Genetically engineered Newcastle disease virus expressing human interferon-λ1 induces apoptosis in gastric adenocarcinoma cells and modulates the Th1/Th2 immune response

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Abstract. Interferon-λ1 (IFN-λ1), a recently discovered cytokine of the type III IFN family, was found to be a therapeutic alternative to type I IFN in terms of tumors. Using reverse genetics technique, we generated a recombinant Newcastle disease virus (NDV) LaSota strains named as human IFN-λ1 recombinant adenovirus (rL-hIFN-λ1) containing human IFN-λ1 gene and further evaluated the expressing of IFN-λ1 in human gastric adenocarcinoma cell line SGC-7901 after infected with rL-hIFN-λ1 by using western blot analysis, RT-PCR and immunofluorescence analyses. IFN-λl specific receptor IFNLR1 was detected on several gastric tumor cell lines including SGC-7901 and AGS and on PBMCs. The expression of the IFN-λ1 proteins reached a high level detected in the supernatant harvested 24 h after the infection of tumor cells. The proliferation changes of SGC infected with rL-hIFN-λ1 was significantly inhibited compared with NDV-infected group. Apoptosis was significantly induced by rL-hIFN-λ1 in gastric cancer cells compared with NDV virus tested by TUNEL assay, western blot analysis and Annexin V flow cytometry. Due to the high dose of IFN-λ1 expressed by the rL-hIFN-λ1-infected tumor cells, the immune study showed that rL-hIFN-λ1 increased IFN-γ production [the T helper cell subtype 1 (Th1) response] and inhibited interleukin (IL)-13 production [the T helper cell subtype 2 (Th2) response] to change the Th1/Th2 response of tumor microenvironment which inhibited tumor growth. This study aims at building recombinant NDV rL-hIFN-λ1 as an efficient antitumor agent.

Introduction

In recent decades, an integrated approach to cancer treatment that includes surgery, chemotherapy, radiotherapy, gene therapy and immune therapy, has become a reasonable therapeutic strategy (1). Oncolytic virotherapy using agents such as Newcastle disease virus (NDV) is one of the new biological strategies for gene and immune therapy and has been tested against many different cancers including gastric cancer, skin tumors and several solid cancers (2-4). NDV, which is a member of the Paramyxoviridae family, is a non-segmented negative-strand RNA virus (NNSV). The NDV genes encode six major structural proteins, including nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large (L) RNA-dependent RNA polymerase, in the following order 3'-NP-P-M-F-HN-L-5'. Oncolysis, apoptosis and enhancing innate immunity are the key mechanisms capacitating NDV as an agent against malignant cells (5). NDV virus can be propagated in embryonated chicken eggs, harvested from the allantoic fluid, and quantified by hemagglutination, NDV has been actively developed and evaluated as a vaccine vector for the control of human and animal diseases, these characteristics made the NDV virus easy medium to obtain (6-9).

Interferon-λ1 (IFN-λ1), also known as interleukin (IL)-29, is a recently discovered cytokine of the type III IFN family (10,11). It is thought to have biologic properties similar to the type I IFNs. Unlike IFN-α, the receptor for IFN-λ1 expresses on a limited number of normal cells including dendritic cells, T cells, and intestinal epithelial cells (12,13). Leukemia cells and colon, prostate, pancreatic, lung, hepatoma, glioblastoma, and breast cancer cells have also been shown to express IFNLR1 receptor (14-18). It is widely accepted that

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cytokines associated with a T helper cell subtype 1 (Th1) exhibit tumour-suppressive activity, whereas T helper cell subtype 2 (Th2) cytokines can promote tumour progression (14,19-21). IFN-λ1 primarily inhibits IL-13 production and gives rise to IFN-γ production in PBMCs (22). IFN-λ1 receptor IFNLR1 expresses on both naive and memory CD4$^+$ T cells (23). It is also reported when cultured with IL-29 the cytokine tumor microenvironment is skewed towards a Th1 response that results in reducing tumor growth (24).

In the present study, we used an established reverse genetics system (8) to generate a recombinant NDV LaSota viruses named as human IFN-λ1 recombinant adenovirus (rl-hIFN-λ1), we incerted IFN-λ1 gene into a lentogenic recombinant LaSota (NDV) genome at the position between the P and M genes. We believe that this new recombinant virus can express IFN-λ1 protein stably allowing us to consider it to br a promising agent for cancer therapy. Our group studied the antitumor effect of the rL-hIFN-λ1 on gastric adenocarcinoma cell lines compared with NDV. Furthermore, in this study we also investigated whether the rL-hIFN-λ1 is capable of regulating the IFN-γ secretion (the Th1 response) and the IL-13 secretion (the Th2 response) indicating the change of Th1/Th2 balance in the tumor microenvironment that results in inhibiting tumor growth.

Materials and methods

Cells and viruses. The BSR cells (Harbin Veterinary Research Institute) for virus rescue, and the human gastric adenocarcinoma cell lines SGC-7901 and AGS were purchased from the Cancer Cell Repository (Shanghai Cell Bank, Shanghai, China; 2010-02-20) and the American Type Culture Collection (ATCC; Rockville, MD, USA). Gastric adenocarcinoma cells SGC-7901 and AGS cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) at 37˚C in a humidified 5% CO_2 incubator. Cell culture reagents were acquired from Gibco (Grand Island, NY, USA). When the cells reached 50-70% confluence, they were infected with rL-hIFN-λ1 or NDV for further study. The NDV LaSota strain and the recombinant viruses were propagated and titrated in 9-day-old SPF embryonated chicken eggs. The NDV strain LaSota, SPF eggs and anti-NDV serum was provided by the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Human PBMCs were isolated from peripheral blood of volunteer donor with density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA). Cells were collected and washed twice in RPMI-1640 medium, and then were resuspended at a final density of 10^6/ml in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) both from Invitrogen. Con-A stimulations were performed in 24-well plates using 1x10^6 whole PBMCs with 5 µg/ml Con-A (Sigma-Aldrich) in a final volume of 2 ml.

Materials. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). Hoechst 33342 were obtained from Sigma-Aldrich. All PCR primers were purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). Total RNA from viral or tumour cells were extracted with TRIzol reagent (Invitrogen). Anti-cleaved caspase-3 was from ImmunoWay Biotechnology (Newark, DE, USA). Anti-IFN-λ1 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-IFNλ-R1 was from R&D Systems, Inc. (Minneapolis, MN, USA). HRP-conjugated goat anti-rabbit, HRP-conjugated goat anti-mouse and FITC-conjugated goat anti-mouse antibodies were purchased from CWBio (Shanghai, China). HRP-conjugated goat anti-rabbit, HRP-conjugated goat anti-mouse, and FITC-conjugated goat anti-mouse antibodies were purchased from CWBio. HRP-conjugated rabbit anti-chicken antibody was from EarthOx Life Sciences (Millbrae, CA, USA), and Cy3-conjugated rabbit anti-chicken antibody was purchased from KPL, Inc. (Gaithersburg, MD, USA). The polyvinylidene difluoride (PVDF) membrane and Luminata were provided by Millipore (Billerica, MA, USA). Trypsin, and EDTA-2Na were offered by Gibco. A mitochondrial transmembrane potential analysis kit was from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). All other supplies for cell culture were obtained from Corning Costar (Corning, NY, USA).

Construction of full-length NDV plasmid and virus rescue. To construct a full-length recombinant genomic cDNA. The cDNA of the IFN-λ1 genes was amplified from a previous study (25). The primers for amplification of human IFN-λ1 gene are shown in (Table I). These primers included the gene start and termination sequences of the NDV genome and the Kozak sequence. The amplified human IFN-λ1 gene was inserted into plasmid pBluescriptII Ks(+) then tested with nucleotide sequence analysis, the newly recombinant plasmid was named as pBS. IFN-λ1. The amplified human IFN-λ1 gene was digested by *Pme*I and then inserted into the NDV genome cDNA through a unique *Pme*I site in the P-M intergenic region at nucleotide position 3165 of the NDV genome (8) to build the new recombinant plasmid named pBRN.hIFN-λ1 (Fig. 2).

The rescue of recombinant NDV viruses from cloned cDNA was as follows: BSR cells infected with A modified virus expressing T7 RNA polymerase at a multiplicity of infection (MOI) of 1.0 and then transfected with pBowing in a six-well plate with pBRN.hIFN-λ1 together with 3 helper plasmids [pBS-NP (0.4 µg), pBS-P (0.2 µg), and pBS-L (0.2 µg)]. After 16 h of incubation at 37˚C, the medium was replaced with 2 ml Opti-MEM (Invitrogen) containing 0.5 µg of TPCK-trypsin and the cells were incubated for another 3 days at 37˚C. The supernatant was then inoculated into the allantoic cavities of 9-day-old embryonated SPF eggs. After 72 h of incubation at 37˚C, the allantoic fluid was harvested and the virus was identified by hemagglutination assay using 1% chicken red blood cells. The resultant recombinant viruses were designated as rL-hIFN-λ1.

Growth characteristics of the recombinant viruses. Virus growth was determined both in cell culture and embryonated chicken eggs. Confluent monolayers of SGC cells were infected with rl, rL-hIFN-λ1 in 96-well plates at a MOI of 1.0. Every 12-h post-infection, the cellular monolayers of SGC cells were harvested by freeze-thawing three times. Virus titers were determined as 50% tissue culture-infective dose (TCID$_{50}$). Two hundred microliters of the recombinant virus dilution containing 10^4 TCID$_{50}$ per ml was inoculated into the allantoic cavity of 9-day-old embryonated chicken eggs. After three

Table I. Primer sequences used in the study.

Gene name	Primer sequence (5'-3')	
	Forward	Reverse
IFN-λ1	GACTgtttaaacTTAGAAAAAATACGGGTA	GACTgtttaaactcaggtggactcagggtgggttg
	GAAgtgccaccatggctgcagcttggaccgt	
IFNLR1	ACCTATTTTGTGGCCTATCAGAGCT	CGGCTCCACTTCAAAAAGGTAAT
NDV-PMEI	GGAAATCAGGAAAATCAAGCGCCT	AGAATCAAAGTACAGCCCAAT

IFN-λ1, interferon-λ1; NDV, Newcastle disease virus.

Table II. The AI of SGC cells infected by rL-hIFN-λ1 and NDV.

Group	AI (n=10)	F-value	P-value
rL-hIFN-λ1	0.278±0.043		
NDV	0.098±0.021	236.45	<0.001
CON	0.022±0.011		

AI, apoptotic index; rL-hIFN-λ1, human interferon-λ1 recombinant adenovirus; NDV, Newcastle disease virus.

days, the allantoic fluids of inoculated eggs were harvested and clarified by centrifugation at 1,000 x g for 10 min. Supernatants were used for titration as described above.

Immunofluorescence analysis. Tumour cells were cultured in 24-well plates for 24 h as previously described, infected with rL-hIFN-λ1 at a MOI of 1.0, treated with NDV and phosphate-buffered saline (PBS) as controls. Then, at 24-h post-infection, the cells were fixed in 4% paraformaldehyde at 4°C overnight, followed by immunofluorescence staining with antibodies against NDV, IFN-λ1 and Hoechst 33342 staining. These stained SGC cells were monitored using immunofluorescence microscopy.

MTT analysis. To measure the proliferation level, the survial of cells was assessed with MTT with six replicates for NDV, rL-hIFN-λ1 and PBS. At 12, 24, 36, 48, 60 and 72-h post-infection, cell survival was determined by incubating the cells with MTT. Absorbance at 490 nm was determined with ELISA microplate readers (BioTek Instruments, Inc., Winooski, VT, USA).

RNA isolation and RT-PCR analysis. Total RNA of virus or tumor cells was extracted from the cultured cells using TRIzol reagent. First-strand cDNA synthesis was performed using oligo(dT) primers and M-MLV reverse transcriptase. The primer set for the IFN-λ1 gene and the IFNLR1 gene were shown in (Table I). All the procedures were based on manufacturer's instructions.

TUNEL assay. Cells were cultured in 24-well plates with slides and 10% FBS DMEM at 37°C with 5% CO_2. Then, the SGC cells were infected with rL-hIFN-λ1 and NDV within the

logarithmic growth phase and fixed in 4% paraformaldehyde. After fixing, the cells were stained according to the manufacturer's instructions. The slides were observed and imaged under an optical microscope. The apoptotic index (AI) was calculated as the number of apoptotic cells/(the number of apoptotic cells + the number of non-apoptotic cells) x 100%.

Enzyme-linked immunoabsorbent assay. Gastric adenocarcinoma cells in six-well plates were infected with NDV or rL-hIFN-λ1 virus at MOI of 1.0. Then the supernatant was harvested at 0, 6, 12, 24, 36 and 48-h post-infection. The expression levels of IFN-λ1 in the supernatant were measured by means of enzyme immune assay as described by human IFN-λ1 platinum kit (eBioscience, San Diego, CA, USA). The OD450 of the samples were determined and plotted as a standard curve. The standard curve was generated by serially diluting the stock enzyme IFN-λ1 in dilution buffer supplied by the kit.

To measure the cytokine protein, cytokine levels within supernatants was measured with ELISA. Antibodies for IFN-λ1, and IFN-γ were purchased from eBioscience and for IL-13 from R&D Systems, Inc. Manufacturer's instructions were followed. Standards and culture supernatants were plated in triplicate.

Western blot assay. SGC and AGS cells with or without treatment were lysed in RIPA lysis buffer with a protease inhibitor cocktail (Santa Cruz Biotechnology, Inc.). The protein concentrations were measured using a BCA kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). An equal amount of protein from each sample was loaded onto a 10% polyacrylamide gel and separated by electrophoresis. Then, the proteins were transferred to PVDF membrane (Millipore, Temecula, CA, USA). The membranes were blocked for 1 h in 5% BSA, incubated with primary antibodies against specific proteins (i.e., caspase-3 for apoptosis, anti-NDV for infection, anti-hIFN-λl for expression of recombiant gene, anti-IFNLR1 for detection of IFNλ-l receptor components and β-actin as a control) and then incubated with HRP-conjugated secondary antibodies. The protein bands were scanned using a Typhoon 9400 variable mode imager (Amersham Biosciences, Buckinghamshire, UK) and detected by Pierce ECL Plus substrate (Thermo Fisher Scientific, Inc.).

Flow cytometry assay using Annexin V. Annexin V/propidium iodide (PI) double-staining was used to detect membrane
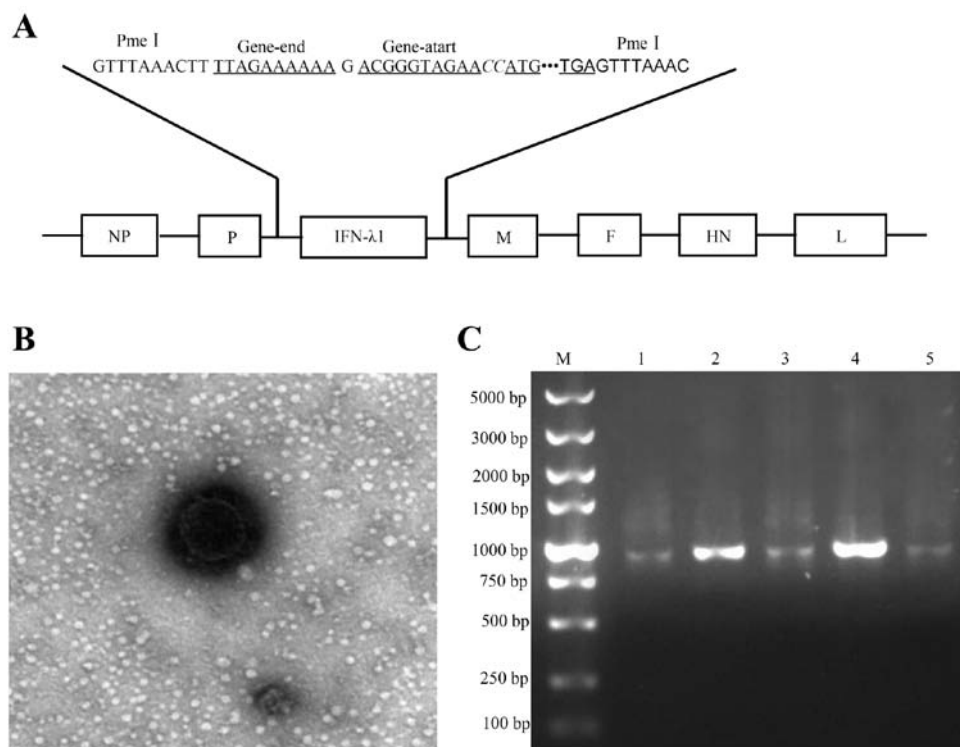
Figure 1. Generation of recombinant Newcastle disease virus (NDV) expressing the interferon-λ1 (IFN-λ1) gene. (A) Schematic representation showing the IFN-λ1 gene was cloned into the intergenic region of NDV genome at the position between the P and M genes. The NDV gene start and the end signal sequences and the IFN-λ1 sequences are boxed or underlined. (B) Electron microscopy of recombinant virus particles. NDV or rL-RVG propagated in eggs was prepared and partially purified by centrifugation through 20% sucrose. Viruses were stained with IgG purified from mouse naïve serum (AB, antibody), and with goat anti-mouse IgG conjugated with colloidal gold and then negatively stained. Transmission electron microscope showing two 200 nm conjugate human IFN-λ1 recombinant adenovirus (rL-hIFN-λ1) virus (→) at 80.0 kV. (C) Reverse transcription-polymerase chain reaction analysis of the presence of IFN-λ1 in the recombinant virus rL-hIFN-λ1. Lane M, DL5000 DNA marker; lanes 1-5, sequences around 1,000 dp cloned from the rescued viral total RNA and then amplified to sequence by nucleotide sequence analysis.

events according to the manufacturer's instructions (Biotech, Beijing, China). Flow cytometric analysis of labeled cells was performed using a FACSort flow cytometer (FACSCalibur) and data were analyzed using CellQuest software (both from BD Biosciences, Franklin Lakes, NJ, USA). The cytogram of the four quadrants in the figure was used to distinguish normal (Annexin V$^-$/PI$^+$), early apoptotic (Annexin V$^+$/PI$^-$), late apoptotic (Annexin V$^+$/PI$^+$), and necrotic cells (Annexin V$^-$/PI$^+$). The sum of early and late apoptosis was presented as total apoptosis. Finally, staining of the cells was observed with a fluorescence microscope. All experiments were carried out in triplicate.

Immunoelectron microscopy. Purified virus particles were bound to 200-mesh Formvar carbon-coated nickel grids (Electron Microscopy Science, Hatfield, PA, USA). For immunolabeling, grids were blocked in PBS containing 2% globulin-free BSA (Sigma-Aldrich) and incubated with chicken anti-NDV polyclonal IgG. Grids were then washed in blocking solution and incubated in goat anti-chicken IgG conjugated to 10-nm gold beads (Sigma-Aldrich). The grids received a final wash, followed by negative staining with 1% phosphotungstic acid. They were examined under a model H7500 transmission electron microscope (Hitachi High Technologies America, Inc., Schaumburg, IL, USA) at 80 kV. Images were obtained by using an XR100 digital camera system (Advanced Microscopy Techniques, Danvers, MA, USA).

Statistical analysis. The data comparisons were performed using one-way analysis of variance (ANOVA) in SPSS v17.0 software. $p<0.05$ and $p<0.01$ were considered statistically significant. All experiments were repeated at least three times.

Results

Generation of recombinant NDV LaSota strain expressing human IFN-λ1 gene. Recombinant NDV expressing the human IFN-λ1 was generated by inserting the IFN-λ1 gene in the genomic cDNA of the NDV LaSota strain (Fig. 1A). For virus recovery, the heterogeneous gene carrying plasmid was transferred into 9-11 days old SPF embryonated chicken eggs for efficient virus propagation. After incubation for 5 days, the allantoic fluids were harvested and analyzed in a rapid plate HA test using chicken erythrocytes and observed by electron microscopy (Harbin Veterinary Research Institute) (Fig. 1B). The obtained viruses were named as rL-hIFN-λ1. The presence and accuracy of the inserted IFN-λ1 gene, the sequences from rL-hIFN-λ1 genome were confirmed by nucleotide sequence analysis (data not shown), the sequences contained whole IFN-λ1 genome were cloned by RT-PCR (Fig. 1C) by primers NDV-PMEI (Table I) set above and blow the PMEI set on NDV. The results showed they represented the correct sequences successfully inserted into the NDV genome, and mutation did not occur in rL-hIFN-λ1.

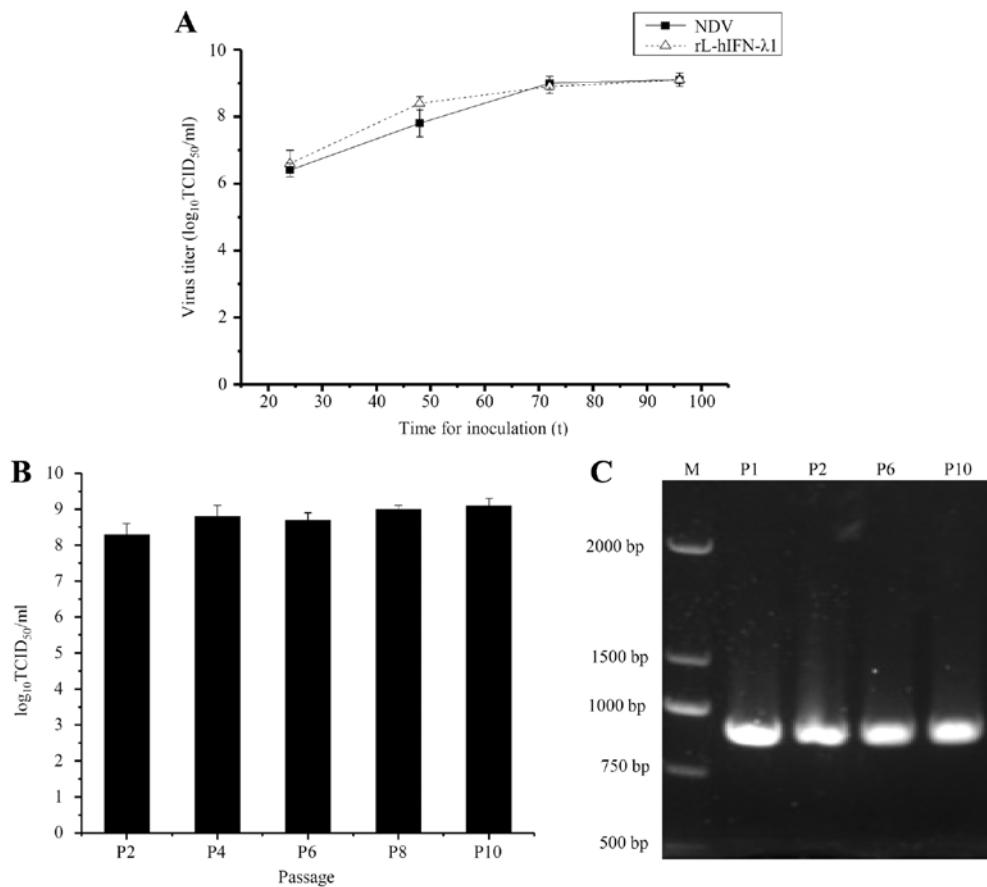Figure 2. Growth characteristics for Newcastle disease virus (NDV) and human interferon-λ1 recombinant adenovirus (rL-hIFN-λ1). (A) The SGC cells were infected with NDV or rL-hIFN-λ1 at a MOI of 0.01. Every 24-h post-infection, the cells were harvested. Virus titers at each time-point were determined by TCID$_{50}$ titration in SGC cells. (B) The allantoic fluid of SPF eggs inoculated with 100 TCID$_{50}$ of recombinant virus were harvested after 4-day inculation. The amount of virus was determined by end-point titration. The results represented mean values from three independent experiments. (C) mRNA expression in some of the generation of rL-hIFN-λ1 virus. Lane M, DL2000 DNA marker; lanes P1, P2, P6, P10, some of the generations of rL-hIFN-λ.

Growth characteristics of the recombinant virus. To compare the growth characteristics of the recombinant viruses rL-hIFN-λ1 and NDV, viral titers of the supernatants collected from infected SGC cells at 0, 24, 36, 48 and 72 h were determined by TCID$_{50}$. The growth properties of rL-hIFN-λ1 in the SGC cells were similar to those of the parental virus NDV (Fig. 2A). The result demonstrated that the recombinant viruses had mostly retained the growth characteristics of the parental virus in the SGC cells.

The allantoic cavities of 10-day-old embryonated SPF chicken eggs were inoculated with 10^4 TCID$_{50}$ of rL-hIFN-λ1. The allantoic fluid was harvested after 4-day incubation. The viral titers was determined in duplicate by end-point titration. The results of the replication of rL-hIFN-λ1 indicated that the amount of the recombinant virus remains similar after 10 passages (Fig. 2B). After 10 passages, the presence of the human IFN-λ1 gene was confirmed by RT-PCR (Fig. 2C) and nucleotide sequence analysis (data not shown).

Human gastric carcinoma cell lines and human peripheral blood mononuclear cells express IFN-λ1 specific receptor. To determine the expression of IFN-λ receptor specific receptor IFNLR1 in human gastric cancer cell lines. We used RT-PCR assays and western blot analysis to analyze mRNA and protein expression of IFNLR1, respectively. The IFNLR1

was expressed in all examined gastric cancer cell lines, while the expression level of mRNA or protein was divided vividly among cell lines (Fig. 3), the SGC and AGS gastric cells expressed more IFNLR1 compared with other gastric tumor cells. For this reason we chose these two cell lines for our further study. The IFN-λ receptor IFNLR1 expressed in gastric cancer cell lines showed potential as a target for IFN-λ1 antitumor therapy. We found that PBMCs (human peripheral blood mononuclear cells) also expressed IFNLR1, which allowed us to study the antitumor immune response of rL-hIFN-λ1.

Expression of IFN-λ1 by rL-hIFN-λ1-infected gastric adeno-carcinoma cells. To define whether the IFN-λ1 gene integrated in the NDV genome, the infected gastric tumor cell lines SGC were tested for production of the protein IFN-λ1. The presence of IFN-λ1 genes in the viral genome and expression in virus-infected cells were confirmed as positive for all passage generations using indirect immunofluorescence (Fig. 4A) and western blot analysis (Fig. 4B). In the indirect immunofluorescence the IFN-λ1 protein (green) was highly expressed in the rL-hIFN-λ1-infected group, while the NDV protein (red) was expressed in both the rL-hIFN-λ1-infected group and NDV-infected group. Neither IFN-λ1 nor NDV protein was expressed in the control group.
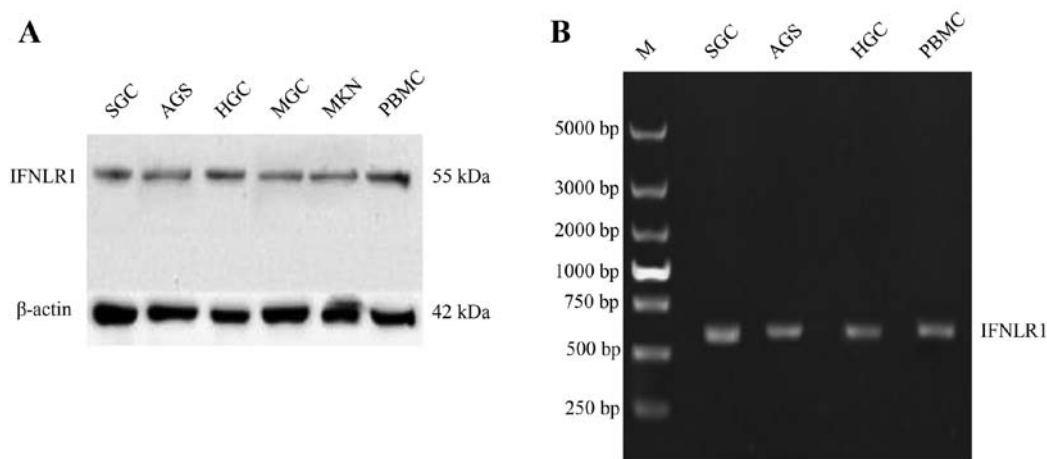
Figure 3. Human gastric cell lines express interferon-λ1 (IFN-λ1) receptor IFNLR1. (A) The expression of IFNLR1 was measured by western blot analysis in a panel of five human gastric tumor cell lines and human PBMCs. (B) mRNA expression of IFNLR1 was evaluated by RT-PCR for the expression of IFNLR1 on three gastric tumor cell lines SGC, AGS and HGC and on PBMCs.
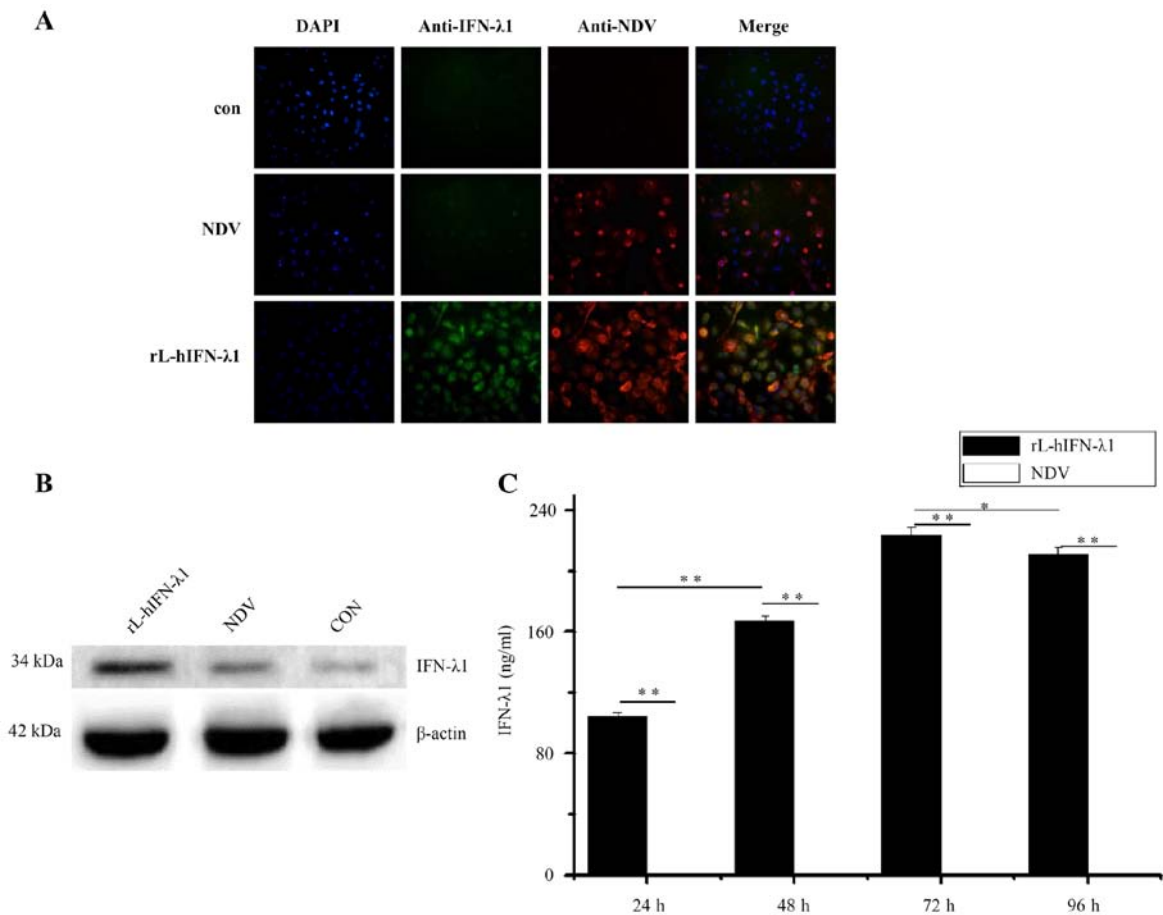

Figure 4. Expression of viral genes and proteins in infected gastric adenocarcinoma cells. (A) Indirect immunofluorescence staining of human interferon-λ1 recombinant adenovirus (rL-hIFN-λ1)-infected SGC cells with chicken anti-Newcastle disease virus (NDV) serum and anti-IFN-λ1 antibody observed with immunofluorescence microscope (x200 magnification). (B) IFN-λ1 protein expression in SGC cells was detected by western blot analysis. The IFN-λ1 protein was highly present in the rL-hIFN-λ1-infected group compared with NDV group and control group. (C) SGC tumor cells were infected with rL-hIFN-λ1 or NDV for 1 h and washed, After culture for 24, 48, 72 and 96 h, the supernatants were collected and analyzed for IFN-λ1 by ELISA; *p<0.05, **p<0.01.

To define whether the IFN-λ1 gene integrated in the NDV genome leads to the production of biologically active IFN-λ1 in supernatants of rL-hIFN-λ1-infected gastric tumor cells, we measured the quantitation of IFN-λ1 by ELISA. The supernatants of tumour cells were harvested 24, 48, 72 and 96 h after the infection. The IFN-λ1 expression reached the highest level at 72 h to 223 ng/ml in the supernatants of rL-hIFN-λ1-infected SGC cells, whereas the SGC cells infected with NDV did not release any IFN-λ1 into the supernatants (Fig 4C).
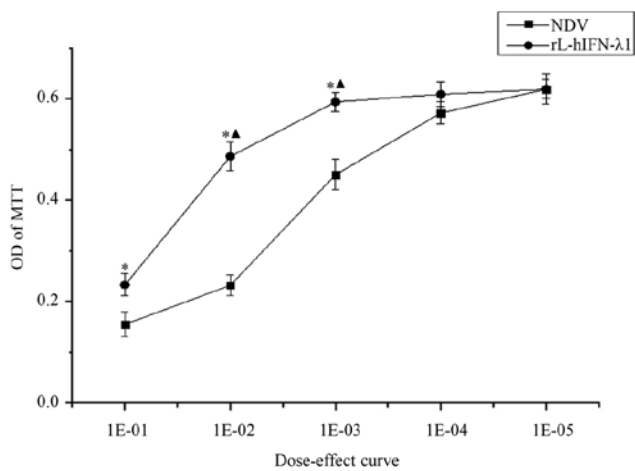
Figure 5. Proliferation changes in SGC cells after viral infection. Dose-response curve of SGC cells after viral infection at 24 h. The OD of MTT after dilution (n=5). The cells infected with human interferon-λ1 recombinant adenovirus (rL-hIFN-λ1) for 24 h showed significantly decreased absorbance when compared with OD of the Newcastle disease virus (NDV)-infected group (*p<0.05). A significant difference was observed between different dilutions of the same virus ($^▲$p<0.05).

The proliferation changes of SGC cells infected with rL-hIFN-λ1. To elucidate the proliferation changes of rL-hIFN-λ1-infected gastric adenocarcinoma cells, MTT assay was utilized to portray dose-response curves. After 24 h of rL-hIFN-λ1 or NDV infection, the gastric tumour cells were assessed by MTT assay. OD of MTT in the rL-hIFN-λ1-infected group was weaker compared with that in the NDV-infected group, suggested that the rL-hIFN-λ1-infected group had a greater inhibition ratio. Moreover, the inhibition ratio increased over the dose after infection (Fig. 5).

rL-hIFN-λ1 induces apoptosis in gastric tumor cells. The recombinant virus rL-hIFN-λ1 was designed for cancer therapeutics, and apoptosis was measured in gastric carcinoma cell lines.

TUNEL assay kit was used for monitoring apoptosis. The results revealed that the number of apoptotic cells and the AI was much higher in the rL-hIFN-λ1 compared with NDV group, the negative control group showed much less apoptosis (p<0.01) (Fig. 6A).

Furthermore, the levels of cleaved caspase-3, a key protein to indicate apoptosis, were measured by western blot analysis at 24 h after virus infecting tumour cell lines. The expression levels of cleaved caspase-3 proteins were upregulated in the rL-hIFN-λ1 group compared with the other NDV group and the negative control group (Fig. 6B).

Flow cytometry analysis showed that early apoptosis of cells was higher in the rL-hIFN-λ1-infecting group than that in NDV group, early apoptosis was not detected in the control group (Fig. 6C).

rL-hIFN-λ1 modulates the human Th1/Th2 response in the tumor microenvironment. Th1/Th2 balance is confirmed to play an important role in tumor growth, the high expression of Th1 response and reduction is capable of inducing antitumor immunity to prevent cancer cell lines in preclinical study (14,27) and gastric cancer in clinical research (28). To determine whether rL-hIFN-λ1 could elevate the secretion of chemokines of tumor microenvironment to promote antitumor immune responses, SGC cells were infected with rL-hIFN-λ1 or NDV, after cultured for 72 h, supernatants were collected to stimulate PBMCs for 5 days, then the production as quantified of T-cell cytokines IFN-γ (representative of Th1 responses) and IL-13 (representative of Th2 responses) by ELISA everyday. Our studies revealed that the Th1 cytokine IFN-γ increased significantly and the Th2 cytokine IL-13 was highly decreased
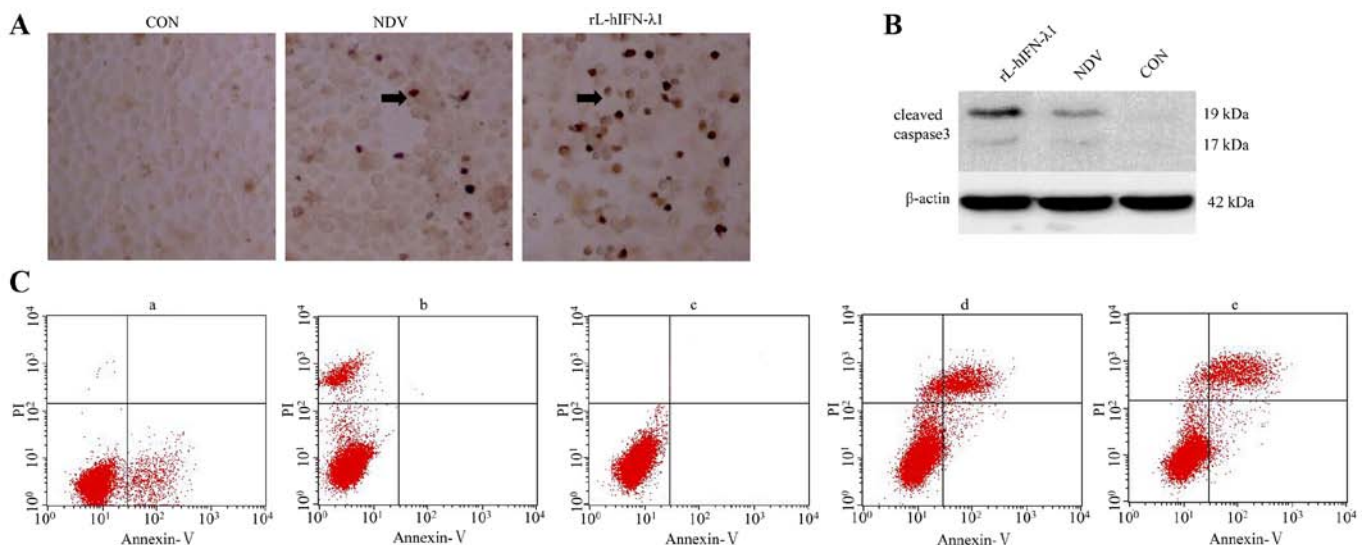

Figure 6. Apoptosis in SGC cells at 24 h after infection. (A) TUNEL analysis of apoptosis in SGC cells. Apoptosis of virus-infected cells was obvious compared with the controls. The apoptotic cells (→) in the human interferon-λ1 recombinant adenovirus (rL-hIFN-λ1) groups were more abundant compared with those in the Newcastle disease virus (NDV) group. (B) The expression of apoptosis-associated proteins in SGC cells was detected by western blot analysis. The protein cleaved caspase-3 had higher expression levels in rL-hIFN-λ1 group compared with those in the NDV group and control group. (C) Flow cytometry analysis results of 24 h early apoptosis cells. (C-a) Annexin V positive control. (C-b) Propidium iodide (PI) positive control. (C-c) SGC cells without treating with virus as a negative control. (C-d) SGC cells were infected with NDV, the apoptotic rate produced by NDV was 9.43%. (C-e) SGC cells were infected with rL-hIFN-λ1, the apoptotic rate produced by rL-hIFN-λ1 was 11.07%.
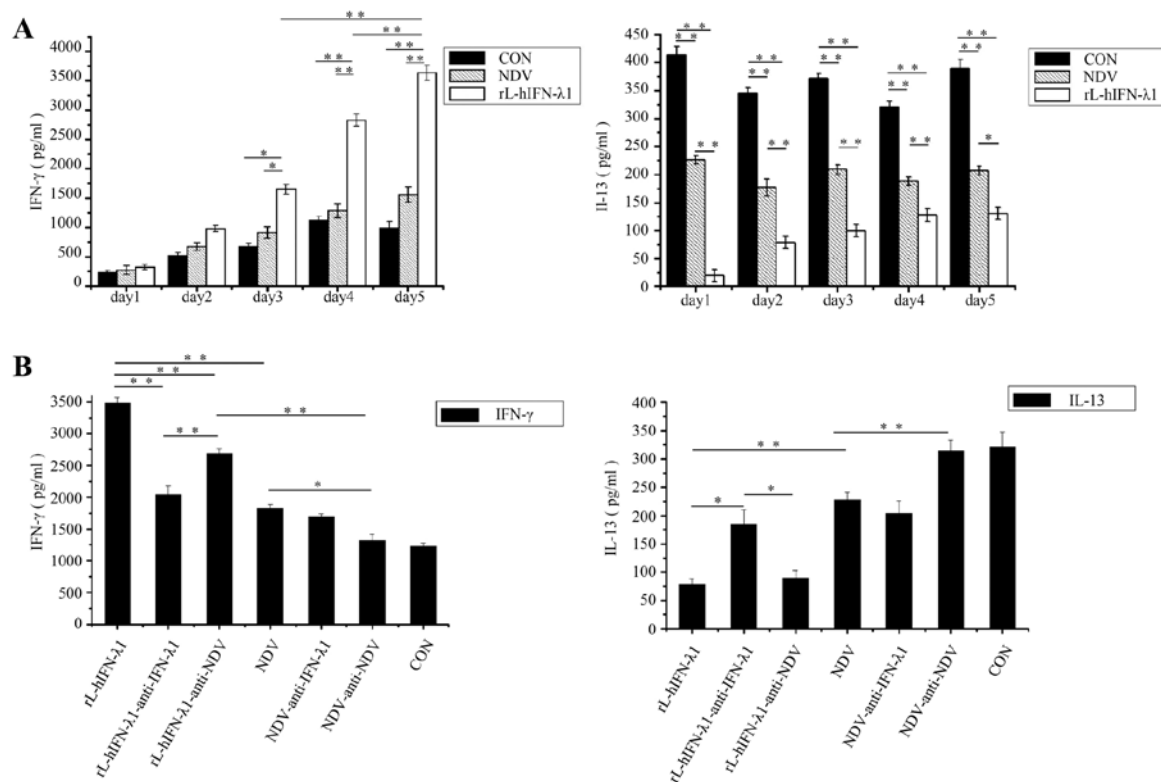
Figure 7. Effect of human interferon-λ1 recombinant adenovirus (rL-hIFN-λ1) on T helper cell subtype 1 (Th1) (IFN-γ) and Th2 [interleukin (IL)-13] production by Con-A-stimulated PBMCs. (A) PBMCs stimulated with Con-A (5.0 mg/ml) were co-cultured for 1-5 days with the supernatants of SGC cells collected after 3 days infection with rL-hIFN-λ1 or NDV. The cytokine protein levels were measured by ELISA. (B) Anti-IFN-λ1 (anti-29) or anti-NDV was added into the former supernatants collected as described to detect whether the virus itself or the cytokine protein produced by the viral infection tumor cells played a dominant role in the changing of Th1/Th2 immuno-response. The left panel shows a significant decrease in the expression of IFN-γ in the anti-IFN-λ1 added group compared with group added with anti-NDV. The right panel showed that the IL-13 has a higher increase in anti-IFN-λ1 added group than the anti-NDV group ($^*p<0.05$) ($^{**}p<0.01$).

in the rL-hIFN-λ1 group, as compared with NDV and control groups (p<0.01) (Fig. 7A). To confirm whether the Th1/Th2 immune response change was related to cytokine IFN-λ1 or the NDV viral, anti-IFN-λ1 or anti-NDV was added during the cell culture with the former supernatants, then cytokine levels were tested after PBMCs cultured for 5 days. The Th1 response was enhanced, and the Th2 response inhibited much more in the anti-IFN-λ1 group than in the anti-NDV group, NDV group and control group showed no significant change. The results demonstrated that the tumor microenvironment cytokine skewed towards a Th1 response duo to the high dose of IFN-λ1 expressed by the rL-hIFN-λ1-infected tumour cells (Fig. 7B).

Discussion

Oncolytic viral therapy is a method that harnesses the natural ability of a virus to infect, duplicate and lyse a host cell as part of its natural life cycle (29). NDV, an oncolytic virus, has been confirmed to possess the capablity to inhibit malignant cells via multiple mechanisms (30). In recent years, NDV has been shown to induce the immune system to eliminate tumor cells (31), and NDV strains can selectively replicate up to 10,000 times better in tumor cells than in normal cells (32). With the development of reverse genetics, modification of the viral genome for NDV as well as introduction of foreign sequences has become possible (33).

IFN-λ1 (IL-29), a recently discovered cytokine, accompanies type I IFN in the signaling pathways and differs wildly in tissue responsiveness. It is known that the epithelial cells of most tissues express IFNLR1 and are responsive to IFN-λ in mice (34,35). Most of the tumor cells are epithelial in origin. The tumor cells express IFNLR1 and are responsive to IFN-λ1. On the other hand, IFN-λ1 receptor also expressed in both naive and memory CD4$^+$ T cells (22). T cells are capable of responding to IFN-λ1 with altering cytokine production, it allows IFN-λ1 to alter the balance of Th1 and Th2 immune responses, which could be potential agents for cancer therapy (27,36,37). IFN-λ1 has also been shown to exert antitumor effects in both murine and human models (24).

Due to the above advantages of tumour therapy, we inserted human IFN-λ1 gene into the NDV viral genome, in such a way that the viral infection of tumor cells leads to the expression of IFN-λ1. We propose that the recombinant virus rL-hIFN-λ1 may have antitumor therapy potential. At the start of this study, there was no study on genetically engineering recombined NDV expressing IFN-λ1 genes to improve the effect of cancer therapy.

In the present study, we confirmed the stability of growth characteristics of our recombinant virus was as much as the NDV virus passing 10 generations in the embryonated SPF eggs. The results have also shown the high dose stable expression of IFN-λ1 gene in rL-hIFN-λ1 infected tumour cell lines at 24 h reaching the highest levels at 72 h.

In recent years, some studies have confirmed apoptosis as the dominant key to NDV-related cell death (29,38-41). It was demonstrated in our previous study that NDV caused gastric adenocarcinoma SGC-7901 and AGS cell death via apoptosis (42). The mechanism behind NDV oncolytic effect have been investigated. It was recently found that its apoptosis inducing effect is presumably exerted through ER-mediated cellular stress mechanism (43-45). Our previous study also found in addition to apoptosis, that NDV can also induce autophagy, ERs and mitochondrial dysfunction (42).

The new generated recombinant NDV LaSota strain is designed as a potential candidate for a viral vector in cancer therapy in human, we further found that the high levels of IFN-λ1 receptor IFNLR1 was expressed in the gastric tumor cell lines SGC and AGS, which indicated that gastric cancer could be a potential target for IFN-λ1 and rL-hIFN-λ1 therapy. The TUNEL assay, western blot assay and the Annexin V flow cytometric analysis all confirmed that the number of apoptotic cells and the AI were markedly higher in the rL-hIFN-λ1 and NDV groups compared with control group, and rL-hIFN-λ1 group exhibited a higher AI compared with NDV groups.

The high dose of cytokine expressed by the rL-hIFN-λ1 infecting tumour cells had pro-tumor Th2 immune responses and in favor of antitumor Th1 responses in PBMCs, which generate an antitumour environment making the rL-hIFN-λ1 a protential antitumor agent for immunotherapeutic approaches.

In conclusion, rL-hIFN-λ1 inhibited the growth of gastric cancer cell lines which contained the IFNλ-R1 receptors and accelerated apoptosis to a certain extent. The recombinant rL-hIFN-λ1 virus modulated Th1/Th2 immune response to change the tumor microenvironment to an antitumour cytokine which led to the reducing of tumor growth. The present study is expected to provide an experimental basis for further mechanism research and clinical application of rL-hIFN-λ1 in gastric adenocarcinoma therapy, considered that the recombinant NDV LaSota expressing human IFN-λ1 named as rL-hIFN-λ1 could be a potential viral agent for gastric tumor therapy.

Acknowledgements

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