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Changes in apoptosis, proliferation and T lymphocyte subtype on thymic cells of SPF chickens infected with reticuloendotheliosis virus

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ABSTRACT

Reticuloendotheliosis virus (REV), an avian retrovirus is able to infect a variety of birds and can cause immunosuppression. The aim of this study was to investigate the relationship of thymic lymphocytes apoptosis, proliferation and T cell subtype with immunosuppression. In this study, a hundred and twenty one-day old SPF chickens were randomly divided into control groups (group C) and a REV infection groups (group I). The chickens of group I received intraperitoneal injections of REV with $10^{4.62}$/0.1 ml $TCID_{50}$. On day 14, 21, 28 and 35 post-inoculation, the chickens of C group and I group were sacrificed by cardiac puncture blood collection, and the thymic lymphocytes was sterile collected. The proliferation ability of lymphocytes was tested by Cell Counting Kit-8. Flow cytometry was performed to detect apoptosis, cell cycle stage and the change in T cell subtype. The RNA genome copy numbers of REV virus were detected using real-time PCR. Real-time PCR and western blotting were performed to analyze the expression of CyclinD1 and Bcl-2. Our results showed that REV genome copy number steadily declined, the proliferation potential of thymic lymphocytes was inhibited, lymphocytes apoptosed, the ratio of CD4+/CD8+ decreased and the expression of CyclinD1 and Bcl-2 were firstly inhibited, then rapidly recovered. Thus, immunosuppression lead by REV is closely related to the change of T cell subtype, apoptosis, and proliferation of thymic lymphocytes.

1. Introduction

Reticuloendotheliosis virus (REV) causes immunosuppression, oncogenesis, and growth retardation in many avian hosts worldwide (Xue et al., 2017; Yang et al., 2017). The avian species including chicken, turkey, duck, geese, mallards, pigeons and other birds (Bohls et al., 2006; Jiang et al., 2014; Lin et al., 2009; Zhai et al., 2016). In recent years, REV has been widely distributed around the world and has contaminated a number of vaccines, especially vaccines for Gallid herpesvirus-2, fowl pox virus, and Marek's disease (Awad et al., 2010; Li et al., 2015; Wozniakowski et al., 2015). REV infections were very common in chicken flocks in China (Cui et al., 2009). It has been reported that a great amount of contaminated poultry vaccines in China were contaminated by REV (Wei et al., 2012). In addition, the immunosuppression caused by REV infection increases the susceptibility to secondary infections without other pathogenies (Yoshida et al., 1981).Early REV infection can also result in poor immune responses to vaccines against avian influenza virus (Sun et al., 2009). Therefore, REV is causing significant damage to the poultry industry.

Cell proliferation and apoptosis are very important and common self-regulatory mechanisms that are crucial in the development of the body and the maintenance of homeostasis (Lockshin and Zakeri, 2007; Perez-Garijo, 2018). Many viruses can induce abnormality in host cells. It is reported that some viral infections are closely related to apoptosis and tumorigenesis (Fulda, 2009; Liu et al., 2015; Saha et al., 2010).Critical to the avian immune response to viral infection, chicken T lymphocytes have been shown to control antigen specific control of viral infection (Pei et al., 2003; Seo and Collisson, 1997). Therefore, the number and function of T lymphocytes affect the immune function of REV-infected chickens. As the site of development and maturation of T lymphocytes, the thymus is crucial in body immunity (Takada et al., 2017). In chickens infected with REV, atrophy of the thymus causes the

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development of the T lymphocyte to be affected and leads to immunosuppression (Liang et al., 2013). Whether the immunosuppression caused by REV is related to the apoptosis and proliferation of T lymphocytes is the main question we discuss in the study?

Our previous experiments demonstrated that the apoptotic factors Caspase-6 and Caspase-7 were significantly increased in the immune organs of REV infected SPF chickens at 21 days. Early REV infection could cause severe inhibition of antibody responses and inhibit production of immune-related cytokines (Sun et al., 2006; Xue et al., 2013). However, there is no study on the relationship between the apoptosis and proliferation of thymus T lymphocytes with the immunosuppression induced by REV infection. In this study, we tested the dynamic changes of the virus replication, T lymphocyte subtype, proliferative capacity, the apoptosis state and Bcl-2 and CyclinD1 expression of thymus T lymphocytes in REV-infected SPF chickens. Our findings provide new clues for understanding the pathogenesis of REV infection.

2. Materials and methods

2.1. Ethics statement

All the procedures involving the chickens were in accordance with the Animal Care and Use Committee of Heilongjiang Province (SYXK (Hei) 2012-2067). The study was carried out in strict accordance with the Animal Ethics Committee of Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. All efforts were exerted to minimize the suffering of the chickens.

2.2. Virus and chickens

The REV T strain (No. CACCAV107) was obtained from China Veterinary Culture Collection Center (CVCC). The $TCID_{50}$ of REV was $10^{4.62}$/0.1 ml. All SPF chickens were fed in negative-pressure-filtered air isolators as recommended.

2.3. Experimental design and thymic lymphocytes collection

A hundred and twenty one-day old SPF chickens were randomly divided into a control groups (group C) and a REV infection groups (group I). The chickens of group I received an intraperitoneal injection of REV with $10^{4.62}$/0.1 ml $TCID_{50}$. On day 14, 21, 28 and 35 post-inoculation, 15 C group and 15 I group chickens (lymphocytes extracted from every 5 chickens were randomly mixed into one sample and 3 different samples per group) were sacrificed by cardiac puncture blood collection. The thymus was sterile collected. To prepare thymic lymphocytes, the sterile thymi were filtered through a sieve screen using a syringe plunger to obtain a single cell suspension in tissue culture medium (RPMI 1640, Gibco BRL NY, USA). Cell suspensions were overlaid onto a Histopaque-1077 (Sigma Aldrich, St. Louis, MO, USA) density gradient medium and centrifuged at 1900 rpm for 10 min at 20℃. The lymphocytes were collected from the interface and washed two times in RPMI 1640.

2.4. Thymic lymphocytes proliferation assay

The thymic lymphocytes of both C (non-infected) and I (infected REV) groups were seeded in 96-well plates at a density of 5×10^5 cells per dish and cultured in the presence of 10 μg/ml Con A (Sigma–Aldrich, St. Louis, MO, USA) for 48 h. Each well was then treated with 10 μL Cell Counting Kit-8 (Beyotime Biotechnology, Jiangsu, China) for 4 h at 37 °C. The OD450 values were measured by an ELx800 microplate reader (Bio-TEK). Independent experiments were performed in triplicate and repeated three times. Each experiment was based against a blank control.

2.5. Thymic lymphocytes cell-cycle analysis

The thymic lymphocytes of both C and I groups were washed with cold PBS and fixed in 70% ethanol at 4 °C for over 18 h. Thereafter, the cells were washed and resuspended in cold PBS. Then, according to the instruction of cell cycle analysis kit (Beyotime Biotechnology, Jiangsu, China), 1×10^6 cells were collected and incubated with PI solution for 30 min in the dark at 37 °C. Subsequently, the cell cycle stage was determined by flow cytometry (BD Biosciences, Franklin Lake, NJ, USA) and the date was analyzed using Modfit LT (Verity Software House, USA) software.

2.6. Analyses of apoptosis

The thymic lymphocytes samples were harvested by centrifugation at 1500 rpm for 5 min, then washed three times with cold PBS 2×10^5 cells were collected. Next, apoptotic cells were detected according to the instruction of the Annexin V-FITC apoptosis detection Kit (Beyotime Biotechnology, Jiangsu, China). 5 μL of Annexin V and 10 μL of PI were added in the 500 μL binding buffer provided. The cells were incubated for 15 min in dark at 4 °C and analyzed by flow cytometry (BD, USA).

2.7. Analyses of T lymphocyte subtype

Cell samples were cleaned as in the apoptosis analysis, with 1×10^6 cells collected. The cells were incubated with mouse anti-chicken CD4-FITC and mouse anti-chicken CD8α-RPE (Southern Biotech, Birmingham, AL, USA) for 30 min in dark at 4 ℃ then washed 2 times with cold PBS and detected by flow cytometry (BD, USA). The negative and single-stained samples were used for compensation controls.

2.8. RNA extraction and replication of the virus in thymic lymphocytes

Total RNA from thymic lymphocytes of REV-infected and virus free control chickens was extracted using Trizol reagent (Invitrogen, Shanghai, China) following the manufacturer's instructions. 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 thymic lymphocytes was quantified using primers specific for the viral long terminal repeat (LTR) region. The LTR primers used were: forward primer: 5′-CCAGCATCCAGGAGGTCTCT-3′ and reverse primer: 5′-CCGTG TAGGCCATGTTGTTC-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 10^2 molecules at the lower limit and 10^8 molecules at the upper limit. A real time-PCR assay was performed in a total volume of 20 μL containing 10 μL of SYBR® Premix Ex TaqTM (2×; Takara, Shiga, Japan), 100 ng of cDNA, 10 pmol of forward primer and 10 pmol of reverse primer. The Real-time PCR reactions of the gene followed the program of 95 °C for 5 min, then 45 cycles of 95 °C for 10 s and 57 °C for 30 s, and 72 °C for 10 s. Each qRT-PCR was carried out in triplicate. The $2^{-\Delta\Delta CT}$ method was used to evaluate. The absolute numbers of REV genome per 10^6 cells were calculated based on the standard curve.

2.9. qRT-PCR assay to examine the gene expression of Bcl-2 and CyclinD1

qRT-PCR was used to examine the gene expression of Bcl-2 and CyclinD1 using the Bcl-2 forward primer:5′-AGCTGGCAGTGTGGTAG CAG-3′, and reverse primer:5′-TGTTCTTGGCAGGCTCGTAA-3′ and the CyclinD1 forward primer:5′-CGCCGCTACCAGAGGGACTT-3′, and reverse primer:5′-C CGGACCCAGTTGACCCCAT-3′. β-Actin was used as the internal reference gene. The β-actin forward primer was: 5′-CGGG ACGGATGAGAAGAA-3′, and reverse primer: 5′-TCGGCGCTCCAGATG TAC-3′. Primers were designed using Primer 5.0 software. The primers were synthesised by Sangon Biotech, Shanghai, China. The specific method of real-time PCR was used as above.

2.10. Preparation of total protein extracts and western blot analysis

The thymic lymphocytes samples were extracted using RIPA buffer (50 mM Tris−HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1%SDS) supplemented with protease inhibitor (1 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin) for 30 min on ice. Total cell lysates were subjected to sonication and centrifuged at 12,000 × *g* at 4 °C for 20 min. Protein concentration was tested using the BCA protein assay (Thermo Scientific, Rockford, IL) using BSA as a standard. Total proteins (30 μg) were electrophoresed on 12% SDS–PAGE gel and then transferred to a nitrocellulose membrane (Pall Corporation, Pensacola, FL). The membranes were blocked for 2 h at room temperature with 5% non-fat milk in Tris-buffered saline (TBS), then rinsed with TBS containing 0.1% Tween-20 (TBS-T) three times. Subsequently, the membranes were incubated with primary antibodies (rat anti-chicken antibodies against cyclinD1, Bcl-2, α-Tubulin) diluted in the 5% non-fat milk TBS-T solution (1:1000) overnight at 4 °C. After rinsing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody in the 5% non-fat milk TBS-T solution (1:4000) for 1 h at room temperature and washed three times with TBS-T. The bands were visualized by ECL Western Blotting detection (Amersham Biosciences, Piscataway, NJ) and quantification was analyzed by Image J software (NIH, Bethesda, MD, USA).

2.11. Statistical analysis

Statistical analyses of significance were performed using two-sample Student's *t*-test to compare two means, using Bonferronni's adjustment to adjust multiple comparisons.

3. Results

3.1. Replication of the virus in thymic lymphocytes cells

To Confirm REV infection in SPF chickens and assess the replication ability of the REV in thymic lymphocytes cells, the RNA genome copy numbers for the virus were detected using real time PCR at 14, 21, 28 and 35 days after infection. The REV genome was detected and peaked at 14 days; thereafter, REV genome copy number steadily declined until the termination of the experiment at day 28 (Fig. 1a). In addition, the infected chicks exhibited typical REV infection symptoms, including depression, loss of appetite, and atrophy of thymus (Fig. 1b).

3.2. T cell functions evaluation by cell proliferative potential and CD4+/CD8+ ratios

We further evaluated the proliferation of both C (non-infected) and I (infected REV) group T cells using the CCK-8 assay. The proliferative potential of I groups T cells was significantly lower than the C groups at 14, 21and 28 days. On other days, the Abs 450 nm value was not significantly different (Fig. 2a).

In addition, 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 cytometry. As is shown in Table1, the CD4+ and CD8+ cells in I groups chickens were less than C groups chickens at 21 and 28 days, however, the CD8+ cells of I groups were significantly higher than the C groups at 14 day. The double positive T cell of I groups were dramatically more than C groups at 21 days. The ratios of CD4+/CD8+ in I groups chickens were markedly lower than C groups chickens at 14 and 21 days (Fig. 2b).

3.3. T cell function evaluation by cell-cycle analysis

To investigate the influence of REV infection on the SPF chicken T lymphocytes cell-cycle, we applied Cell Cycle Analysis Kit and flow cytometry. The G1 phase cells of I groups were significantly higher than C groups at 14 and 21 days and S, G2/M phase cells were lower, however the opposite result was seen at 28 days, and 35 days were not significantly different (Fig. 3).

3.4. T cell function evaluation by apoptosis analysis

To assess the effects of REV infection on SPF chicken T lymphocytes apoptosis, we used Annexin V-FITC/PI Apoptosis Detection Kit and flow cytometry. As is shown in Fig. 4, the apoptosis of I groups T cell was significantly more serious than C groups at 14 and 21 days, whilst other

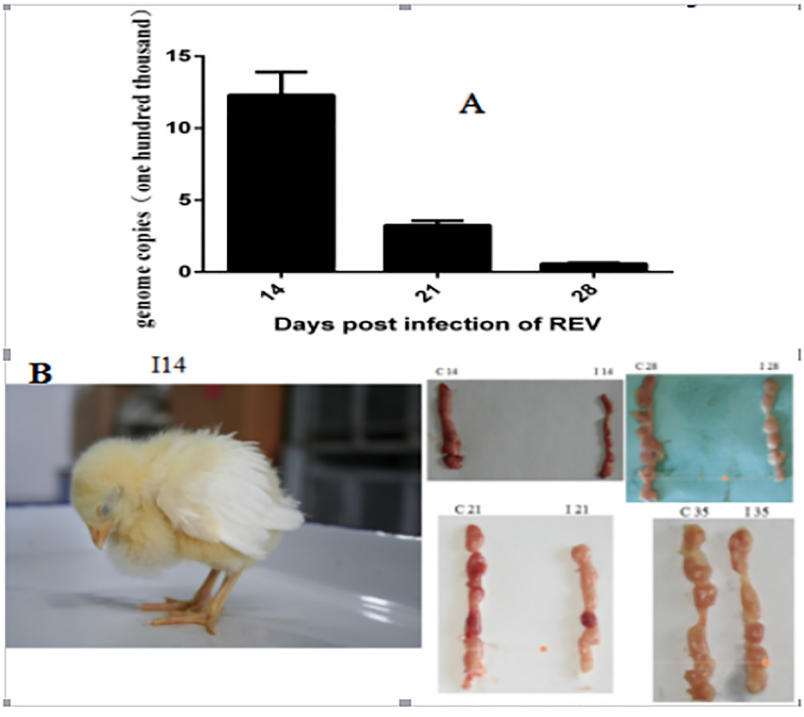

Fig. 1. The RNA genome copy numbers(order of magnitude 10^6), growth retardation and atrophy of thymus.
(A) Y axis represents REV genome copy numbers (order of magnitude 10^6). The genome copies steadily declined until the termination of the experiment at day 28, **(B)** the infected chicks exhibited typical depression. The thymus of chicks: C14 vs I14, C21vs I21, C28 vs I28, C35 vs I35.

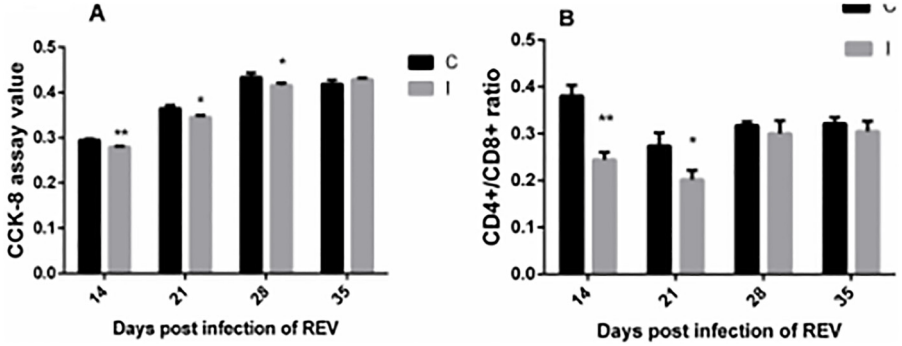

Fig. 2. The proliferative potential and CD4 + /CD8 + ratios of thymic lymphocytes.
(A) The proliferative potential of thymic T lymphocytes. (**B**) The CD4 + /CD8 + ratios of thymic T lymphocytes. Data is expressed as mean ± SD. * means significant difference, ** means extremely significant difference, the same below.

Table 1
Changes of T lymphocytes subtype in the thymus of SPF chickens (%).

Time	Items	CD4 +	CD8 +	DP (CD4 + CD8 +)	CD4 + /CD8 +
	C group	2.87 ± 0.25	7.60 ± 0.59	77.43 ± 1.17	0.38 ± 0.023
14 days	I Group	2.73 ± 0.31	11.17 ± 0.55**	71.50 ± 1.28	0.24 ± 0.016**
	C group	3.60 ± 0.36	13.20 ± 0.56	66.37 ± 1.51	0.27 ± 0.028
21 days	I Group	2.10 ± 0.41**	10.53 ± 0.89*	74.33 ± 2.42**	0.20 ± 0.021*
	C group	5.00 ± 0.27	15.72 ± 0.55	66.26 ± 1.17	0.32 ± 0.008
28 days	I Group	4.27 ± 0.35*	14.27 ± 0.71*	63.13 ± 3.66	0.30 ± 0.027
	C group	4.93 ± 0.38	15.33 ± 0.61	67.47 ± 1.50	0.32 ± 0.013
35 days	I Group	4.84 ± 0.32	15.87 ± 0.75	64.70 ± 2.05	0.31 ± 0.022

Notes: Data is presented with the means ± standard deviation. When compared with the control group, * means significant difference, ** means extremely significant difference.

days were not significantly different. In summary, the above results indicated that REV pre-infection can lead to inhibition of T cell functions, but the process looks like it can be resumed.

3.5. Kinetic changes of Bcl-2 and CyclinD1 mRNA expression in thymic lymphocytes infected with REV

Bcl-2 is one of the most representative factors for study of cell apoptosis and CyclinD1 is also vital on cell-cycle research. Thus, to demonstrate the kinetic changes of apoptosis and cell-cycle of REV-infected chicken's thymic lymphocytes from transcriptional expression, Bcl-2 and CyclinD1 were detected by real time PCR. The Bcl-2 expression of I groups thymic cells was significantly lower than C groups at 14 and 21 days, however, Bcl-2 expression increased at 35 day (Fig. 5a). In addition, the transcriptional expression of CyclinD1 was down-regulated in REV-infected chickens at 14 days, but, CyclinD1 expression was up-regulated at 35 days. Other days were not significantly different (Fig. 5b).

3.6. Kinetics of Bcl-2 and CyclinD1 protein expression after infection with REV

To demonstrate the kinetic changes of apoptosis and cell-cycle of REV-infected chicken's thymic lymphocytes from protein expression, Bcl-2 and CyclinD1 were detected by Western blot. The Bcl-2 protein expression of I groups T cells was significantly lower than C groups at 14, 21 and 28 days, however, Bcl-2 protein expression increased at 35 day (Fig. 6a). In addition, the protein expression of CyclinD1 was down-regulated in REV-infected chickens at 21 and 28 days, but other days were not significantly different (Fig. 6b).

4. Discussion

REV is a common immunosuppressive and oncogenic virus, and early infection can cause severe growth retardation and immunosuppression, marked by the inability to mount immune response to vaccination whilst also increasing the susceptibility to concurrent or secondary bacterial or viral infections (Witter et al., 1979; Xue et al., 2017). The immunosuppression caused by REV infection is closely related to atrophy of immune organs such as the thymus (Wang et al., 2012). In that way, the number and function of thymus T lymphocytes should go hand in hand with REV infection. In this study, we found that the load of virus first increased and then decreased or even disappeared in the thymus. The volume of the thymus firstly atrophied and gradually recovered, and the proliferation potential of thymus lymphocytes was also first reduced and then raised. Furthermore, our date showed that the depletion of lymphocytes in the thymus was associated with apoptosis induced by REV infection and the ratio of CD4 + /CD8 + was lower in REV infected chickens. Thus, REV can suppress the immune function of the thymus significantly through reducing the number and function of thymic lymphocytes.

T lymphocytes are responsible for cell-mediated immunity, most CD4 + T cells are helper T cells, and CD8 + T cells are cytotoxic T cells (Pavelka and Roth, 2015). The CD4 + T cells coordinate immune responses through the expression of key transcription factors and signature cytokines against the invading pathogen (Marshall et al., 2017). Cytotoxic T lymphocytes (CTLs) are vital for the induction of immune responses against viral infection or transformed cells (Rauf et al., 2012). Thus, the number of T lymphocytes and CD4 + /CD8 + ratio can reflect the cellular immune status of the body. Studies have shown that, in peripheral blood mononuclear cells (PBMCs), after 2 weeks, the lower ratio of CD4 + /CD8 + caused by REV infection could inhibit the host immune response and cause susceptibility to other pathogens (Xue et al., 2013). Wang et al have found that lymphocytes in spleen

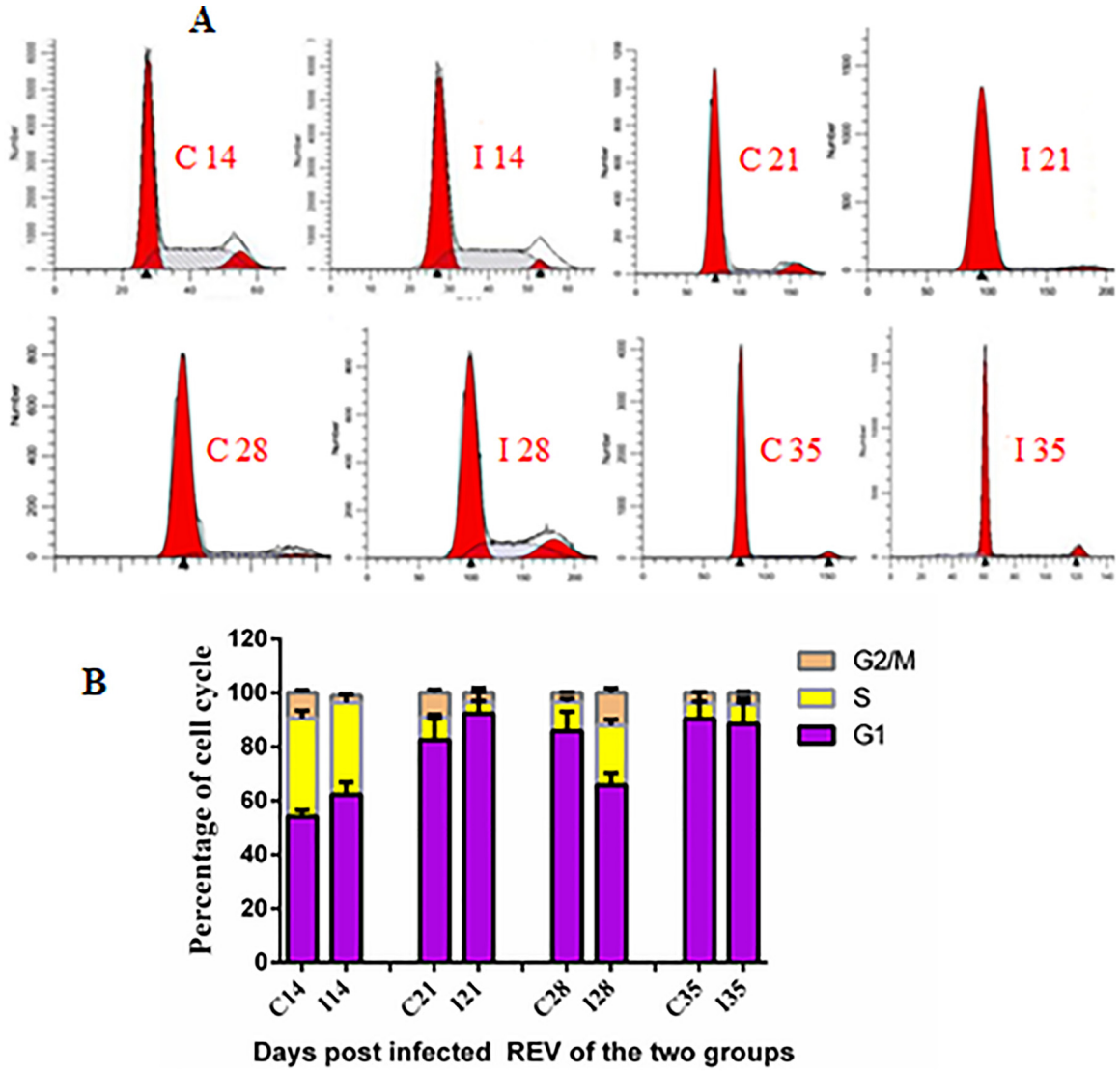

Fig. 3. The cell-cycle analysis of thymic lymphocytes.
(A) The cell cycle of thymic lymphocytes in C14 vs I14, C21 vs I21, C28 vs I28, C35 vs I35. **(B)** The replicate data results statistical column chart of cell cycle of thymic lymphocytes in all time points.

presented significant apoptosis after infection with REV, and thought that depletion of lymphocytes in lymphoid tissues is the main reason for immunosuppression (Wang et al., 2012). Thymus is the place where T lymphocytes develop and mature, then, the mature lymphocytes enter the peripheral (e.g. spleen and PBMC) through blood circulation to exert cellular immune function. Thus, thymic T lymphocytes play an important role in the chicken immune responses to viral infection (Robinson et al., 2014). In that way, how do the changes of T lymphocytes in thymus after infection with REV? In order to examine the interactions of REV with thymic T lymphocytes, we have detected T cell subsets by Flow Cytometry. As shown in Table1, in order to clear REV virus, the levels of thymic CD8+ increased in the early stage of infection. With the cell apoptosis and decline in cell proliferating ability, the levels of CD4+ and CD8+ and the ratio of CD4+/CD8+ decreased. Our results combined with previous studies indicate that REV not only can significantly reduce cell-mediated immunity within in thymus (immature T cell subsets), but also induce immune suppression in the mature T cell population, such as spleen and PBMCs. However, the relationships between REV infection in the thymus and immune suppression within the mature T cell population were not clear. Whether REV infection first causes thymus immunosuppression and then leads to peripheral cell-mediated immunosuppression, which will be further evaluated in future studies.

In the induction of apoptosis, some infectious factors, especially viruses, play an important role, such as chicken infectious bursal disease virus (IBDV), chicken anemia virus (CAV), Marek's disease virus of chicken (MDV), which can induce lymphocyte apoptosis and cause immunosuppression (Lai et al., 2017; Qin et al., 2017; Wan et al., 2016). Atrophy of the thymus is often accompanied by apoptosis of lymphocytes (Gao et al., 2008). In the current study, the REV genome peaked at 14 days, thereafter REV genome copy number steadily declined until the termination of the experiment at day 28 (Fig. 1a). The volume of thymus firstly atrophied and gradually recovered (Fig. 1b), with apoptotic analysis showing that thymus lymphocytes appeared apoptotic following REV infection at 14 and 21 days (Fig. 4). These results probably indicate that like the immunosuppressive viruses mentioned above, REV as an inducing factor causing the apoptosis of thymus lymphocytes, thus causing atrophy of the thymus. With the removal of the virus, apoptosis is controlled, and the thymus gradually restored. In order to verify our point of view, we have conducted further experiments.

Many immunosuppressive viruses can cause lymphocyte apoptosis,

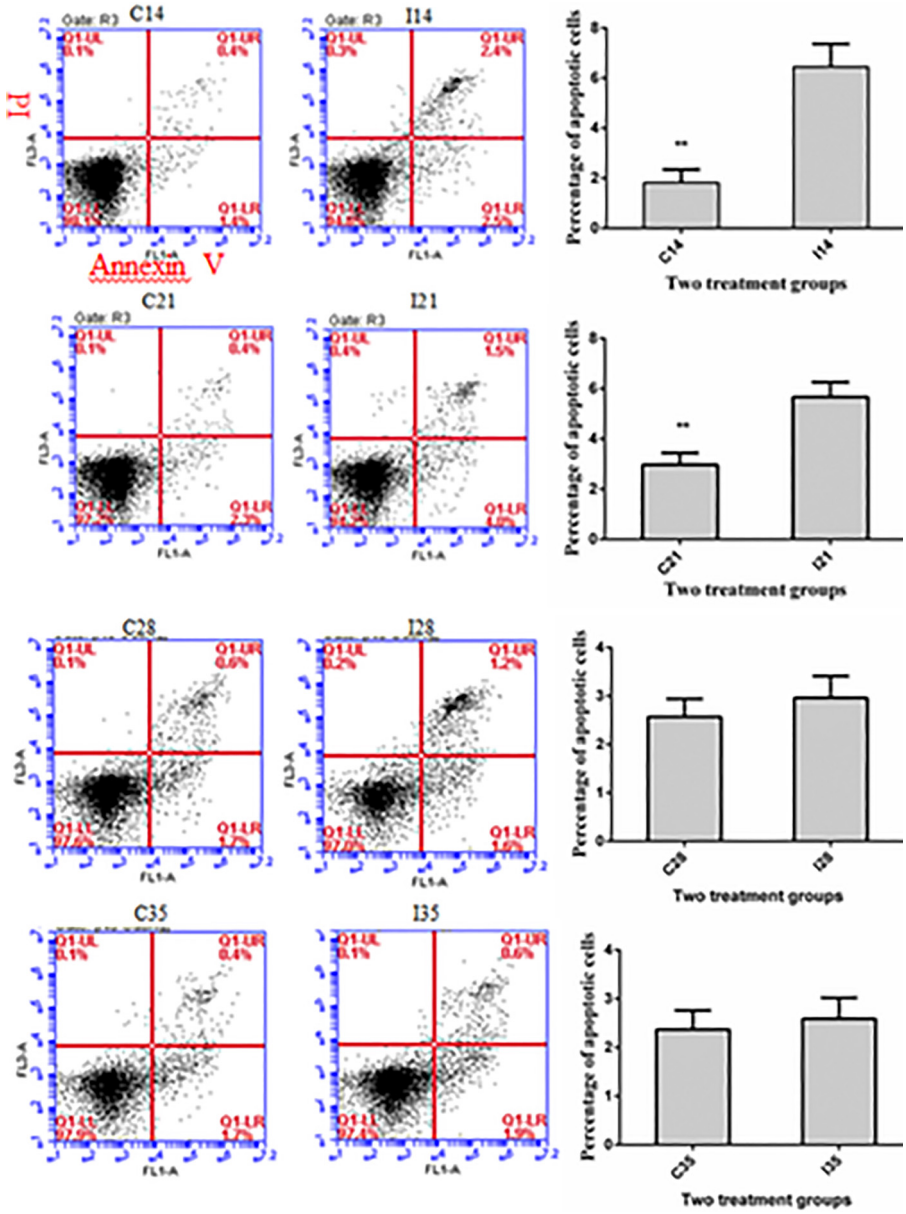

Fig. 4. The apoptotic analysis of thymic lymphocytes.
Annexin V-FITC/PI staining was used to detected thymic lymphocytes, the apoptosis of thymic lymphocytes in C14 vs I14, C21 vs I21, C28 vs I28, C35 vs I35, and the X axis represents Annexin V staining and the Y axis represents PI staining. The replicate data results statistical column chart of apoptosis of thymic lymphocytes, and the X axis represents percentage of apoptotic cell and the Y axis represents two treatment groups.

not only in poultry, but also in other animals. For example, highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) infects piglets and can mediate cell apoptosis through the caspases dependent pathway and significantly increases the expression of Caspase-3 and Caspase-8 in the thymus of piglets (Wang et al., 2015). The expression of Bcl-2 can decrease the apoptosis of CD4T cells in patients with human immunodeficiency virus (HIV) infection (Nardacci et al., 2015). Thus, REV infection induces apoptosis of lymphocytes in immune organs, which also can cause changes in related apoptotic factors. Our previous experiments demonstrated that the apoptotic factors Caspase-6 and Caspase-7 were significantly increased in immune organs of REV infected SPF chickens(Yu et al., 2017).In this study, the Bcl-2 transcriptional expression in thymus lymphocytes was significantly decreased at 14 and 21 days, however increased at 35 days (Fig. 5a). The dynamic changes of Bcl-2 protein expression are similar to transcriptional expression, which also markedly decreased at 28 days (Fig. 6a). The members of the bcl-2 family are a group of crucial regulatory factors in apoptosis (Ismail et al., 2007). Bcl-2 is one of the most representative factors used to study cell apoptosis. The expression of Bcl-2 can aid verification of the occurrence of apoptosis. Apoptosis can not only maintain the body in a stable condition, but also plays an important role in regulating and controlling the number of cells (Heinke et al., 2001). REV infection leads to apoptosis, which results in a decrease in the number of thymus lymphocytes. In summary, the above results indicated that immunosuppression caused by REV infection is closely related to the apoptosis of thymus lymphocytes.

It has been demonstrated that occurrence of some diseases is due to the loss of control of normal apoptosis and the disturbance of the balance between cell proliferation and apoptosis, such as in cancer (Park et al., 2001). Is the immunosuppression caused by REV associated with the destruction of this balance? In order to verify this idea, we further detected the proliferative ability of lymphocytes by analyzing cell proliferation, the cell cycle and CyclinD1 expression of thymic lymphocytes. T lymphocytes are responsible for cell-mediated immunity,

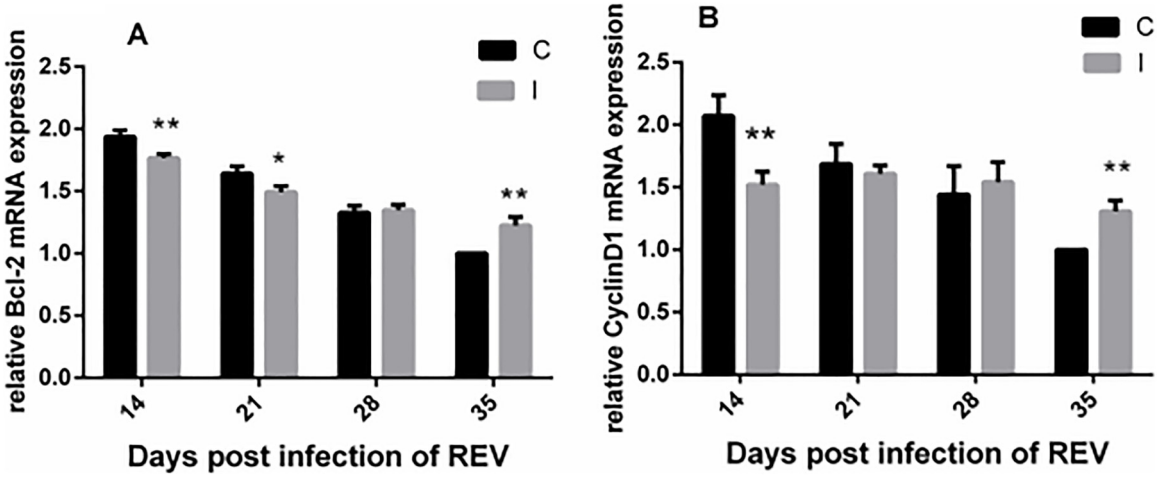

Fig. 5. The kinetic changes of Bcl-2 and CyclinD1 mRNA expression.
(A) The Bcl-2 mRNA expression of thymic lymphocytes. **(B)** The CyclinD1 mRNA expression of thymic lymphocytes. Data are expressed as mean ± SD.

which plays a critical role in the host defense system against infections (Feng et al., 2015). The inhibition of peripheral blood T-cell proliferation induced by REV would enable the virus to down-regulate the host immune response (Xue et al., 2013). Thus, the proliferation potential decline of cells could inhibit cell proliferation of the lymphocytes and reduce cellular immunity. CyclinD1 is an important member of the cyclin family and drives cell cycle progression at the G1/S transition. It's expression is closely related to cell proliferation (Zuryn et al., 2014). Therefore, analysis of the expression of CyclinD1 can be used to understand cell proliferation at the molecular level. Lin Z et al have found that GANT61 decreased CyclinD1 mRNA expression and protein levels, which also induced G1/S arrest and apoptosis of Daoy cells (Lin et al., 2017). In the present study, the proliferative potential of thymus T cells was significantly decreased after REV infection (Fig. 2a). The G1 phase cells were observably increased and S and G2/M phase cells decrease at 14 and 21 days after REV infection, but the opposite result was observed at 28 days (Fig. 3). The CyclinD1 transcriptional expression of thymus lymphocyte was markedly decreased at 14 days, however it increased at 35 days (Fig. 5b). The dynamic change of CyclinD1 protein expression was significantly down-regulated at 21 and 28 days (Fig. 6b). Our results suggest that the inhibition of thymic T-cell proliferation induced by REV would reduce cellular immunity and cause immunosuppression. However, the above results showed that after 28 or 35 days of REV infection, apoptosis was inhibited and the cell proliferation ability and the expression of cyclinD1 were significantly increased. Our previous experiment also showed that the cell cycle related factors c-myc, cyclin A1, and cyclin B2 were significantly increased at 28 days post infection (Yu et al., 2017). Whether the body functions recovery or the tumorigenicity of REV caused this is not known. In this study, necropsy of the sacrificial chickens didn't show neoplastic nodules or grey foci in the internal organs, thus, the specific mechanisms will be further evaluated in future studies.

In the current study, we successfully applied the mode for the pathogenesis of REV infection in SPF chickens and explored the influence of REV infection on T cell immune function in the thymus of SPF chickens and preliminarily linked the relationship of thymic T lymphocytes apoptosis and proliferation with immunosuppression. Apoptosis and the inhibition of lymphocyte proliferation lead to atrophy of the thymus and decrease in the number of lymphocytes. Our results provide further proof that immunosuppression caused by REV is closely related to the apoptosis and proliferation of thymic T lymphocytes.

5. Conclusion

Our results suggest that REV infection causes thymic lymphocytes

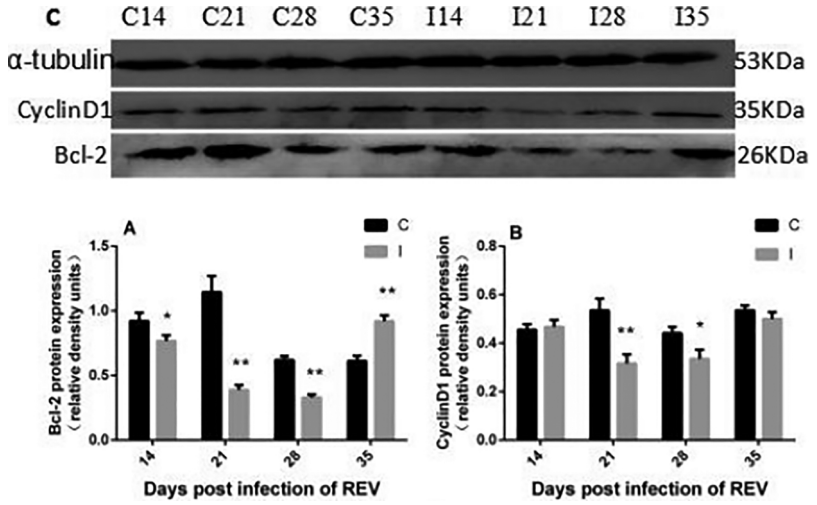

Fig. 6. The kinetic changes of Bcl-2 and CyclinD1 protein expression.
(A) The Bcl-2 protein expression of thymic lymphocytes. **(B)** The CyclinD1 protein expression of thymic lymphocytes. **(C)** The protein expression of α-Tubulin, CyclinD1 and Bcl-2 with western blot. Data are expressed as mean ± SD.

apoptosis, inhibits T lymphocyte proliferation, changes T cell subsets and enhances the immunosuppressive effect. In the present study, several new reasons for immunosuppression caused by REV have been found in infected SPF chickens, but some questions need to be further investigated.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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