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Immunotherapy of Dual-Function Vector with Both Immunostimulatory and B-Cell Lymphoma 2 (Bcl-2)-Silencing Effects on Gastric Carcinoma

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

B 1 Lanying Ma*
CD 1 Mei Han*
CD 2 Zumureti Keyoumu
EF 3 Hua Wang
A 1 Saifuding Keyoumu

1 Department of Digestive System, Xinjiang Medical University Affiliated Tumor Hospital, Urumqi, Xinjiang, P.R. China
2 Department of Preventive Care, Xinjiang Medical University Second Affiliated Hospital, Urumqi, Xinjiang, P.R. China
3 Basic Medical College of Xinjiang Medical University, Urumqi, Xinjiang, P.R. China

* Lanying Ma and Mei Han are the co-first authors of this paper

Corresponding Author: Saifuding Keyoumu, e-mail: saifudingkeyoumu@sina.com
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Background: Tumorigenesis is a kind of pathology marked by infinite proliferation and restrained apoptosis compared with normal cells. The abnormal expression of some proto-oncogenes and apoptosis inhibition are essential for tumor growth, which has been confirmed by molecular biologic and immunologic studies. The hypofunction of the host immune system also drives the development and metastasis of malignant tumors. Bcl-2, which has a critical role in regulating apoptosis, is overexpressed in several cancers.

Material/Methods: In this study, we constructed a dual-function small hairpin RNA (shRNA) vector containing an Bcl-2-silencing shRNA and a TLR7-stimulating ssRNA and examined it effect on tumor cell growth and proliferation.

Results: Stimulation with this bi-functional vector *in vitro* promoted significant apoptosis of MFC cells by regulating the expression of apoptosis-related proteins and induced secretion of type I IFNs. Most importantly, this bi-functional vector more effectively inhibited subcutaneous MFC cell growth than did single shRNA and ssRNA treatment *in vivo*. Natural killer (NK) and CD4+ T cells were required for effective tumor suppression, and TLR7 was shown to play a helper role in the activation of NK cells and CD4+ T cells, possibly by regulating the expression of receptors or secretion of cytokines.

Conclusions: This bi-functional vector that contained ssRNA and shRNA may represent a promising approach for tumor therapy.

MeSH Keywords: Genes, bcl-2 • Stomach Diseases • Toll-Like Receptor 7

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Background

Gastric cancer, from which about 10 percent of patients with cancer suffer every year worldwide, is one of the most common malignant tumor in terms of incidence, and it has the second highest mortality rate [1,2]. Although either classical or novel chemotherapeutic drugs treatment have improved the life quality of gastric cancer patients, the 5-year survival rate still is low [3]. The characteristic of malignant tumors mainly is unrestricted proliferation and apoptosis of tumor cells. The B-cell lymphoma (Bcl)-2 gene family is a significant group of apoptosis-related genes, which can encode not only anti-apoptotic proteins (Bcl-2), but also pro-apoptotic proteins (Bcl-2-associated X protein [Bax]). The expression of Bcl-2 protein in normal cells is relatively low, but abnormally high in tumor cells [4]. Thus, Bcl-2 kinase may be a candidate molecular target for cancer therapy.

Tumor pathogenesis is a result of failure of immuno-inhibition or tumor outgrowth. These events put a functional imprint onto the emerging tumor repertoire because tumor cells arising in the presence of a fully functional immune system are less immunogenic than those that develop in the absence of immunity [5]. Host immunosuppression, mediated by tumor cells, is characterized by incompetence of cytotoxic T lymphocytes (CTLs), massive production of suppressing cytokines (such as IL-10 and TGF-β), and activation of Treg cells, leading to functional deficiencies in CTLs, $CD4^+$ Th1 cells, or natural killer (NK) cells [6–8]. Thus, tumor immunotherapy must restimulate the immune response, in addition to suppressing oncogene expression.

Toll-like receptors (TLRs) are pattern recognition receptors that can recognize conserved structures in pathogens, which results in triggering the innate immune response and priming the antigen-specific adaptive immune response. TLRs are important in protective immunity against cancer and infection [9]. TLR7, as one of the minority TLRs expressed in endosomes, recognizes natural nucleoside structures (for example, viral single-stranded RNA [ssRNA]) and synthetic compounds (such as imidazoquinolines) [10,11]. Binding of TLR7 with its agonists triggers a signaling cascade, which comprises recruitment of MyD88, activation of the NF-κB or IRF7 pathway, and secretion of type I IFN and inflammatory cytokines. In addition, TLR7 stimulation also can prime activation of NK and T cells directly or with the help of activated antigen-presenting cells (APCs), causing antitumor immune responses to emerge [12,13].

In this study, we constructed a dual-function small hairpin RNA (shRNA) vector that contained an shRNA silencing Bcl-2 and a TLR7-stimulating ssRNA. Transfection with this bi-functional vector *in vitro* enhanced significant apoptosis of MFC cells and promoted secretion of type I IFNs. Importantly, the vector more effectively inhibited MFC cell growth than shRNA and ssRNA treatment individually. NK and $CD4^+$ T cells participated in effective tumor suppression. The TLR7 signal pathway is essential for the activation of NK and $CD4^+$ T cells. The bi-functional vector may represent a promising approach for tumor therapy.

Material and Methods

Plasmid construction and lentiviral packaging

The plasmid construction was structured as previously described [14]. Briefly, transcription of shRNA oligonucleotide targeting Bcl-2 (sense-loop-antisense, GATGAAGTACATCCATTAT) was designed by using BLOCK-iT RNAi Designer as a synthetic duplex with overhanging ends identical to those created by restriction enzyme digestion (BamHI at the 5' and EcoRI at the 3'), and was cloned into vector pTZU6+1 vector that contains a U6 polymerase-III (pol-III) promoter. Transcription of each ssRNA oligonucleotide synthetic duplex sequence (sense-terminator, CGGGCAGACAACACACTGAGAAAAAA) was designed using a similar overhanging-ends procedure to the shRNA, and cloned into expression vector pSIREN, which contains a U6 pol-III promoter. To create the dual-function vector, the U6+shRNA in pTZU6+1-shRNA was digested by HindIII and EcoRI, and inserted into pSIREN-ssRNA. pSIREN-control, ssRNA, shRNA, or dual vectors were cloned into a lentiviral pGCSIL-GFP plasmid, and transfected into 293 T cells. Forty-eight hours later, culture supernatant was collected and filtered through a 0.45-mm filter. Viruses (LV-control, LV-ssRNA, LV-shRNA, LV-dual) were harvested by centrifugation at 70,000 × g at 4°C for 2 hours. Harvested viruses were aliquoted and stored at –80°C.

Cell culture

Mouse gastric cancer cell line MFC (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) was maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO/BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS). This cell line was used within 6 months of receipt. Cells were never used for more than 10 passages and were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Animals and treatment

A total of 120 mice (6–8 weeks old; Experimental Animal Center of Beijing University, Beijing, China) were raised under specific pathogen-free (SPF) conditions. The Committee on the Ethics of Animal Experiments of the Xinjiang Medical University Affiliated Tumor Hospital approved all the animal studies. 1×10^6 MFC cells were injected subcutaneously into the right flank of 65 mice. After 2 weeks, LV-control, LV-ssRNA, LV-shRNA, and LV-dual (MOI=50) were administered intratumorally once a week

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for 2 weeks. After another 2 weeks, the mice were sacrificed and the tumor volume was calculated by length × width^2/2.

Cell depletion

NK cell depletion mAbs were purified from PK136 (α-NK1.1) hybridoma cell lines. To deplete cells, mice were injected intraperitoneally with 1 mg of mAb. Three days later the dual vector was administered intravenously. Fewer than 5% splenic or intestinal cells were consistently observed in cell-depleted animals throughout the study, with no significant reductions in other cellular populations. Control mice received 200 µL of Dulbecco's phosphate-buffered saline.

RT-PCR

The expression of BCL-2, IFNα/β, and TLRs in the MFC cell line was measured by RT-PCR. Briefly, total RNA was isolated following manufacturer recommendations with Trizol reagent (Life Technologies, Grand Island, New York, USA); cDNAs were generated from 1 µg of RNA using a MMLV reverse transcriptase, RNAsin RNAse inhibitor, and oligo dT kit (Promega Corporation, Madison, Wisconsin, USA) and stored at –20°C for batch analysis. The sample volume was increased to 25 µL with the solution containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl2, 1 U Taq polymerase, 0.1 mM forward and reverse primers of Bcl-2, IFNα/β, TLRs, and β-actin as internal control (described in Supplementary Table 1) in a DNA Thermocycler (PerkinElmer/Cetus, Boston, Massachusetts, USA). PCR products were electrophoresed through a 2% ethidium bromide-stained agarose gel, visualized by transillumination, and scanned. Densitometry was performed using Image J 1.47 software (National Institutes of Health, Bethesda, Maryland, USA), and the results were expressed as arbitrary units normalized to β-actin expression. Each assay included a DNA minus control and a standard curve performed with serial dilutions of control cDNA.

Western blotting

The protein expression of Bcl-2, TLR7, and related signaling molecules was determined by Western blotting. MFC cells were washed with plain prewarmed phosphate-buffered saline (PBS) after infection. Cells were solubilized in lysis buffer (BestBio, China) and a protease inhibitor cocktail (BestBio, China). Whole cell extracts were mixed in Laemmli loading buffer, boiled for 5 min, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were blocked with 5% non-fat milk for 2 h, transferred onto nitrocellulose membranes, and blotted overnight at 4°C with antibody or anti-β-actin (Santa Cruz Biotechnology, California, USA) (at a dilution of 1: 2000). The membranes were washed with Tris-buffered saline with Tween 20 (TBST) three times and incubated with horseradish peroxidase (HRP)-conjugated second antibody for 1 h. Protein bands were visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, USA) and examined with Alpha Ease FC software.

MTT assay

The proliferation of MFC cells after transfection was measured by the 3-(4,5-dimethyl-2-thiazolyl)-2,5–diphenyl-2H-tetrazolium bromide (MTT) assay [15]. Treated cells were resuspended in DMEM medium and seeded into 96-well plates (10,000 cells/well). The plates were incubated for different times, and 0.1 mg of dissolved MTT was added for another 4 h of incubation. Dimethyl sulfoxide 200 µL was added to dissolve the remaining formazan precipitate, and the absorbance at 570 or 630 nm (A570/630) of each sample was determined with a microplate autoreader (Bio-Rad).

ELISAs

At specified end points, culture supernatants was removed and centrifuged at 10,000 rpm for 3 min. Cytokine analysis was performed on supernatants using the following ELISA kits: Duoset murine IFN-α/β (R&D Systems). All ELISAs were performed according to the manufacturer's protocols. Plates were read using a SAFIRE plate reader (450 nm).

Flow cytometry

Immunofluorescence staining was performed following standard protocols. In brief, cultured cells were washed twice in PBS with 5% FCS and resuspended in 100 µL of staining buffer, which was PBS containing 0.5% bovine serum albumin and 0.1% NaN3, at 10^6/mL. Cells were added to the 96-well plates and blocked with mouse serum for 30 min at 4°C. The antibodies used were FITC-conjugated NK1.1 and PerCP-Cy5.5-conjugated CD3e (BD Biosciences); FITC-conjugated CD4 and MHC-I, PerCP-Cy5.5-conjugated CD8, PE-conjugated CD69, APC-conjugated NKG2D, and PE-conjugated NKG2A (eBiosciences); and FITC-conjugated MULT-1, PE-conjugated H-60, and PE-conjugated IFN-γ mAb (R&D Systems). After incubation for 30 min at 4°C, cells were washed three times in washing buffer and fixed with PBS containing 1% (w/v) paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA). The samples were measured with flow cytometry FACScan (Becton Dickinson, Mountain View, California, USA), and the data were analyzed using the software WinMDI (Scripps Research Institute, La Jolla, California, USA).

NK cytotoxicity assays

The ability of spleen lymphocytes to kill MFC cells was evaluated by CFSE/7-AAD flow cytometry assay, as previously described

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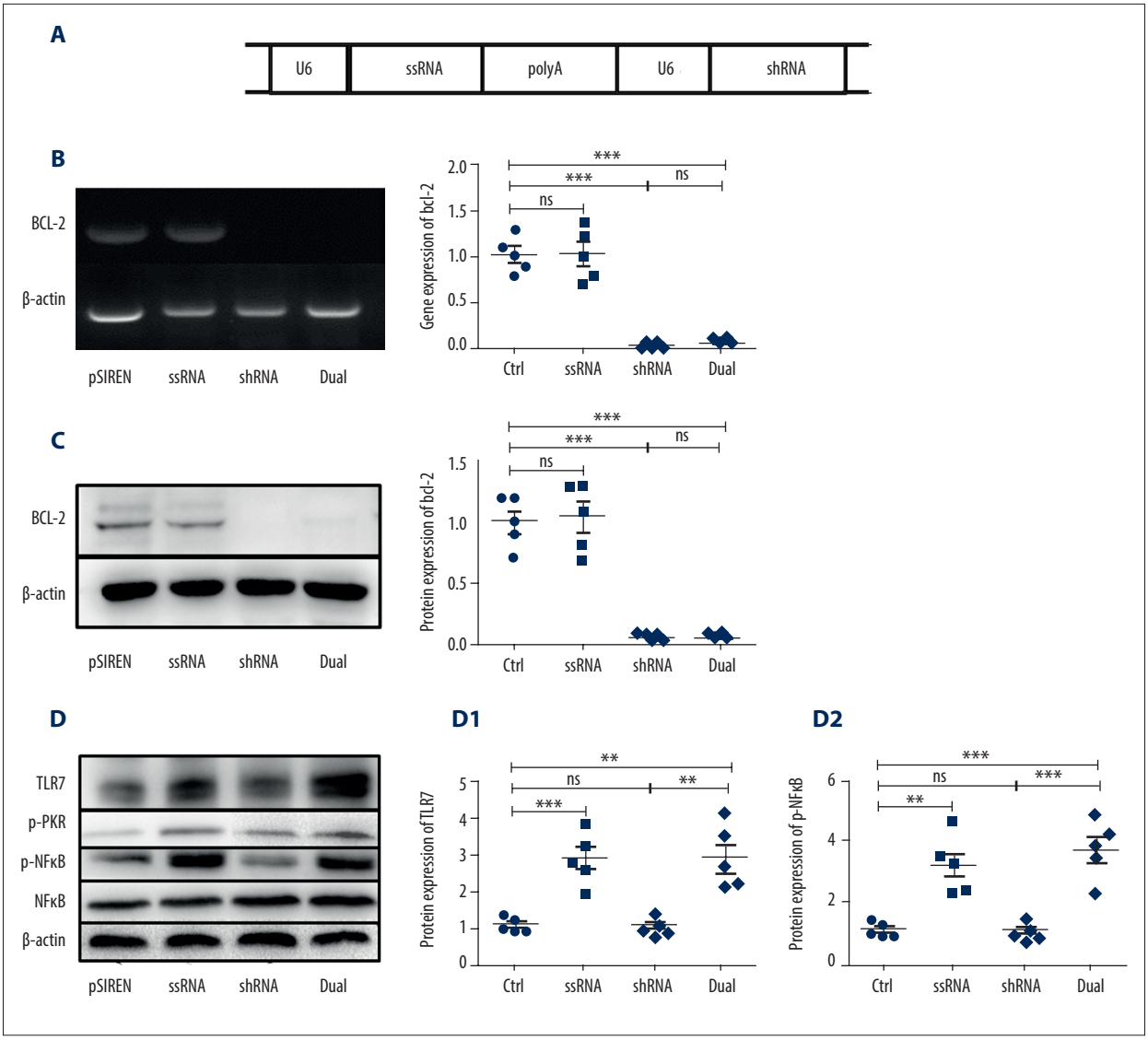

Figure 1. The construction of a bifunctional vector bearing ssRNA and BCL-2-specific shRNA. (**A**) Schematic of the construction of the dual-functional vector. The expression of BCL-2 in the MFC cell line was measured by RT-PCR (**B**) and Western blotting (**C**) after 24 hours post-transfection with indicated vectors. (**D**) Western blotting of the expression of TLR7 and related signaling molecules in MFC cells after transfection *in vitro*. Data are means ±SD of TLR7 (**D1**) or p-NFκB (**D2**) from 5 independent experiments. *** $P<0.001$ and ** $P<0.01$ compared with the pSIREN transcription group.

[15]. Briefly, MFC cells were incubated with 1 mL of CFSE (2 mmol/L; Molecular Probes) for 10 minutes at 37°C and then washed. Spleen lymphocytes were isolated and added to the target cells at effector/target ratios of 50: 1, 25: 1, and 12.5: 1, respectively, for 4 hours. Following a further wash, cells were labeled for 15 minutes with 7-AAD (optimized at 0.25 mg/mL; Sigma-Aldrich) to identify dead cells. The cells were then analyzed via flow cytometer (FACScalibur). Cytotoxicity was calculated as follows: percent lysis = (CFSE/7-AAD double positive cells/CFSE positive cells) ±100%.

Measurement of apoptosis

Apoptosis can be detected by translocation of phosphatidyl serine to the cell surface using an annexin V-FITC antibody. The percentage of cells that were Annexin V-positive represented the proportion of apoptotic cells. Alternatively, apoptosis was also measured by TUNEL staining using a One Step TUNEL Apoptosis Assay Kit (Beyotime) [16]. Nuclear staining was evaluated under a light microscope via DAPI staining (Beyotime).

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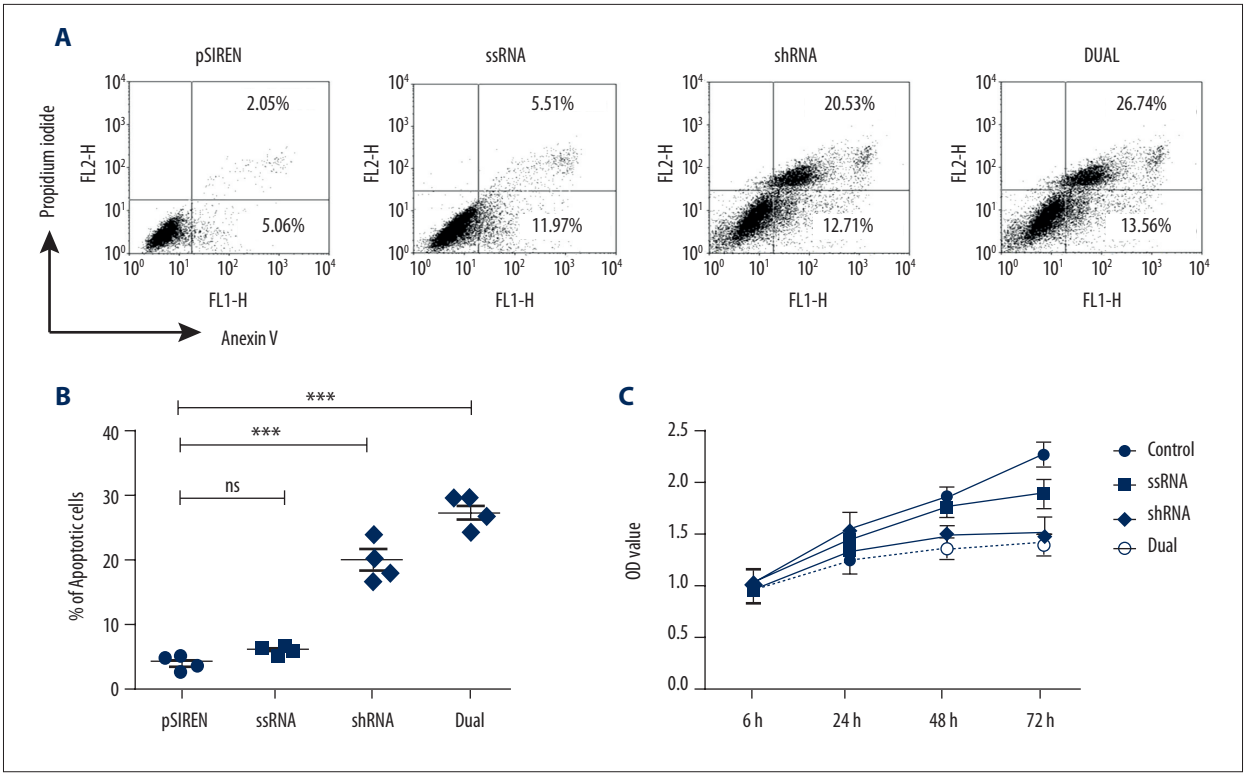

Figure 2. Transfection with shRNA and bi-functional vector promotes the apoptosis of MFC cells *in vitro*. Flow cytometric analysis of apoptosis in MFC cells after transfection for 24 hours was done with the indicated vectors using Annexin V/PI double staining, and results are shown as representatives (**A**) or means ±SD (**B**). The proliferation of MFC cells after transfection was measured with the MTT assay (**C**). Data are shown from at least 3 independent experiments. ** $P<0.01$ compared with the pSIREN transcription group.

TLR7 inhibition

To inhibit the function of TLR7, IRS661 (5'-TGCTTGCAAGCTT GCAAGCA-3'; TAKARA), an oligonucleotide (ODN) inhibitor of TLR7 signaling that interferes with the combination of TLR7 and ssRNA [17,18] was administered intravenously before LV-dual vector treatment.

RNA-mediated interference

Scrambled RNA (control) or small interfering RNA (siRNA) pools targeting human TLR7 (Dharmacon, Pittsburgh, Pennsylvania, USA) were used to measure the activation of NF-κB after TLR7 was stimulated by R837. Concentrations of siRNA were 50–100 nM and were delivered by the use of X-tremeGENE siRNA transfection reagent (Roche). Subsequent tests were carried out after 72 hours post-transfection.

Statistical analysis

Experimental results were plotted and analyzed for statistical significance with Prism5 software (GraphPad Software Inc., California). A *P* value of 0.05 was considered significant.

Results

Construction of a dual-function vector with both TLR7-stimulatory ssRNA and Bcl-2-silencing shRNA

First of all, we detected the expression of Bcl-2 in mouse gastric carcinoma cell line MFC. PCR and Western blotting showed that Bcl-2 was highly expressed in the MFC cell line compared with the normal gastric carcinoma cell line (data not shown). To clarify the role of Bcl-2 in cell proliferation and apoptosis and to stimulate an immune response and silence Bcl-2 expression simultaneously, we constructed a dual-function vector containing a TLR7-stimulating ssRNA and a Bcl-2-gene-silencing shRNA (Figure 1A). The annealed siRNA oligonucleotides were knocked into the expressing vector pTZU6+1 to construct shRNAs. Four different ssRNA oligonucleotides were designed and inserted into the pSIREN plasmid (ssRNAs). The shRNA and ssRNA vectors were transfected into MFC cells separately to test the Bcl-2-silencing and TLR7-stimulating effect.

Then the ability of the bi-functional vector to downregulate Bcl-2 expression was confirmed. Data show that Bcl-2 mRNA and protein levels were downregulated after transfection with the

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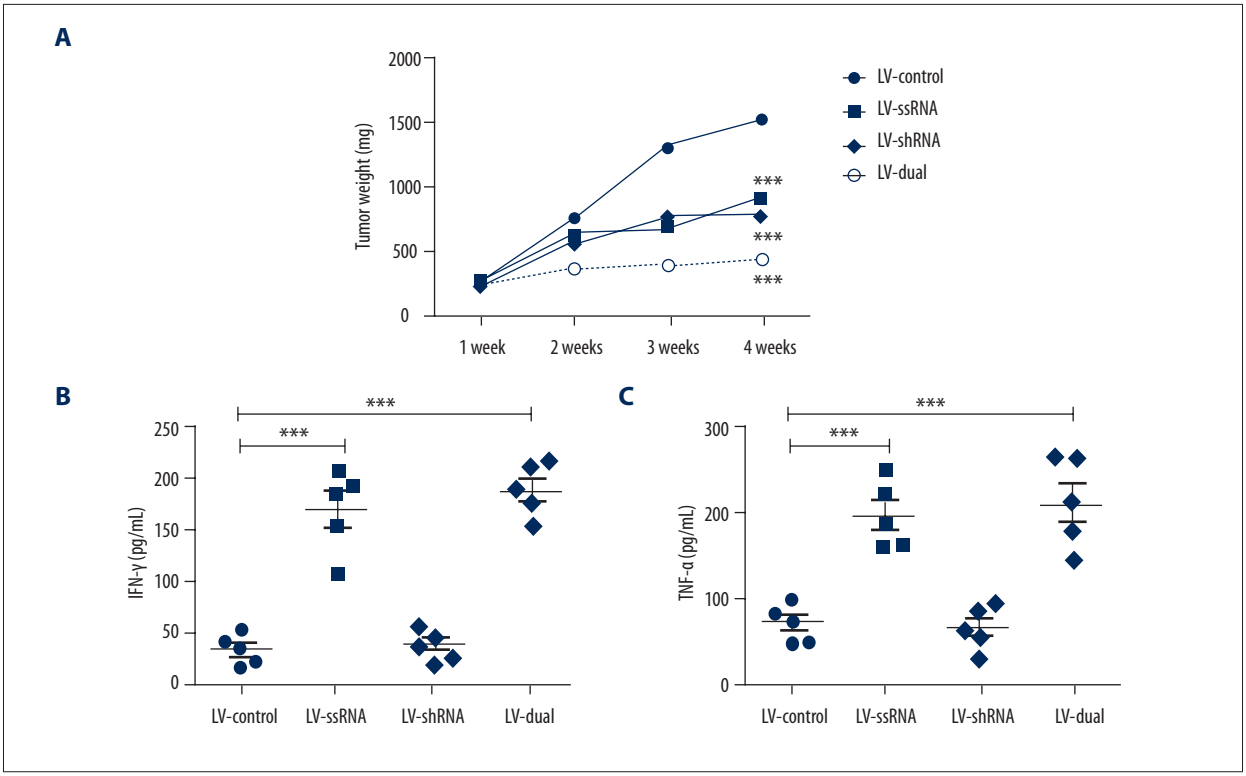

Figure 3. Treatment with bifunctional vector delays tumor growth *in vivo*. A total of 65 mice were subcutaneously challenged with 1×10^6 MFC cells, and 42 mice were chosen to participate in the follow-on experiment. LV-control (11 mice), LV-ssRNA (10 mice), LV-shRNA (10 mice), and LV-dual (11 mice) (MOI=50) were administered intratumorally 2 weeks later for 14 days once a week. Tumor volumes were calculated (**A**). (**B, C**) The levels of IFN-γ or TNF-α in the serum of mice were measured by ELISA after 24 hours post-transfection with indicated vectors. Data are representative of 5 independent experiments with at least 3 mice per group. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ compared with the LV-control group.

Table 1. Percentages of NK and T cells in splenic lymphocytes from bearing mice (%, n=3).

	Control	ssRNA	shRNA	Dual
NK cells	8.29±0.47	17.00±0.89**	8.28±0.41^{ns}	29.06±0.79***
CD4+T cells	12.62±0.41	22.87±0.64***	10.66±1.74^{ns}	29.28±1.22***
CD8+T cells	22.50±2.46	22.20±0.87^{ns}	23.14±0.94^{ns}	25.97±1.23^{ns}

ns $P>0.05$; ** $P<0.01$;*** $P<0.001$, compared with the control group.

bi-functional vector compared with the pSIREN or ssRNA transfection group (Figure 1B, 1C). The ssRNA is regarded as a ligand of TLR7, and ssRNA stimulation can activate the TLR7 signal pathway, leading to the production of type I IFN and inflammatory factors [10,11,19]; therefore, we detected whether the bifunctional vector could stimulate the TLR7 pathway by measuring the protein level of TLR7 and related signaling molecules. There was an increase of the mRNA and the protein expression of TLR7 in MFC cells post-transfection with the ssRNA and bifunctional vector (Figure 1D). Collectively, these results indicated a successfully constructed dual-function ssRNA-shRNA vector with both Bcl-2 silencing and TLR7 pathway stimulation effects.

The dual-function ssRNA-shRNA vector promotes the apoptosis and inhibits the proliferation of MFC cells

Bcl-2, which is well known as an oncogene related to cell apoptosis, is overexpressed in tumor cells and promotes the unlimited proliferation of cells and carcinogenesis [20–24]. Silencing of Bcl-2 significantly promoted apoptosis of MFC cells, as detected by Annexin V/PI double staining (Figure 2A, 2B). Importantly, the dual-function vector showed a more significant pro-apoptotic effect than the shRNA vector. Meanwhile, after transfection, the dual-function vector was more effective at inhibiting the proliferation of MFC cells than the shRNA vector (Figure 2C).

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These results suggest that transfection with the Bcl-2 shRNA and dual-function vector promotes apoptosis of gastric carcinoma cells by silencing Bcl-2, resulting in the activation of the apoptosis-related signal pathway, and the proliferation of MFC cells was apparently held back.

Bi-functional vector could inhibit subcutaneous tumor growth and activate NK and T cells *in vivo*

To probe the antitumor effect of the dual-function vector *in vivo*, 1×10^6 MFC cells were administered subcutaneously to 65 mice. Two weeks later, the mice that were dead or failed to develop tumors were rejected. The remaining 42 mice were divided randomly into four groups, and LV-control, LV-ssRNA, LV-shRNA, and LV-dual (MOI=50) were administered separately via intratumoral injection once a week for two weeks. Tumor volume was calculated at four weeks. Treatment with ssRNA, shRNA, and dual-function LV-vector significantly suppressed tumor growth, with the dual-vector treatment emerging as having the most significant effect (Figure 3A). These results indicated that silencing of Bcl-2 and stimulation of the TLR7 contribute to the antitumor activity of the bi-functional vector *in vivo*.

The more efficient tumor inhibition of the bi-functional vector compared with shRNA suggested that the immunostimulatory effect exerted by the ssRNA plays an important role in inhibiting tumor growth. To explore the mechanism of immune stimulation in suppression of tumor growth, we observed the activation of immune responses induced by the bi-functional vector in mice. The proportion of splenic NK and $CD4^+$ T cells, but not $CD8^+$ T cells, increased significantly in both LV-ssRNA-treated and LV-dual-treated mice (Table 1). Also, the density of IFN-γ and TNF-α, which were secreted increasingly after TLR7 was activated and played an important role in tumor killing in mouse serum that was treated with LV-ssRNA or LV-dual, increased dramatically (Figures 3B, 3C and Supplemenatry Figure 1).

Activation of NK cells is mediated by the bi-functional vector in an NKG2D-dependent manner

To explore the mechanism of NK cells in the suppression of the growth of MFC during dual-function vector administration, we isolated splenic NK cells from tumor-bearing mice and tested their ability to kill MFC cells as targets. The cytotoxicity of NK cells from mice treated with both LV-ssRNA and LV-dual was higher than that of NK cells from LV-control-treated mice. Also, the LV-dual group had the highest cytolysis (Figure 4A). We then detected the expression of NKG2D, NKG2A, and intracellular cytokine IFN-γ by FACS. The expressions of NKG2D and IFN-γ as activating marker obviously increased, whereas the inhibitory receptor NKG2A was suppressed in both the ssRNA and dual-function vector treatment groups, with larger changes observed in the dual-function vector treatment group (Table 2). We measured the expression of NKG2D ligands MULT-1, H-60, and MHC-I on MFC cells via FACS. MULT-1 and H-60 expressions were upregulated after transfection with both ssRNA and bi-functional vectors *in vitro*, but no significant difference was found in MHC-I expression (Figure 4B–4D). To verify the role of NK cells in the suppression of gastric carcinoma mediated by the bi-functional vector, the monoclonal antibody specific for NK cells was invited to clear the NK cells in mice (Figure 5A). The mice received MFC cells by subcutaneous administration after injection with NK cell antibody. There was no difference between the mice injected with antibody and those that were treated with equivalent PBS. However, the bi-functional vector had less ability to inhibit the growth of tumor in the absence of NK cells (Figure 5B). Even the secretion of IFN-γ and TNF-α in serum of mice bearing tumor was held back in the above model (Figure 5C, 5D). These results showed that treatment with ssRNA and bi-functional vector induced NKG2D expression and IFN-γ production, at the same time reducing the expression of NKG2A and PD-1, both of which promoted NK cell activation together. In addition, ssRNA and bi-functional vector treatment also augmented the expression of NKG2D ligands, and the interaction of NKG2D and its ligands contributed to the enhanced NK lysis.

The TLR7 signal pathway is important for the antitumor function mediated by the bi-functional vector

TLR7, also known as the "nucleic acid-sensing TLR," was originally identified as recognizing ssRNA derived from RNA viruses and imidazoquinoline derivatives such as imiquimod and resiquimod (R-848), and guanine analogs such as loxoribine [25,26]. To further explore the mechanism whereby ssRNA recognition leads to increased NK activation during dual-vector treatment, and confirm the role of TLR7 in the dual vector-induced immunostimulatory effect and tumor suppression, we administered IRS661, a TLR7 inhibitor, to tumor-bearing mice intravenously before LV-dual vector treatment. The data showed that IRS661 treatment nearly completely eliminated dual vector-induced tumor inhibition (Figure 6A, 6B). Meanwhile, IRS661 treatment significantly attenuated the activation of NK cells, namely, reduction of expression of IFN-g, NKG2D, and CD69 in/on NK cells (Figure 6C), and the expression of H-60 and Mult-1on MFC cells (Figure 6D). These results revealed that activation of the TLR7 signal pathway is essential for the antitumor effect of the bi-functional vector.

Discussion

Chemical treatment or physical therapy alone can no longer be an effective treatment of tumor because growth and invasion of tumor not only rely on infinite proliferation and evading apoptosis,

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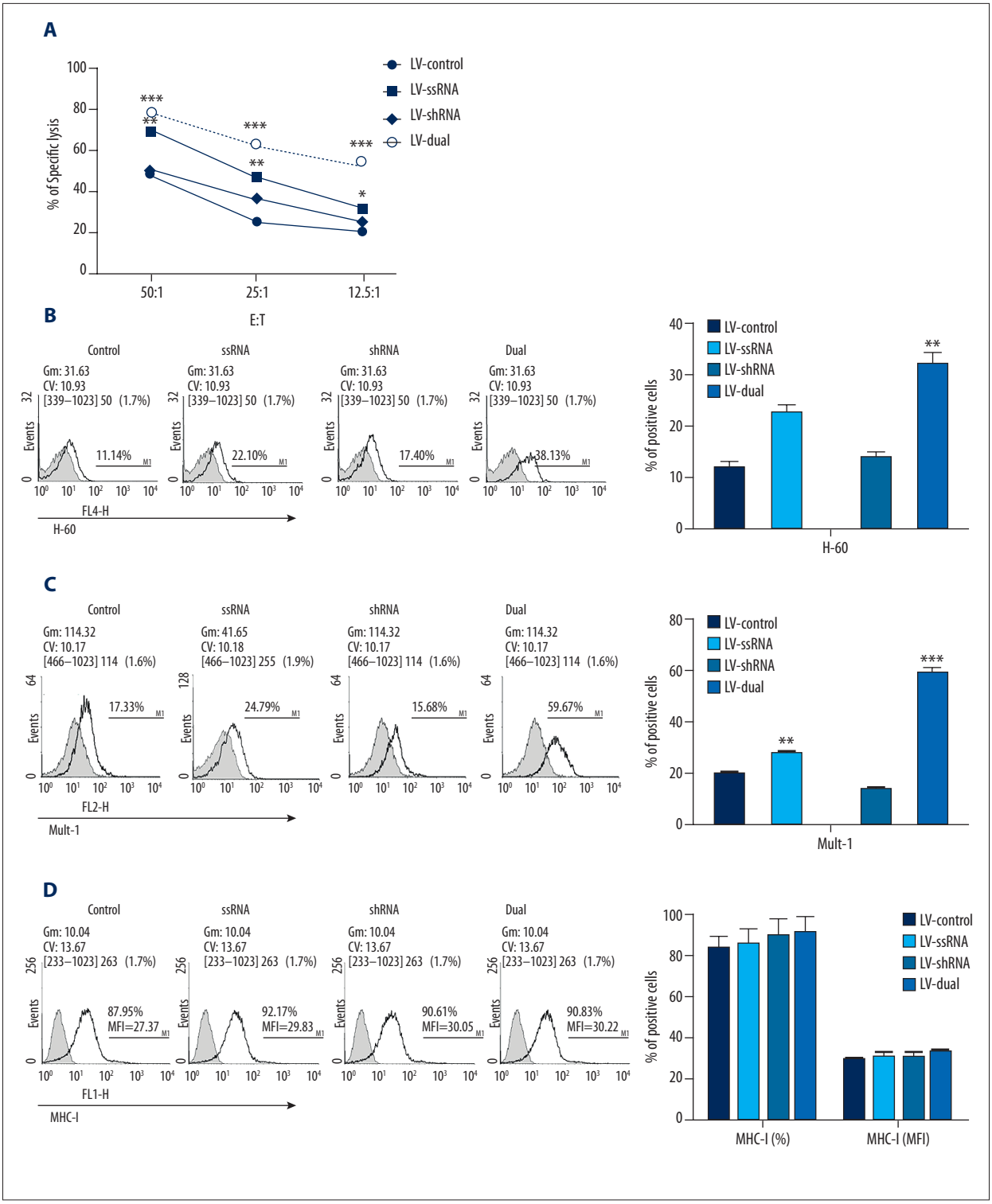

Figure 4. NK cells are involved in the suppression of gastric carcinoma mediated by the bi-functional vector in a NKG2D-dependent manner. (**A**) The cytotoxicity of NK cells was determined by measuring (using CFSE/7-AAD assay) the ability of splenic lymphocytes in treated mice to kill MFC cells. The expression of NKG2D ligands H-60 (**B**), MULT-1 (**C**), and MHC-I (**D**) on MFC cells was confirmed via FACS after transfection with indicated vectors for 24 hours. Data are representatives or means ±SD. * $P<0.05$ and ** $P<0.01$ compared with the LV-control group.

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Table 2. The percentages of NKG2D$^+$, NKG2A$^+$ or IFN-γ$^+$ NK cells (%, n=3).

	Control	ssRNA	shRNA	Dual
NKG2D	29.66±1.89	50.06±1.99**	30.93±1.84^{ns}	70.80±2.89***
NKG2A	41.50±1.84	21.10±1.08***	43.43±0.84^{ns}	14.02±0.63***
IFN-γ	16.94±1.09	37.13±1.08***	16.60±0.76^{ns}	47.89±2.10***

ns $P>0.05$; ** $P<0.01$;*** $P<0.001$, compared with the control group.

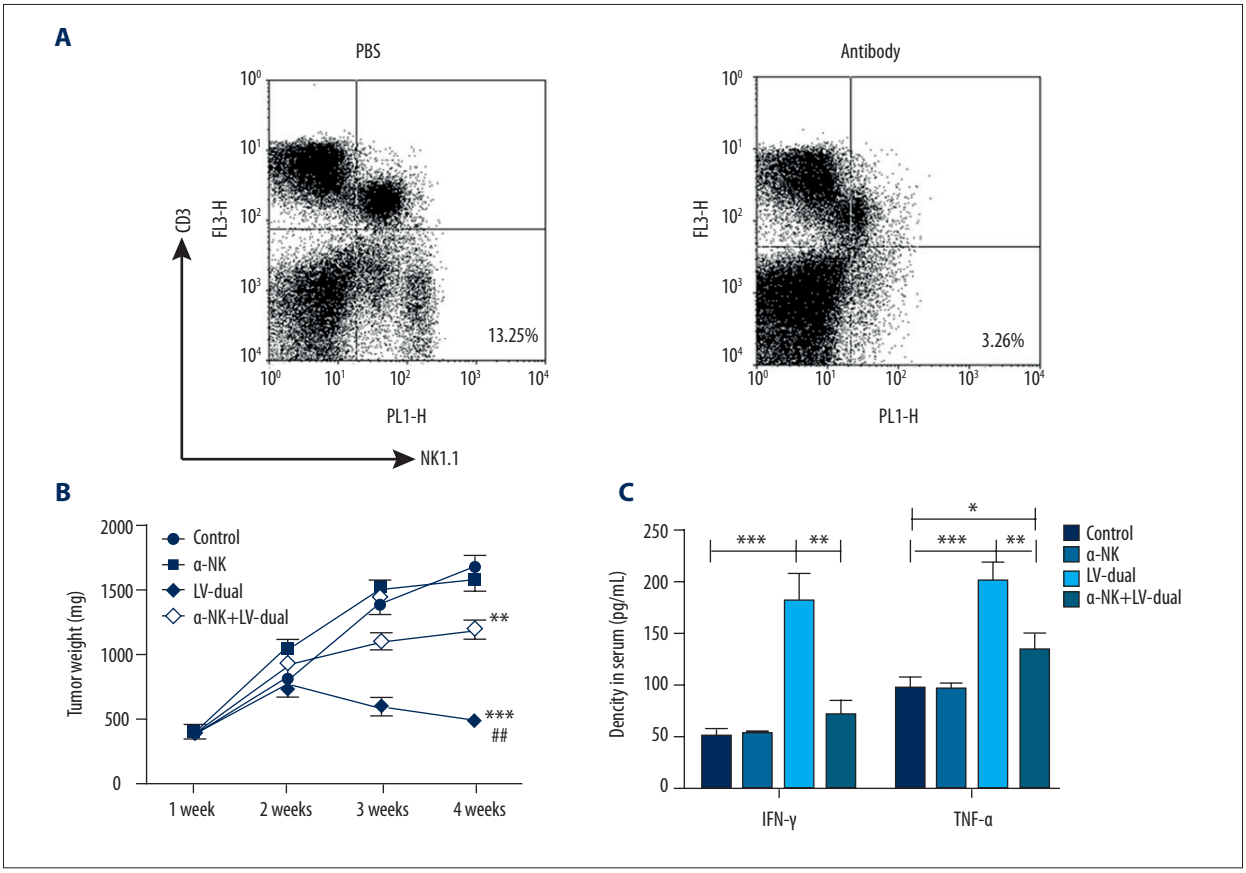

Figure 5. NK cells may be the major effector mediated by the bi-functional vector in this model. Depleting antibody was injected intraperitoneally into mice. Three days later, the removal efficiency was detected by FACS (**A**). Tumor-bearing mice were injected intraperitoneally with 1 mg of depleting antibodies for 3 days to deplete NK cells. The LV-dual vector (MOI=50) was administered intratumorally once a week for 2 weeks. Two weeks later, the growth of tumor was observed and tumor weights were calculated. Data are representative of 3 independent experiments with 5 mice per group. (**B, C**) Tumor volumes and the levels of IFN-α or TNF-α in the serum of mice that developed tumor after injection with antibody, LV-dual, or both of them were measured. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ compared with the control group. ## $P<0.01$ compared with the LV-dual group.

but also on the ability to evade immune recognition and suppress immune reactivity [6,8]. At the same time, the immunosuppressive factors produced by tumor cells can induce their surroundings into an immunosuppression state, which further promotes tumor growth and migration [27]. Therefore, a novel and effective therapeutic strategy will be one that combines silencing of oncogene expression and stimulation of antitumor immune responses.

Apoptosis is a hot topic to biology and medicine researchers. Many chemotherapy drugs and natural extracts are proven to have anti-cancer potential by activating apoptosis of cancer cells [28]. Bcl-2 is one of the Bcl-2 gene families that is related to the functional regulation of cell apoptosis and consists of anti-apoptotic (Bcl-2, Bcl-XL) and pro-apoptotic (Bcl-2 associated protein X, Bax; B-cell homologous antagonist/killer,

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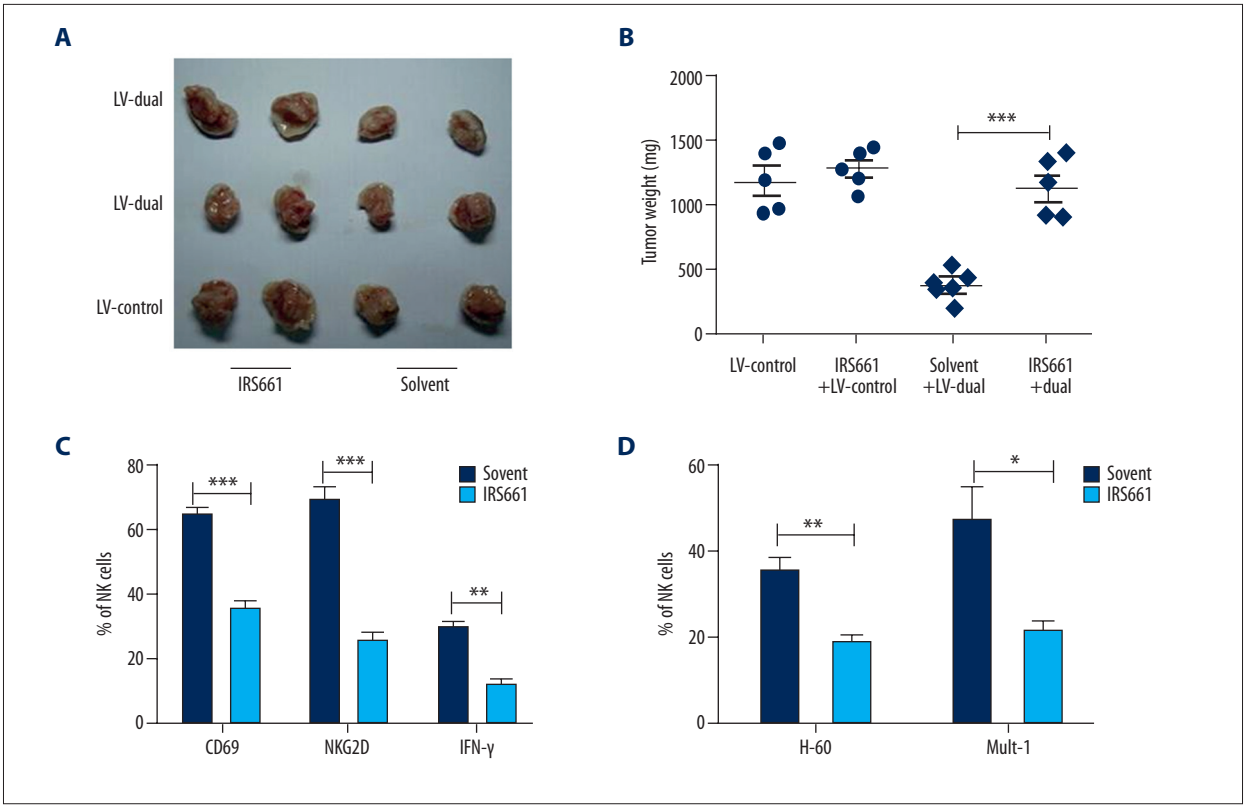

Figure 6. The TLR7 pathway is critical for NK cell activation and the inhibition of the growth of gastric carcinoma resulting from bi-functional vector therapy. (**A**) Tumor volumes were calculated. (**B**) Data are representative of 5 independent experiments with 3 mice per group. The percentages of CD69, NKG2D$^+$, or IFN-γ$^+$ NK cells (**C**) and expression of H-60 and MULT-1 on MFC cells (**D**) were determined via FACS. * $P<0.05$ and ** $P<0.01$ compared with the pSIREN transcription or solvent group.

bak) molecules [29,30]. Bcl-2 is mainly located in the mitochondrial membrane and can maintain normal cells and prevent cell death induced by harmful factors in the external environment. Bcl-2 could promote the expression of an apoptosis-resistant phenotype in cancer cells, and this appearance could make Bcl-2 an interesting therapeutic target for tumor-specific intervention strategies. Studies have shown that down-regulation of Bcl-2 could activate intrinsic apoptosis, eventually inducing cancer-cell apoptosis [31–36].

TLR7 is a important member that recognizes specific viral ssRNA sequences. Activation of the TLR7 pathway leads to secretion of type I IFN and inflammatory cytokines, which further prime both innate and adaptive immune responses [12,19]. Activation of the TLR7 pathway not only functionally activates both CD8$^+$ T cells and NK cells, but also blocks the suppressive function of regulatory T cells and myeloid-derived suppressor cells (MDSCs) [37,38]. Nevertheless, the TLR7 signaling of tumor patients is often suppressed, which suggests that priming TLR7 signaling is a potential therapeutic approach in cancer immunotherapy [39,40]. Based on the previous research, we constructed a dual-function vector containing both a Bcl-2-silencing shRNA and a TLR7-stimulating immunostimulatory ssRNA. This bi-functional vector not only promoted apoptosis of tumor cells by silencing Bcl-2, but also induced production of type I IFN by activating TLR7 signaling. This is the first bi-functional vector that inhibits the growth of gastric cancer by promoting tumor cell apoptosis via Bcl-2-silencing and stimulating TLR7-dependent anti-immune responses.

To determine the mechanism by which the bi-functional vector inhibits the growth of gastric tumor cells, we detected the change of NK, CD4$^+$ T, and CD8$^+$ T cells in mice that were subcutaneously challenged with MFC cells. We determined that both NK and CD4$^+$T cells are required for effective tumor suppression (Figure 3C), whereas NK cells showed enhanced cytotoxicity against hepatoma via augmented NKG2D-NKG2D ligand interaction (Figure 4). Surprisingly, the TLR7 inhibitor IRS661 completely abrogated vector-induced tumor regression (Figure 6). We assume that TLR7 activation is the first important issue for immune cell activation-induced tumor suppression. Most studies showed that the activation of NK cells through TLR7/8 recognition requires the help of APCs [23,41], so the exact mechanism of these effects needs to be further investigated.

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Conclusions

As is known, stimulation by ligands for TLRs induces a state of hypo-responsiveness (homo-tolerance) toward subsequent stimulation with the analogous ligands [42]. However, therapy with the dual-function vector provided long-lasting stimulation rather than short-lived immune activation by TLR7 agonists, and thus will avoid the TLR7 tolerance induced by repeated administration [43]. This study might represent a promising therapeutic approach in future therapy for gastric cancer or other solid tumors in which Bcl-2 is aberrantly expressed.

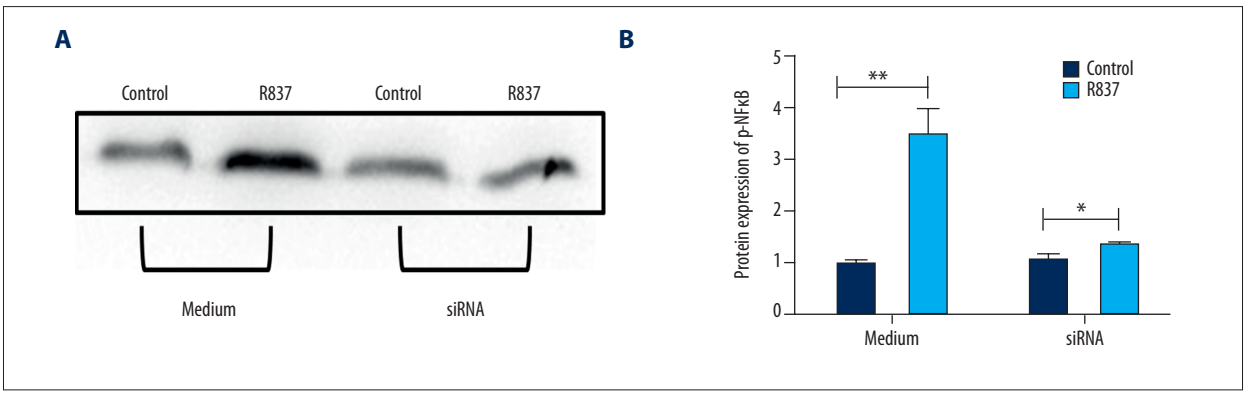
Competing interests

The author(s) declare that they have no competing interests.

Supplementary Materials

Supplementary Table 1. Sequences of primers used for PCR analysis.

Target genes	Sequences	Size (bp)
β-actin	Forward: 5'CTCCTTAATGTCACGCACGATTT3'	539
	Reverse: 5'GTGGGGCGCCCCAGGCACCA3'	
IFN-α	Forward: 5'ATGAAATATACAAGTTATATCTTGGCTTT3'	294
	Reverse: 5'GATGCTCTTCGACCTCGAAACAGCAT3'	
IFN-β	Forward: 5'ACGGCTTCCTGCTGGTGCT3'	101
	Reverse: 5'CGTCCTTAATGTCGCGGTGC3'	
Bcl-2	Forward: 5'CGACTTCGCCGAGATGTCCAGCCAG3'	364
	Reverse: 5'ACTTGTGGCCCAGATAGGCACCCAG3'	
TLR3	Forward: 5'AACCGCAAGCTTCCATACGACAG3'	109
	Reverse: 5'TGTCCGCCACGTATCCTGGCT3'	
TLR7	Forward: 5'CCCAAATAGGAGTGTATGCAGAGG3'	112
	Reverse: 5'GCATGTCTGCACAAGCAATGCTC3'	
TLR8	Forward: 5'GCAGGGGTAACGCACCGTCT3'	103
	Reverse: 5'GCTCACTTTCCTCTGTGAGGGTGT3'	



Supplementary Figure 1. MFC cells were stimulated with R837, agonist of TLR7, or medium after transfection with siRNA targeting human TLR7. Twenty-four hours later, the expression of p-NF-κB in MFC cells was detected by Western blotting. Data are shown as representatives (**A**) or means ±SD (**B**) from 3 independent experiments. ** $P<0.01$ compared with the control group.

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