Knockout of *p53* leads to a significant increase in ALV-J replication

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ABSTRACT Avian leukemia is a common malignant disease, and and its regulatory mechanism is complex. As the most extensive tumor suppressor gene in cancer research, *p53* can control multiple functions such as that of DNA repair, induction of apoptosis, cell cycle arrest and so on. In view of the diversity associated with varied function of *p53*, this study analyzed the possible effect of gene on ALV-J replication and its regulatory mechanism. We successfully constructed a *p53* knockout DF-1 cell line (p53-KO-DF-1 cells) by using CRISPR-Cas9 system. When ALV-J was co-infected with DF-1 and p53-KO-DF-1 cells, it was found that compared with wild-type DF-1 cells, the viral copy number of p53-KO-DF-1 cells infected with ALV-J increased significantly 48 h after infection, whereas the expression of innate immune factors such as Il-2,TNF- α, IFN- γ and MX1 decreased significantly. Detection of *p53*-related tumor genes indicated that after *p53* deletion, the expression of *c-myc, bcl-2*, and *bak* increased significantly, while the expression of *p21* and *p27* was noted to be decreased. The cell cycle distribution and apoptosis of the 2 cell lines was detected by flow cytometry analysis. The results showed that *p53* knockout prevented G0/G1 and G2 M phase arrest induced by ALV-J, and substantially decreased the rate of apoptosis. Overall, the results indicated that p53 gene can effectively inhibits ALV-J replication by regulating important cellular processes, and *p53* gene related proteins involved in cell cycle activity may function as the key targets for the prevention and treatment of ALV-J.

Key words: ALV-J, *p53*, CRISPR-Cas9, cytokines, cell cycle and apoptosis

2021 Poultry Science 100:101374
https://doi.org/10.1016/j.psj.2021.101374

INTRODUCTION

Avian leukemia is among one of the 3 most common tumor diseases found in the birds, which can spread horizontally and vertically in chickens. There are 7 different kinds of avian leukemia virus subgroups that have been identified in infected chickens (A-E/J/K). Among them, avian leukosis virus subgroup J (**ALV-J**) is a recombinant virus derived from endogenous and exogenous virus, which primarily induces hemangioma and myelocytoma, and its pathogenicity and transmission ability has been reported to be significantly higher than those of other subgroups (Li et al., 2017, 2018; Su et al., 2018). ALV-J can promote the development of extensive tumors in the multiple organs of diseased chickens, thus resulting in severe immunosuppression and high mortality.

p53 is an ubiquitous tumor suppressor gene found in various organisms, which is responsible for regulating body homeostasis, and can exhibit diverse functions associated with DNA repair and senescence, regulation of cell cycle, promoting apoptosis and inhibiting tumorigenesis (Laptenko and Prives, 2006). In healthy cells, *p53* has been found to be negatively regulated by *Mdm2* and other genes, thus maintaining a low level in vivo and also exhibiting minimal activity (Michael and Oren, 2003). However, when tumor invasion occurs, *p53* is rapidly activated and *p53* protein has been found to be highly expressed in the nucleus. It can exhibit a change in the conformation from monomeric to tetrameric form and can play a role as a potential tumor suppressor after activation by acetylation and phosphorylation (Kamada et al., 2016). The main role of *p53* as a putative transcription factor, is to control the activation of downstream target genes (such as *p21, PUMA, Bcl-2* family genes) to cause cell cycle arrest,

Received May 7, 2021.
Accepted June 30, 2021.

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apoptosis and thus leading to significant tumor inhibition (Valente et al., 2013; Kim et al., 2017). At the same time, *p53* protein can also effectively interact with other proteins directly or indirectly. For example, under the influence of stress signals, cytoplasmic *p53* can translocate into the mitochondria and competitively interact with antiapoptotic proteins such as *Bcl-xL* and *Bcl-2*, thereby releasing various pro-apoptotic proteins such as Bax and Bak to activate the mitochondrial apoptosis pathway (Estaquier et al., 2012). Reactivation of *p53*-mediated *p21* expression can inhibit the occurrence of the breast cancer (Morrison et al., 2016). Additionally, activation of *p53*-mediated mitochondrial apoptosis pathway can reduce the invasive potential of metastatic pancreatic cancer (Cheng et al., 2019); and enhancement of *p53* phosphorylation as well as acetylation can significantly retard the tumor growth (Cordani et al., 2020).

Therefore, in the process of tumor development, *p53* inactivation serves as a common phenomenon that can promote aberrant tumor transformation and unlimited proliferation. A number of previous studies have shown that when ALV-J occurs, *p53* gene will be accompanied by mutation or deletion and other abnormal modifications (Takagi et al., 2006; Yue et al., 2015), thereby suggesting that there may be a possible relationship between *p53* as well as the occurrence and development of ALV-J. However, in the exact role of *p53* in controlling ALV-J-induced tumor has not been reported in the literature. In this study, the CRISPR-Cas9 system was used to knock out *p53* from DF-1 cells and successfully construct a *p53* knockout cell line (P53-KO-DF-1). By infecting normal DF-1 cells and p53-KO-DF-1 cells with ALV-J, the possible effects on ALV-J replication and innate immunity, cell cycle regulation and apoptosis of DF-1 cells were also compared and analyzed. Our results confirm the antiviral activity of *p53* gene against ALV-J infection and has elucidated the regulatory mechanisms by which *p53* can potentially play an inhibitory role in tumorigenesis.

MATERIALS AND METHODS

Cells, Virus, and Antibodies

DF-1 cells (chicken embryo fibroblast cell) were cultured and maintained in Dulbecco's modified Eagle's medium (**DMEM**) containing 10% (v/v) fetal bovine serum (**FBS**, Gibco, Grand Island, NY), 1% (v/v) penicillin-streptomycin (Solarbio life science, Beijing, China) in a humidified incubator with 5% CO2 at 37℃. ALV-J NX0101 strain(GenBank:DQ115805)was propagated and titrated in DF-1 cells and stored at −80℃. The virus was kindly provided by Professor Zhizhong Cui of Shandong Agricultural University. The various antibodies used were purchased from the following companies: anti-P53 (1:1000, 610183, BD, Franklin Lakes, NJ), anti-GAPDH (1:5000, AT0002, CMCTAG, San Diego, CA), horseradish-peroxidase-conjugated anti-rabbit (1:10000, West Grove, PA).

Knockout of p53 in DF-1 Cell Lines Using the CRISPR-Cas9 System

For *p53* knockout, 2 different pairs of sgRNA were designed according to the conservative region of chicken *p53* exon by using an online CRISPR Design Tool (http://crispr.mit.edu/). The primer P53-KO-312-Fwd (Forward) and *P53*-KO-312-Rev (Reverse) were phosphorylated and annealed to form P53-KO-312, and then inserted into Px459M (PX459 pSpCas9-2A-Puro-MCS) vector at BbsI site. *P53*-KO-625-Fwd and *P53*-KO-625-Rev were phosphorylated and annealed to form *P53*-KO-625, and then inserted into EZ-Guide-XH vector at BbsI site. Therafter, the EZ-Guide-XH-*P53*-KO-625 was inserted into PX459M-P53-KO-312 vector at XhoI and HindIII sites from EZ-Guide-XH vector to form PX459M-P53-KO. The plasmids were then sequenced to confirm the correct insertion.

DF-1 cells were transfected with the plasmid Px459M-*P53*-KO. After 48-h transfection, the cells were treated with puromycin at a concentration of 1 μg/mL for 4 d. Then, the single cell suspension was seeded into 96-well plates to continue the culture in DMEM with 20% (v/v) FBS, 1% (v/v) penicillin-streptomycin and puromycin (1 μg/mL), and culture in 24-well plates. DNA sequencing of *p53* protein was used for confirmation by PCR and sequencing, and knockout of *p53* protein was evaluated by western blot analysis. The RT-PCR primers used in this study have been listed in Table 1.

Real-Time PCR Analysis

Total RNA was extracted from cells using Trizol (Takara, Tokyo, Japan) and reverse transcription was performed using ReverTra Ace qPCR RT Kit (Toyobo, FSQ-101, Osaka, Japan). The samples were amplified by SYBR Green Realtime PCR Master Mix (Toyobo, QPK-201, QPK-201T, Osaka, Japan) and Real-time PCR quantitative analysis was carried on Roche real-time PCR software (LightCycler96). The experiment was repeated for three times and three different groups of repeated samples were used each time. The virus copy number of ALV-J was quantified using a standard curve. The relative transcriptional levels of the various genes were determined by 2-ΔΔCT (in which CT is threshold period) method analysis. The details of various amplification primers used have been listed in Table 2.

Western Blot Analysis

The cells were washed with PBS for 3 times, scraped and the cell lysates were prepared after lysing the cells

Table 1. Primers used in p53 knockout experiment.

Name	Primer sequence (5'-3')
p53-KO-312-Fwd	CACCGATCCACGGAGGATTATGGGG
p53-KO-312-Rev	AAACCCCCATAATCCTCCGTGGATC
p53-KO-625-Fwd	CACCGCAGGCGCGTTACCACGACGA
p53-KO-625-Rev	AAACTCGTCGTGGTAACGCGCCTGC

Table 2. Primers used for Real-time PCR assay.

Name	Primer sequence (5'-3')
pol-Fwd	CTAACGAGGCGAGGGAATG
pol-Rev	TTGGTGGGTTGGGTGGAGA
p21-Fwd	CTGTCTGTACGAAGCAATG
p21-Rev	TTCCAGTCCTCCTCAGTC
c-myc-Fwd	TTCTTTGCCCTGCGTGACC
c-myc-Rev	GCCTCAACTGCTCTTTCTCTGC
mdm2-Fwd	TGATGTTCCTGACTGTAAGA
mdm2-Rev	CGTAGATGGCTGAGAATAATC
P27-Fwd	CGCTTCTCAGGGAATCTC
P27-Rev	TTCTGTTGTGTTGGCTCTT
bcl-2-Fwd	CGCTGGTGGACAACATTG
bcl-2-Rev	GGCTCAGGATGGTCTTCA
bak-Fwd	ACCCGGAGATCATGGAGA
bak-Rev	GATGCCTTGCTGGTAGACG
β-actin- Fwd	GAGAAATTGTGCGTGACATCA
β-actin- Rev	CCTGAACCTCTCATTGCCA
IL-2-Fwd	GCTTATGGAGCATCTCTATCATCA
IL-2- Rev	GGTGCACTCCTGGGTCTC
IL-6-Fwd	AGGACGAGATGTGCAAGAAGTTC
IL-6- Rev	TTGGGCAGGTTGAGGTTGTT
TNF-α-Fwd	GACAGCCTATGCCAACAAGTA
TNF-α- Rev	TTACAGGAAGGGCAACTCATC
IFN-γ-Fwd	ACACTGACAAGTCAAAGCCGC
IFN-γ- Rev	AGTCGTTCATCGGGAGCTTG
IRF-7-Fwd	CAGTGCTTCTCCAGCACAAA
IRF-7- Rev	TGCATGTGGTATTGCTCGAT
MX1-Fwd	AACGCTGCTCAGGTCAGAAT
MX1-Rev	GTGAAGCACATCCAAAAGCA

with lysis buffer containing PMSF, and the whole process was carried on ice. The proteins in the sample were separated by SDS-PAGE and followed by blotting onto a PVDF membrane. The membrane was incubated with *p53* specific antibodies overnight at 4℃, then co-incubated with secondary antibody at room temperature for 1 h, and chemiluminescence detection reagents were used to observe the signal bands.

Cell Counting Kit-8 Assay

DF-1 cells and *P53*-KO DF-1 cells were plated at a density of 5×10^4 cells/well in 96-well tissue culture plates and cultured in the complete medium (Solarbio, Beijing, China) for 7 d. According to the instructions of Cell Counting Kit-8 (**CCK-8 kit**) (TransGen Biotech, Beijing, China), 100 μL PBS containing 10% CCK-8 reagent was added to each well after every 24 h instead of the culture medium and cultured at 37℃ for 2 h. The absorbance was measured with a Bio-Tek Instruments EL310 Microplate Autoreader at 450 nm. The experiment was repeated for at least 3 times for each group of the cells.

Comparison of Proliferation Ability of ALV-J in the Different Cell Lines

DF-1 cells and P53-KO-DF-1 cells were inoculated in 6-well cell culture plates at a density of 4×10^5/mL, respectively. The confluence degree was 70% in the conventional medium, and the NX0101 strain (GenBank: DQ115805) was infected with a virus titer of 1,000 $TCID_{50}$. After incubation at 37℃ for 2 h, the virus was discarded and replaced with a cell culture medium containing 1% FBS. The supernatant of ALV-J *P27* group specific antigen was detected by using a Subgroup J ALV Antibody Test Kit (IDEXX, China) at 0, 24, 36, 48, 60, 72 h, respectively. The assay was conducted in accordance with the manufacturer's instructions, and the absorbance (OD) of each sample was measured with a Bio-Tek Instruments EL310 Microplate Autoreader at a wavelength of 450 nm. At the same time, Trizol was used to extract the RNA, and real-time RCR was performed to detect the viral load of the 2 cell lines.

Attachment and Internalization of ALV-J

The kinetics of ALV-J attachment was studied by adding the virus at 1000TCID50 to DF-1 and p53-KO-DF-1 cells. After incubation for 4 h at 4℃, the cells were washed extensively with chilled PBS and then lysed. To allow internalization of the attached viruses, the cells were washed extensively with chilled PBS following attachment for 4 h and the temperature was shifted to 37℃. At 48 and 72 h postinternalization, the cells were washed with acidic PBS (pH 2.5) and then lysed. The samples were counted before cell lysis.

Flow Cytometric Analysis of Cell Cycle and Apoptosis

The percentages of cell cycle distribution were evaluated by PI staining. The cells were washed with precooled PBS and fixed overnight with 70% ethanol at 4°C. After rewashing with precooled PBS, 5% propidium iodide (Beyotime, China) was used for labelling the cells and incubated at 37℃ in dark for 30 min. The cell cycle was then analyzed by flow cytometer. Apotosis detected using an Annexin V fluorescein isothiocyanate kit according to the protocol. The cells were resuscitated with 100 μL 1× binding buffer, and the cell concentration was kept at about 1×10^6 cells /mL. Annexin V-PE and 7-AAD staining (BD) were added to the 1× binding buffer to prepare the cell staining solution (Annexin V-PE: 7-AAD: 1× binding buffer = 1:1:20). The cells were thereafter stained in the dark for 15 min, and analyzed by flow cytometry within 1 h. All samples were analyzed employing FACSCalibur (BD) with appropriate software (ModFit LT; BD, Topsham, ME) and the FlowJo (Ashland, OR) software were used for data analysis.

Statistical Analysis

Three different biological repeats were set up in each group of experiments. The data has been represented as mean ± SD. The data was compared and the differences were determined by one-way analysis of variance and least significance difference (**LSD**). The differences were considered as statistically significant when $P < 0.05$.

RESULTS

Establishment of p53 Knockout Cell Line

In order to study the possible role of *p53* protein in ALV-J infection, 2 guide RNAs of *p53* protein were inserted into px459M and EZ-Guide-XH vectors (Figure 1A), respectively. The double knock out plasmid Px459M-p53-KO was constructed and transfected into DF-1 cells. The positive clone cells were then screened by puromycin selection. Moreover, upon using *p53* specific primers to verify knockout efficiency, the results showed that the size of *p53* sequence in the normal cells was 1104bp, but that in knockout cell lines was 589bp (Figure 1B). However, after DNA sequencing, it was proved that the deletion fragment was located at site 115-630 bases (Figure 1C). At the same time, western blot analysis was also used to detect the expression of *p53* protein. The results clearly indicated that as compared with the normal DF-1 cells, *p53* protein was not expressed in knockout cell line at all (Figure 1D).

p53 Inhibits ALV-J Infection and Replication

DF-1 and p53-KO-DF-1 cell lines were seeded in 96-well plates respectively. The growth activity of these two cell lines was then measured by CCK-8 every 24 h for168 h. The results showed that *p53* knockout significantly promoted the growth of p53-KO-DF-1 cell line (Figure 2A). In order to verify the potential effect of *p53* on ALV-J infection and replication, DF-1 and p53-KO-DF-1 cell lines were simultaneously inoculated with ALV-J to analyze and compare the virus replication. Thereafter, RNA, was extracted from cells at then different time points and then reverse transcribed as a template to amplify the *pol* gene of ALV-J virus by qPCR.

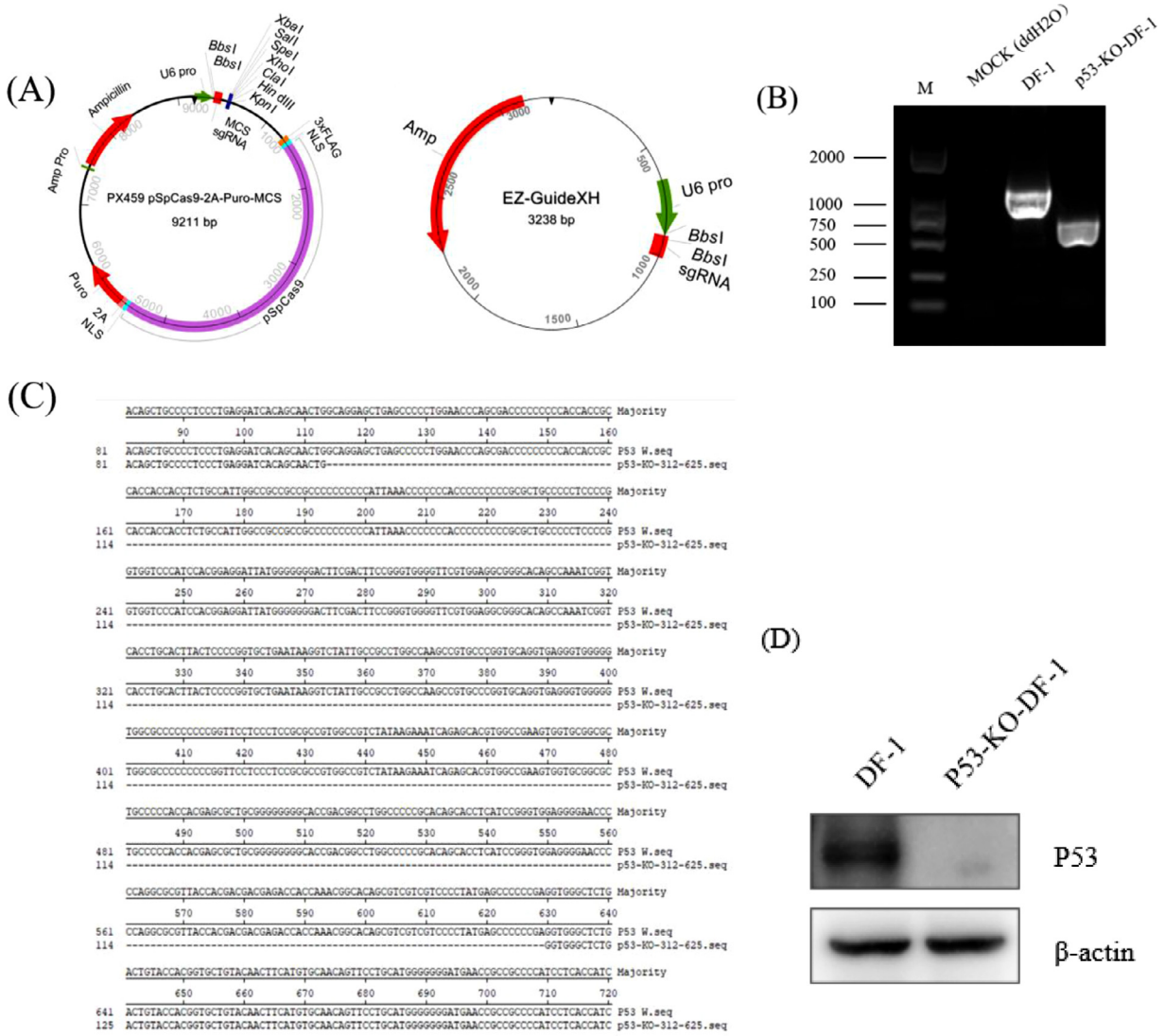

Figure 1. Establishment of *p53* knockout cell line. (A) The plasmid map of PX459M (PX459pSpCas9-2A-Puro-MCS) and EZ-Guide-X. (B) Trizol reagent was used to extract total RNA from DF-1 and P53-KO-DF-1 cells, then, the full length of *p53* gene was amplified by RT-PCR. The target bands were separated on 1% agarose gel. Lane 1, negative control (ddH2O), Lane 2, parental DF-1 cells (1104 bp), Lane 3, DF-1-KO-p53 cells (750 bp). (C) The correctly cloned target fragments were sequenced and compared with gene of DF-1 cells. (D) Western blotting analysis of *p53*. DF-1 and P53-KO-DF-1cells. The were inoculated in a 6-well plate for 24 h. Protein samples were extracted and western blot analysis was performed against *p53* antibody as per the procedure described in "Materials and Methods" section.

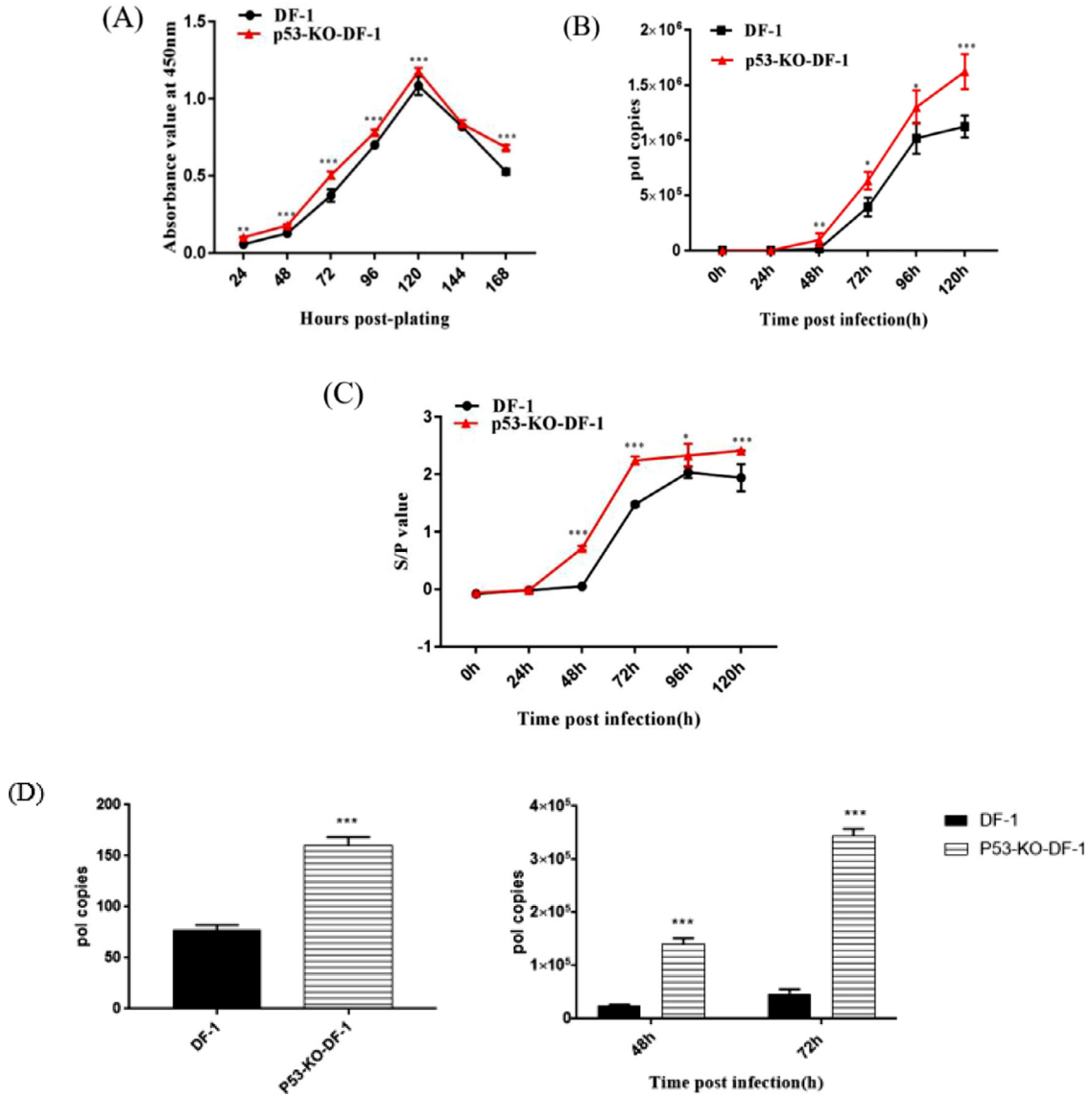

Figure 2. *p53* inhibits the infection and replication of ALV-J. (A) CCK-8 assay was performed to analyze proliferation in DF-1 cells and *P53*-KO-DF-1. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (B) qRCR and (C) ELISA analysis of virus proliferation. DF-1 and DF-1-KO-*p53* cells were seeded in 6-well plates. The cells were infected for 24, 48, 72, 96, and 120 h with ALV-J. Trizol reagent was used to extract RNA for qPCR analysis, while the cell supernatant was collected for the analysis of ALV-J *P27* antigen by ELISA. (D) Attachment and internalization of ALV-J. The DF-1 cell line and p53-KO-DF-1 cell line infected with ALV-J were placed at 4℃ for 4 h for virus adsorption, at 37℃ for 48 and 72 h for virus internalization. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Abbreviations: ALV-J, avian leukosis virus subgroup J; CCK-8, Cell Counting Kit-8; ns, not significant.

The results indicated that the replication peak began to appear in both the cell lines 24 h after ALV-J infection, but the replication efficiency of ALV-J in p53-KO-DF-1 was significantly higher than that in DF-1 cells, and it also increased with time (Figure 2B). The *P27* antigen of ALV-J was detected using ELISA and the results were consistent to that obtained by qPCR. The expression of *P27* antigen in p53-KO-DF-1 was observed to be significantly higher than that in DF-1 cells (Figure 2C). Next, we investigated whether p53 affected the adsorption and internalization of ALV-J. The DF-1 cell line and p53-KO-DF-1 cell line infected with ALV-J were placed at 4℃ for 4 h for virus adsorption, at 37℃ for 48 and 72 h for virus internalization. In order to eliminate effects of cell proliferation, each sample collected is counted to control for the same number of cells. As shown in Figure 2D, the copy number of virus in p53-Ko-DF-1 cells was 2 times higher than that in DF-1 cells after low temperature adsorption. At the same time, p53 knockout increased the percentage of internalized ALV-J by 5 to 10 times. These results indicate that P53 is involved in the adsorption and internalization of ALV-J, and plays a certain inhibitory role on the replication of ALV-J.

P53 Inhibits ALV-J Infection and Replication Through Affecting Innate Immune Factors

A number of previous studies have confirmed that ALV-J can primarily target the macrophages during the process of infection, and induce host innate

immune response by interacting with the macrophages to establish latent infection within the macrophages to evade host immune response. In order to explore whether *p53* can potentially inhibit ALV-J replication by affecting related innate immune factors, the 2 cell lines were infected with ALV-J virus and the expression of multiple innate immune factors was detected (Figure 3). The results of qPCR showed that the expression of innate immune factors was relatively low 72 h before ALV-J infection, but with an increased rate of virus replication, the expression of various immune factors also increased significantly after 72 h, and was found to be positively correlated with the virus replication rate. At the same time, it was observed that after knockout of *p53*, the expression of IL-2, TNF-α, and MX1 stimulated by ALV-J decreased by 5.8 to 7.8 times, whereas the expression of IFN-γ and IRF-7 decreased by 1.4 to 3.2 times, and that of the tumor promoting factor IL-6 increased by 1.26 times. In summary, these results indicate that ALV-J can induce a wide range of innate immune responses, and the presence of p53 can prevent ALV-J from antagonizing innate immunity.

p53 Can Affect ALV-J Replication by Regulating the Expression of Tumor-Related Genes

As a tumor suppressor gene, plays a critical role as a transcription factor in regulating the expression of various downstream target genes (Riley et al., 2008). The common tumor-related genes regulated by *p53* include cell cycle genes such as *p21, p27* and apoptotic-related gene *bcl-2, bak* (Deng et al., 2006; Morrison et al., 2016). We examined the expression of these genes (Figure 4). When DF-1 cells and p53-KO-DF-1 cells were co-infected with ALV-J, it was noticed that the presence of *p53* could promote the expression of *p21* and *p27*, predominantly in the peak period of virus replication (72 −120 h).The expression trend of Bcl-2 and Bak was basically positively correlated, and both of them increased after the deletion of p53, which may be related to the properties of the complex formed by both of them to inhibit cell apoptosis.

We also examined the role of *Mdm2* and *c-myc* genes in affecting ALV-J infection and replication. The main function of upstream gene *Mdm2* is to potentially degrade *p53* protein by ubiquitination mediated by

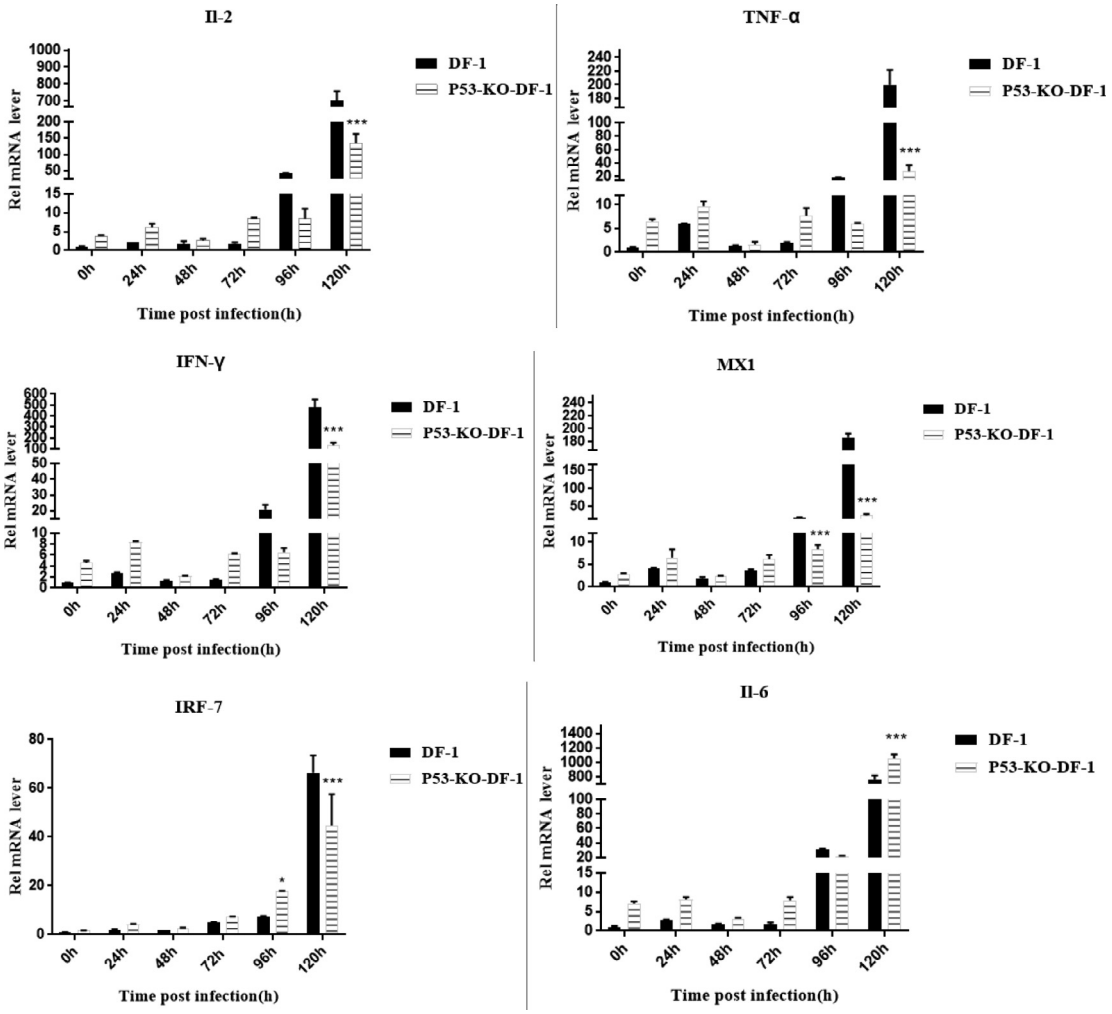

Figure 3. *p53* inhibits the replication of ALV-J through natural immune factors. DF-1 and *P53*-KO-DF-1cells were seeded in 6-well plates. 1000 $TCID_{50}$ ALV-J was used to infect the cells for 24, 48, 72, 96, and 120 h. The transcript levels of IL-2, TNF-α, IFN-γ, MX1, IRF-7, and IL-6 was analyzed by qPCR. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.00$. Abbreviation: ALV-J, avian leukosis virus subgroup J.

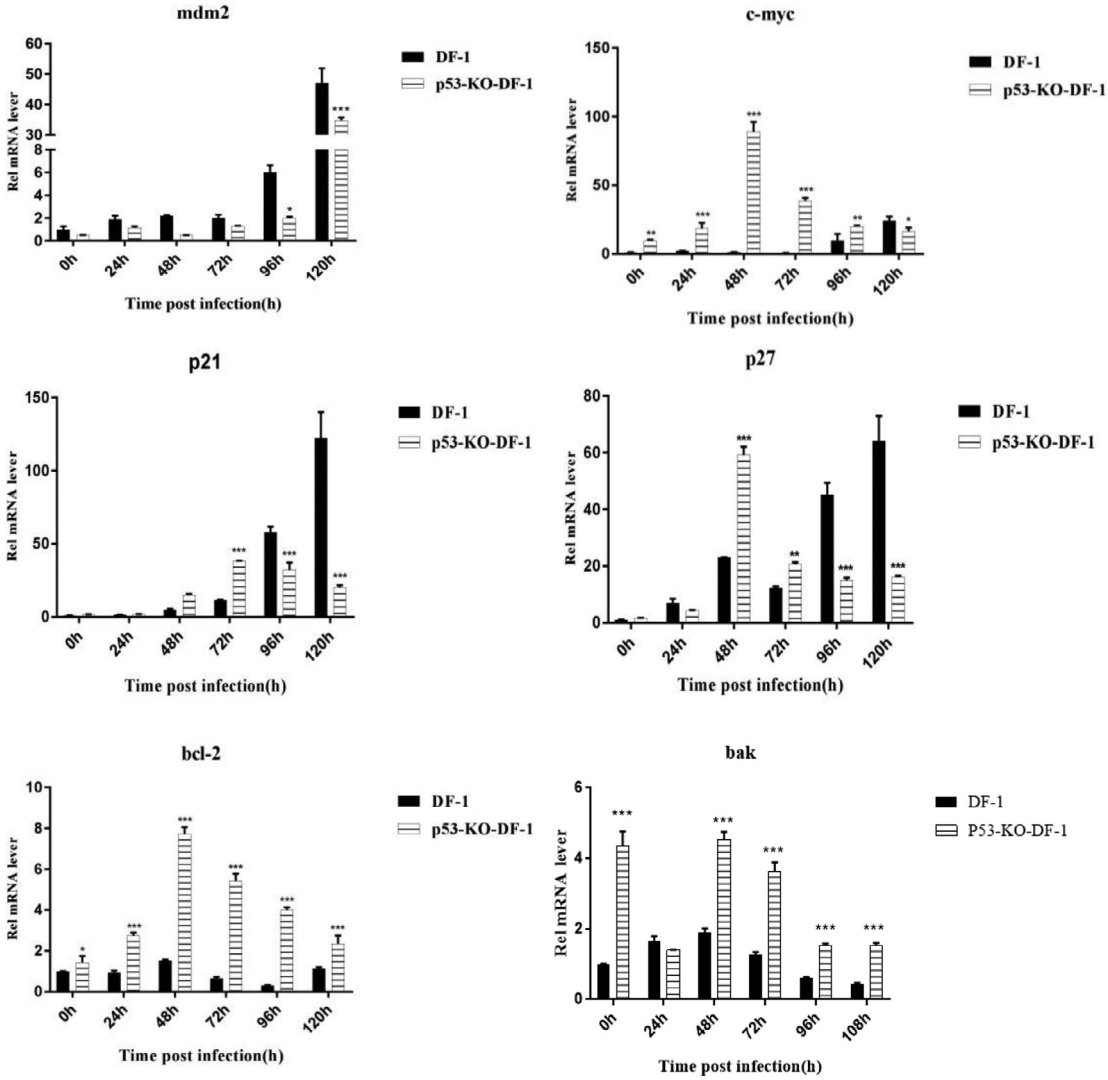

Figure 4. *p53* affects ALV-J replication by regulating the expression of various tumor-related genes. DF-1 and *P53*-KO-DF-1cells were seeded in 6-well plates. 1000 $TCID_{50}$ ALV-J was used to infect the cells for 24, 48, 72, 96, and 120 h. The transcript levels of *c-myc, p21, p27, bcl-2, bak* and *mdm2* was analyzed by qPCR. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Abbreviation: ALV-J, avian leukosis virus subgroup J.

proteasome and thus maintain the dynamic balance of level in vivo. Oncogene *c-myc* can affect the genomic stability and thus promote tumorigenesis (Wu et al., 2020). The results showed that when *p53* was knocked out, the expression of *Mdm2* decreased significantly, indicating that there was a feedback regulation system between *p53* and *mdm2*. The expression of *c-myc* increased significantly when *p53* was knocked out both during the early stage of ALV-J replication (0−48 h) and at the peak time period of 72 h. Thus, in the presence of *p53, c-myc* was maintained at a low level, which indicated that *p53* could possibly prevent ALV-J replication by inhibiting the expression of other oncogenes.

p53 Inhibits ALV-J Infection and Replication by Causing G0/G1, G2/M Phase Arrest, and Promoting Apoptosis

When the cells are damaged by stress, *p53* as a tumor suppressor gene can generally protect the cells by halting the cell cycle and inducing cell apoptosis (Lane, 1994). In order to verify whether *p53* can potentially affect the infection and replication of ALV-J by regulating the processes of cell cycle and apoptosis, the cells were collected at 0 to 120 h after inoculation with ALV-J, and analyzed for the cell cycle progression and apoptosis by flow cytometry. The results showed that most of the *p53*-KO-DF-1 cells were in S phase initially with an increase in virus concentration. While DF-1 cells in the early stage of viral replication (0−48 h) were mostly distributed in G0/G1 phase, and that in the late stage of viral replication (72−120 h) were found in G2/M phase (Figures 5A and 5B). Figure 5B depicts the quantitative analysis of Figure 5A. We also analyzed the apoptosis of cells from 0 to 96 h and found that *p53* knockout significantly inhibited the apoptosis induced by ALV-J infection and thereby reduced the apoptosis rate by up to 20% (Figures 5C and 5D).

DISCUSSION

p53 is a well-known tumor suppressor gene, which primarily acts as a potential transcription factor to induce

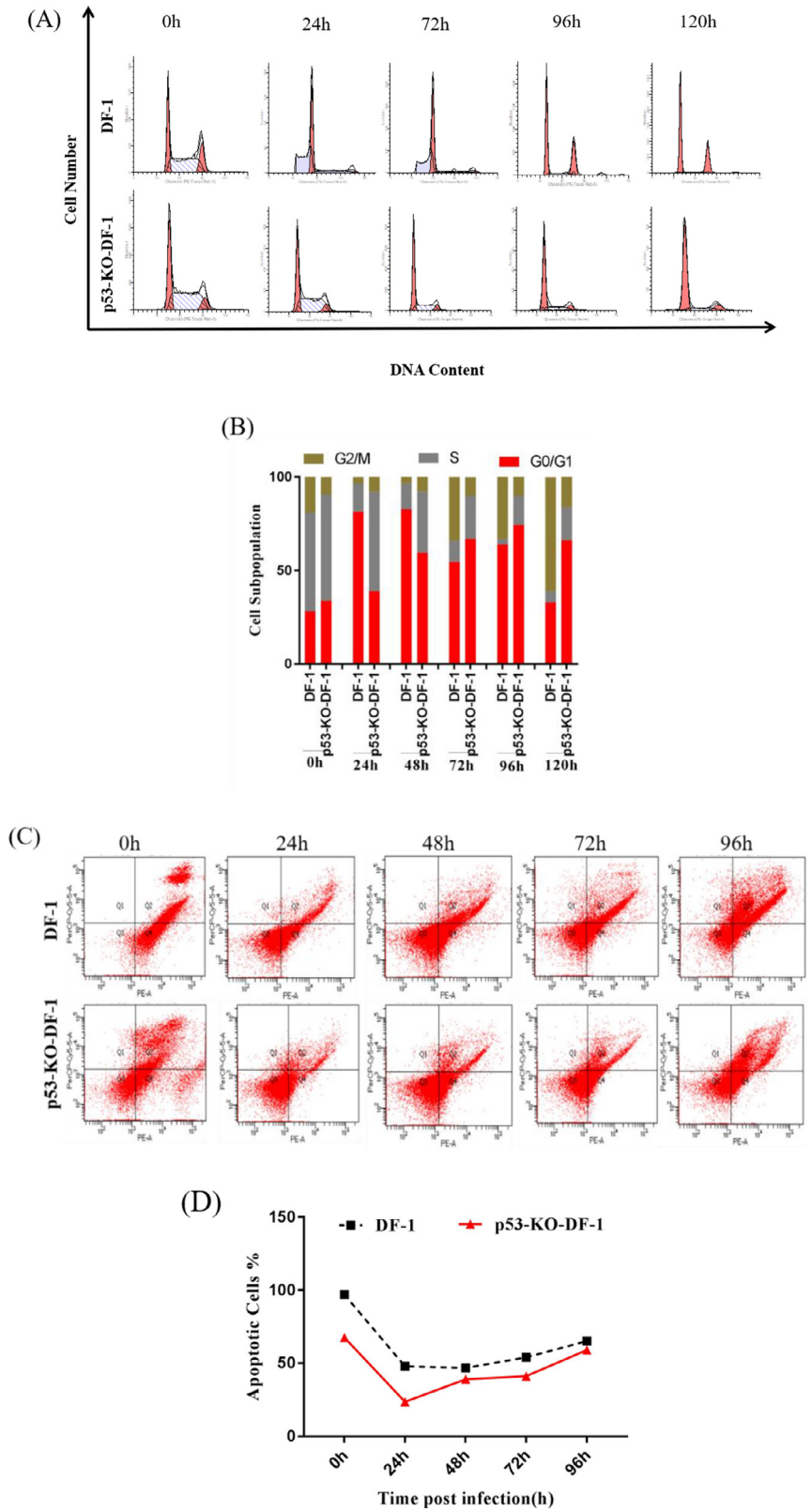

Figure 5. *p53* inhibits ALV-J replication through affecting cell cycle progression and apoptosis. (A) Representative results of cell cycle analysis and (B) statistical analyses of cell cycle distribution analyzed by flow cytometer in DF-1 and *P53*-KO-DF-1 cells after infection with ALV-J for 24, 48, 72, 96, and 120 h. (C) Representative results of apoptosis studies and statistical analyses of cell apoptosis analyzed by flow cytometer in DF-1 and *P53*-KO-DF-1 cells after infection with ALV-J for 24, 48, 72, and 96 h. Abbreviation: ALV-J, avian leukosis virus subgroup J.

cell protective responses by blocking the progression of the cell cycle and causing apoptosis in the cells exposed to the stress signals (such as DNA damage, hypoxia, oncogene activation, etc.) (Kastan, 1996; Meek, 2015). An abnormal expression of *p53* is often used as an indicator of carcinogenesis in human diseases (Duffy et al., 2017). For instance, it has been reported that about 50% of cervical cancer cases are accompanied by *p53*

overexpression, which may promote both the occurrence and development of cancer (Sahu et al., 2002). In view of the important role of *p53* in the development of various malignancies, the possible effect of *p53* pathway on ALV-J replication in infected DF-1 cells was explored in this study. Firstly, a *p53* knockdown cell line (P53-KO-DF-1) using CRISPR-Cas9 system was successfully established, and expression of *p53* was verified both at mRNA and protein level. Subsequently, the replication rate of ALV-J in normal DF-1 cell lines and *p53* knockdown cell lines was compared, and it was found that knocking down this gene could significantly increase the replication rate and the adsorption and internalization of ALV-J, thus suggesting that *p53* could effectively inhibit the replication of ALV-J in DF-1 cells.

To understand the possible mechanism(s) through which *p53* could act as a tumor suppressor in the process of ALV-J infection additional experiments were performed. Innate immunity plays an important role in the host's defense against viral infections, which mainly depends on orchestration of response mediated by the various cytokines (Kabelitz and Medzhitov, 2007). Since avian leukemia caused by ALV-J is an immunosuppressive disease, the expression of several cytokines related to tumorigenesis was analyzed. For example, IL-2 can induce T cell proliferation, differentiation and activation (Choudhry et al., 2018). IL-6 is a typical tumor-promoting cytokine, which can promote tumorigenesis by regulating a variety of signaling pathways (Kumari et al., 2016). TNF-α is a proinflammatory cytokine produced by macrophages/monocytes during acute inflammation, resulting in cell necrosis or apoptosis (Sung et al., 2001). IFN-γ can resist viral infections, inhibit cell proliferation, participate in immune regulation and also exhibit diverse proinflammatory activities (Kak et al., 2018). Additionally, both IRF-7 and MX1 are gene products related to the expression of interferon (Engelhardt et al., 2001; Honda et al., 2005). Upon comparing the levels of various cytokines in two cell lines after exposure to *p53*, it was found that the expression of IL-2, TNF- α, IFN-γ, and *MX1* was significantly increased under the action of p53, while the expression of tumor-promoting factor IL-6 was significantly decreased. However, the opposite trend appeared after the deletion of p53. This indicates that in the immune response caused by ALV-J infection, *p53* can prevent ALV-J from inhibiting the innate immunity, maintain a high level of immune response, and reduce the damage of ALV-J to the host.

The level of *p53* in normal body is low and does not generally exert its activity (Blagosklonny, 1997). However, when the cells are stressed, *p53* is converted into an active tetrameric conformation and can be activated by acetylation and phosphorylation. *p53* activation plays a tumor inhibitory role by regulating the expression of various downstream target genes through its transcriptional activity (Feng et al., 2019; Lucchesi et al., 2016). It has been reported that when DNA is damaged or subjected to other types of stress, the tumor suppressor *p53* is activated, which causes the transient expression of *p21* gene to suppress cell cycle in the G1/S phase (Xiao et al., 2020), and p53 can also inhibit the activation of *bcl-2* promoter by *Brn-3a*, thus inducing neuronal apoptosis (Budhram-Mahadeo et al., 1999). In the course of our study, we found that after infection with ALV-J, *p53* can effectively regulate the expression of various target genes. *p53* upregulates *p21* and *p27* to causes cell cycle arrest, downregulates the levels of *bcl-2* and *bak* to induce cell apoptosis, and simultaneously induces the overexpression of oncogene c-myc, thus may play a certain inhibitory role in tum*ors*.

In the following experiments, cell cycle and apoptosis were detected and analyzed by flow cytometry. G1 phase has been generally considered as the checkpoint of cell cycle, which is responsible for controlling the cells from entering into the S phase (Nojima, 2004). Most tumor cells lose G1/S phase, which can facilitate the cells to enter S phase under abnormal circumstances, carry out DNA replication, and thus resulting in DNA damage (Benedict et al., 2018). We found that when *p53* was present, along with an increase in the cycle blocking genes p21 and p27, the cells in the early stage of viral replication (0−48 h) were mostly distributed in G0/G1 phase, while that in the late stage of viral replication (72 −120 h) were found in G2/M phase, which significantly reduced the S phase of cells thereby significantly reducing the risk of accumulating mutations.

Apoptosis plays a protective role in the process of tumorigenesis. The important step in this process is mitochondrial apoptosis, which primarily depends on the changes in the mitochondrial membrane permeability and can be regulated by *Bcl-2* family complex (Dashzeveg and Yoshida, 2015). Under normal circumstances, p53 competitively interacts with the antiapoptotic protein Bcl-2, destroys the heterodimer complex composed of Bcl-2 and Bax/Bak, activates the mitochondrial membrane space to release various apoptotic protein factors, and induces apoptosis (Czabotar et al., 2014). In our study, with the replication of ALV-J, the expression of *bcl-2* after deletion of *p53* was significantly increased, which was positively correlated with the expression of *bax,* and the apoptosis rate of cells decreased. It suggests that *p53* may regulate the process of apoptosis by regulating the generation of Bcl-2/Bak, but the detailed mechanism(s) need to be deciphered in the future studies.

CONCLUSIONS

We have provided substantial evidence in this study that *p53* could effectively inhibit the replication of ALV-J in DF-1 cells. We found that *p53* can prevent the inhibition of ALV-J on the innate immune response in DF-1 cells when stimulated upon ALV-J infection, and can induce the expression of the key downstream target gene of *p53*, such as *c-myc, p21,p27* ,*bcl-2* and *bak*. We have also proved that *p53* can inhibit ALV-J replication by promoting the expression of oncogene c-myc, inducing cycle gene *p21* to cause G1 and G2 phase arrest, and

affecting apoptosis-related gene *bcl-2* and *bak* to promote apoptosis.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation project under Grant [grant number 31672518].

DISCLOSURES

Authors declare no conflict of interest.

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