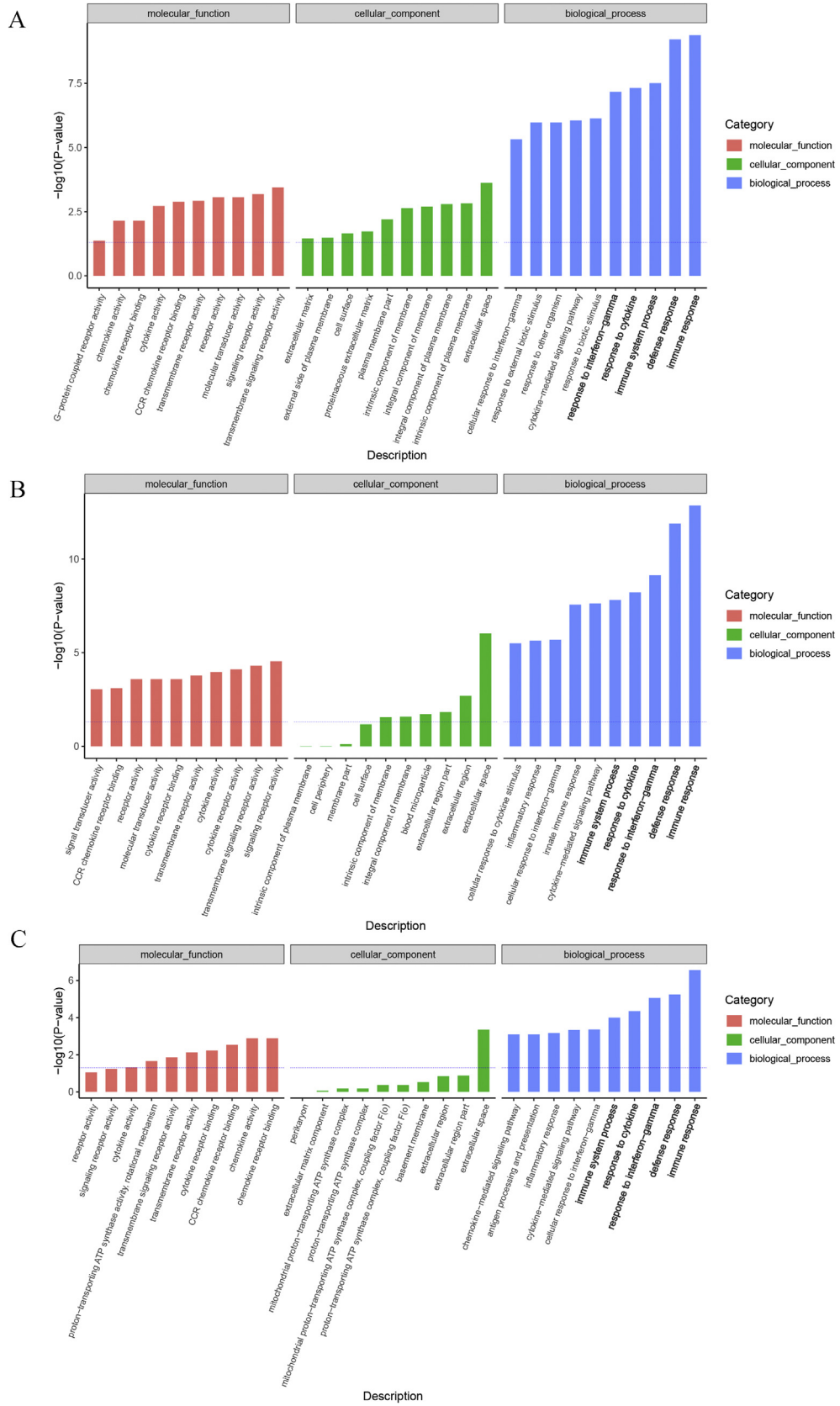
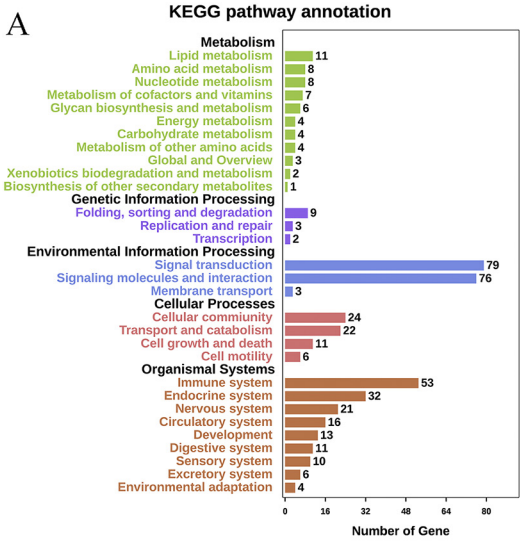
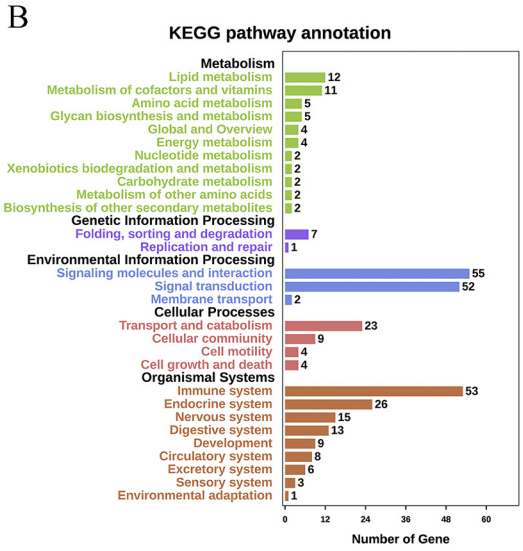
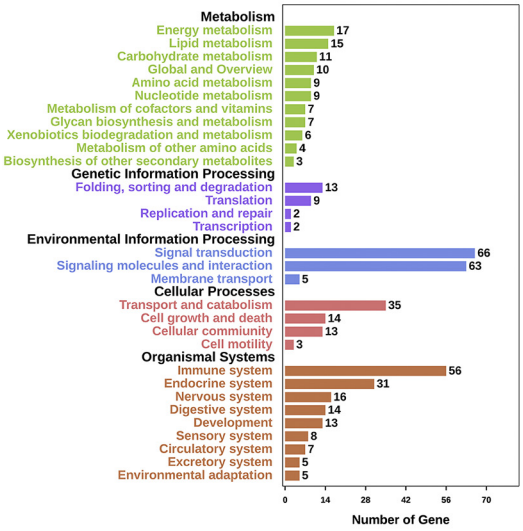
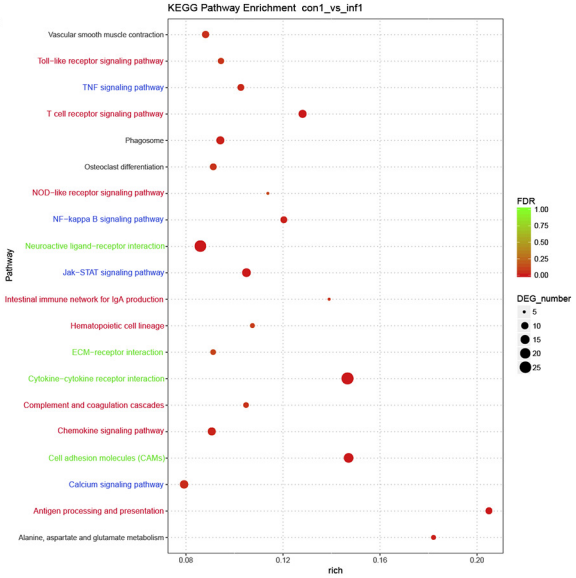


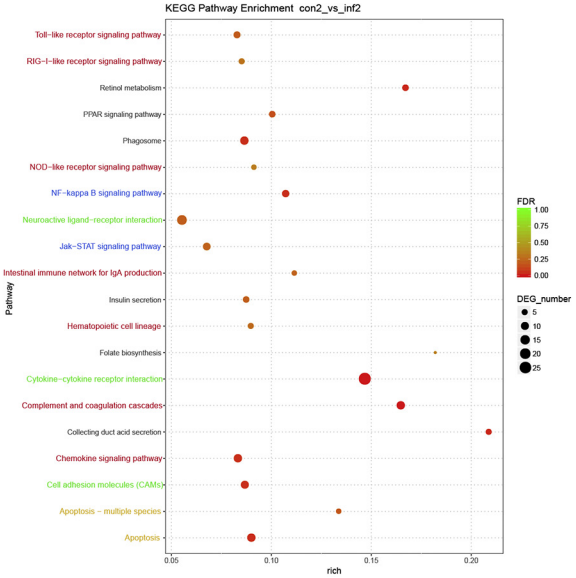

**Fig. 4.** Gene Ontology (GO) analysis of differentially expressed genes according to 7 dpi (a), 14 dpi (c), and 21 dpi (c).
Each colour represents a different biological process. The x-axis shows the description and the y-axis is the −log10 (p-value). The top five enriched GO terms are marked in black bold font.



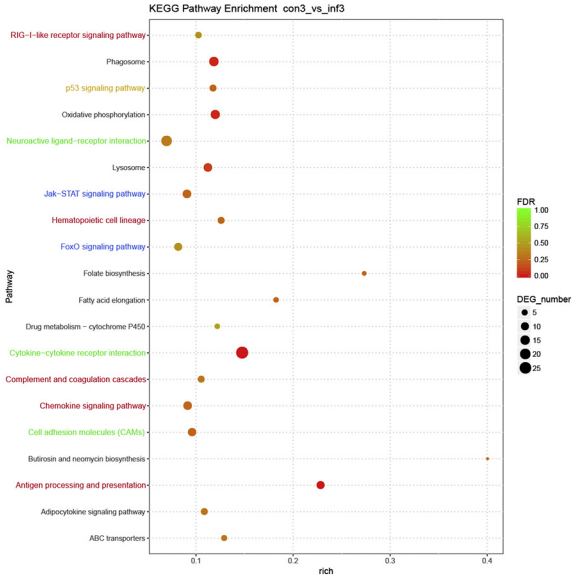




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**Fig. 5.** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed genes according to 7 dpi (a and d), 14 dpi (b and e), and 21 dpi (c and f).
Note: (a–c) In the ordinate, the black font is the first category of pathway and the colour font is the second category. The ordinate represents the number of differentially expressed genes (DEGs) enriched in this category of pathway. (d–f) Take the KEGG pathway as the ordinate and the rich factor as the abscissa. The size of the dot indicates the number of differential genes annotated to the pathway, and the colour indicates the p-value significance of the pathway. The figure shows the 20 most significant pathways in the results. The red, blue, green, and yellow marks represent the signaling pathways of the immune system, signal transduction, signaling molecules and interactions, and cell growth and apoptosis categories, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

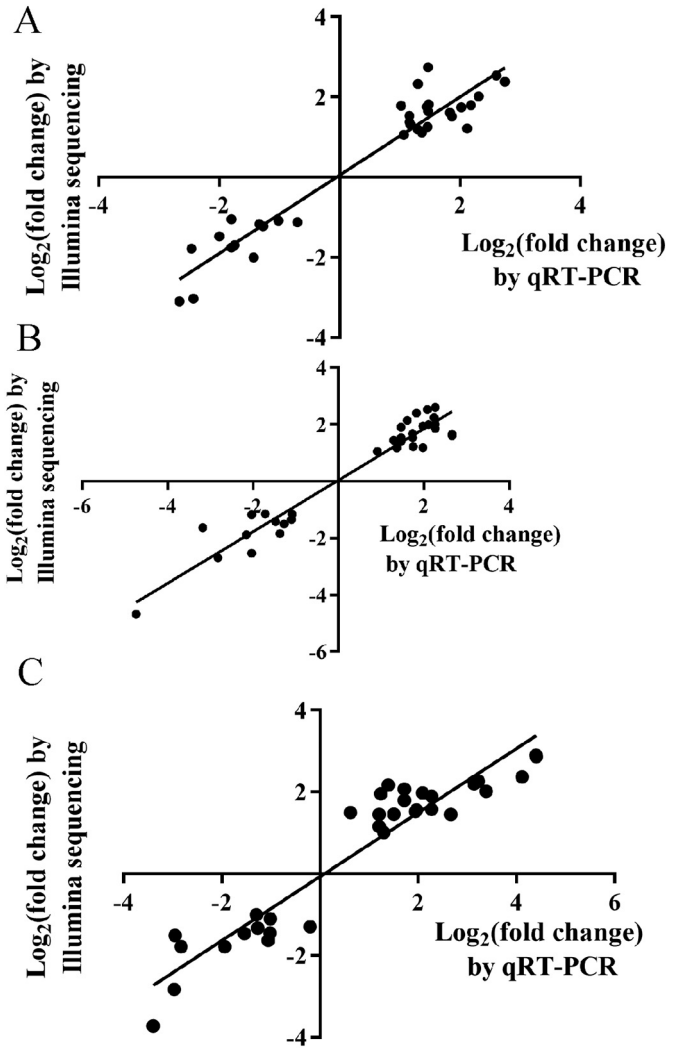


**Fig. 6.** qRT-PCR validation of differentially expressed mRNA at 7 dpi (a), 14 dpi (c), and 21 dpi (c).
The x-axis shows the log2(fold change) obtained by qRT-PCR, and the y-axis is the log2 (fold change) obtained by next-generation sequencing (NGS).

upregulated at all three time points, which might indicate that the host overexpressed TRIM25 to inhibit the replication and expression of REV to maintain the body's homeostasis. As the closest molecule to RIG-I (Kang et al., 2002), MDA5 was independently identified as a binding target for V proteins of paramyxoviruses (Andrejeva et al., 1965), which inhibited the dsRNA-induced activation of the IFN-β gene through MDA5 (Yoneyama et al., 2005). Other researchers found that MDA5 acted as a positive regulator in the virus-induced activation of type I IFN genes (Andrejeva et al., 1965) and there is evidence that RIG-I and MDA5 transmit an identical signal leading to the activation of IRF-3, IRF-7, and NF-κB (Yoneyama et al., 2005). In this study, the relative expression of MDA5 was upregulated at all three time points, which was likely due to the fact that large amounts of long dsRNA produced by REV invasion increased the expression of type I IFN induced by the MDA5 gene to resist and remove REV.

We also found that interferon-stimulated genes (ISGs) are differentially expressed in infections, such as IFI27L2, IFI35, IFI6, IFIH1, IFIT5, IFITM3, IRF1, ISG15, MX1, NMI, OASL, STATs, TRIM25, and USP18. The expression of ISGs may be related to the survival of the virus, a phenomenon similar to that of human retrovirus and avian retrovirus infections (Bosinger et al., 2004; Hang et al., 2014). These differentially expressed ISGs were expressed at relatively high levels in the pre-infection phase, but these ISGs showed moderate or no differential expression at 21 dpi, such as IFI35, IRF1, STAT2, and STAT4. We speculate that REV gradually breaks through the host's defense system.

Antimicrobial peptides are highly evolved parts of the innate immune system and play important roles in congenital defense (Lai and Gallo, 2009). In the present study, we found that CATHL2, CATHL3 and Gal5 are upregulated, and we speculate that these antimicrobial peptides have antiviral effects in the early stages of REV infection. However, these genes were differentially expressed only at 14 dpi, probably due to immature immune systems. This also indicated that REV slowly penetrated the host's defense system.

Adaptive immunity recognizes the specific molecular structure of an antigen through an antigen recognition receptor, thereby eliciting immune responses (Hansson Göran et al., 2002). Several genes related to immune cells were upregulated, such as LY6E, LY75, LY96, CD14, CD164, CD28, CD4, CD6, CD8B, TCF7, TRAT1, GZMA, LAG3, and RSFR. LY6E plays an important role in the differentiation and activation of T cells (Liu et al., 2003). LAG3 competes with CD4 for binding to MHC II (Lundmark et al., 2006). These results indicate that white blood cells and lymphocytes are important in controlling REV infection. At the same time, some downregulated CD molecules and lymphocyte antigens such as LY86, CD101, CD163, CD163L1, CD36, and CD69 were also observed. CD69 is critical for the generation and maintenance of professional memory Th lymphocytes, which can efficiently help humoral immunity in the late phase (Shinoda et al., 2012). CD36 has a number of functions related to immune responses, inflammation, and angiogenesis (Miller et al., 2011). CD36 has been shown to associate with TLR2 and TLR4 to provide ligands for their activation (Stewart et al., 2009). These may be part of the evidence that REV escapes host antiviral immunity.

A few genes related to apoptosis in this study were identified as differential expression genes, such as IRF1, PDCD1, MTFR2, FABP3, CDKN1A, GZMA, WNT5A, TNIP3, BIRC2, ZC3HAV1, and BIRC7. It was shown that IRF1 regulated tumor formation and the clearance of precancerous cells by apoptosis in previous studies (Schwartz et al., 2011). The cytoplasmic immunoreceptor tyrosine-based inhibitory motif of PD-1, encoded by the gene PDCD1, was activated by an interaction between PD-1 and its corresponding ligands, PDL-1 (B7-H1) and PDL-2 (B7-DC), which induces the inhibitory signal to inhibit the proliferation of T and B cells to maintain peripheral tolerance (Lee et al., 2006; Prokunina et al., 2002). The relative expression levels of IRF1 and PDCD1 were upregulated in this study. After REV infection, the host may promote apoptosis in tumor cells by increasing the expression of some apoptotic factors to resist tumor formation. However, REV also reduces the expression of some apoptotic factors to inhibit tumor cell apoptosis, which is beneficial for the formation of tumors.

Some cytokines related to inflammatory responses in this study were identified as DEGs. Chemokines have the role of collecting

granulocytes, mononuclear cells, and lymphocytes to the site of infection (Teixeira et al., 2002). In this study, the expression levels of CCL4, CCL19, CCL1, CCR2, IL6, IL18, TNFRSF18, CCL5, TNFRSF6B, IL12B, and CCR7 were all upregulated, and the expression of these genes induces immune cells to migrate to the infected sites to play a role of resistance to infection. IL-18 synergistically interacts with IL-12 to enable NK (natural killer) cells to produce IFN-γ and chemokines including CCL4, which might in turn recruit and fully activate macrophages, leading to the development of inflammatory foci presumably necessary for efficient microbial eradication (Sawaki et al., 2007). In this study, CCL4 had the highest relative expression level at 21 dpi and increased at other times, which may well indicate that macrophages and NK cells secrete more CCL4 to resist virus infection with the infection getting worse. However, these cytokines do not inhibit REV replication. This may be the cause of the occurrence of “cytokine storms”, which is a phenomenon that overproduces a variety of inflammatory cytokines against pathogens. Overexpressed cytokines can also cause significant damage to the host against pathogen invasion (Livolsi et al., 2001; Rothman, 2011). To maintain developmental and metabolic stability, the host utilizes anti-inflammatory factors to reduce or eliminate inflammatory damage caused by infection (Hang et al., 2014). In the current study, we found that several anti-inflammatory genes with anti-inflammatory functions were upregulated, e.g., CDKN1A, CDKN2A, CDKN2B, IL10, SOCS3, F13A1, F3, F7, FABP3, and FABP7. The balance between these pro-inflammatory and anti-inflammatory factors may be critical for REV immunosuppression.

## 5. Conclusion

In summary, our data revealed the transcriptomic profile in the spleen of REV-infected SPF chicken for the first time. We identified 1507 DEGs at 7, 14, and 21 dpi using NGS technology. Through the GO enrichment and KEGG pathway analysis of these DEGs, it was found that some signaling pathways, such as those of PRRs, chemokines, T cell receptors, JAK-STAT, etc., play an important role in the tumorigenic and immunosuppressive regulation of REV. A series of DEGs associated with inflammatory factors, apoptosis, innate immunity, and adaptive immunity were also discovered. These findings provide new insights and research directions for the elucidation of the pathogenesis of REV infection and the interaction between REV and the chicken immune system.

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## Declaration of Competing Interests

The authors declare that they have no competing interests.

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