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

Lanying Ma*
Mei Han*
Zumureti Keyoumu
Hua Wang
Saifuding Keyoumu

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

Corresponding Author:
Saifuding Keyoumu, e-mail: saifudingkeyoumu@sina.com

Source of support:
Departmental sources

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

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/900418
Background

Gastric cancer, from which about 10 percent of patients with cancer suffer every year worldwide, is one of the most common malignant tumors 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 reestablish the innate 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’s shRNA in pTZU6’1-shRNA was digested by HindIII and EcoRI, and inserted into pSIREN-ssRNA. pSIREN-control, ssRNA, 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% CO₂.

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. 1×10⁶ 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.
for 2 weeks. After another 2 weeks, the mice were sacrificed and the tumor volume was calculated by length × width²/2.

**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-tetrazoli-um 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% Na3, at 10⁶/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 CD3ε (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.
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 phosphatidylserine 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).
To inhibit the function of TLR7, IRS661 (5’-TGCTTGCAAGCTTGCAAGCA-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 pSIREN transcription group.

**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.
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 bi-functional 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 bi-functional 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.

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).

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±0.41ns  | 29.06±0.79***|
| CD4+T cells | 12.62±0.41  | 22.87±0.64***| 10.66±1.74ns | 29.28±1.22***|
| CD8+T cells | 22.50±2.46  | 22.20±0.87ns  | 23.14±0.94ns | 25.97±1.23ns |

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

The dual-function ssRNA-shRNA vector promotes the apoptosis and inhibits the proliferation of MFC cells.
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, 1×10⁶ 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 Supplemenetary 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-1 on 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,
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.
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,
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 an 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.
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’CTCCTTAATGTACACCGCATTT3’ | 539 |
|              | Reverse: 5’GTGGGCCCACCCACCCCA3’ |   |
| IFN-α        | Forward: 5’ATGAAATATACAAGTTATATCTTGGCTTT3’ | 294 |
|              | Reverse: 5’GATGCTCTTGCAGCTCGAAACAGCAT3’ |   |
| IFN-β        | Forward: 5’ACGGCTTCCTGCTGGTGCT3’ | 101 |
|              | Reverse: 5’CGTCCTTAATGTCGCGGTGC3’ |   |
| Bcl-2        | Forward: 5’CGACTCTGCCGCCAGATGTCCAGCCAG3’ | 364 |
|              | Reverse: 5’ACTTTGGCCAGATAAGGCCACCCAG3’ |   |
| TLR3         | Forward: 5’AACCACGGCCCTGACATCGGAGA3’ | 109 |
|              | Reverse: 5’TGTCGCCACGTATCCGTGCT3’ |   |
| TLR7         | Forward: 5’CCCAATAGGAGCTGGATGAGACAGG3’ | 112 |
|              | Reverse: 5’GCATGTCTGACAAGGCTGGCT3’ |   |
| TLR8         | Forward: 5’GCCAGGCTTAAAGGCACCCTGCT3’ | 103 |
|              | Reverse: 5’GCTCAGCTTCTGCTGAGGTGTC3’ |   |

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.
22. Mutlu P, Ural AU, Gunduz U: Differential oncogene-related gene expres
20. Liu M, Feng B, Shi Y et al: Protamine nanoparticles for improving shRNA-
19. Smits EL, Ponsaerts P, Berneman ZN, Van Tendeloo VF: The use of TLR7 and
18. Love AC, Schwartz I, Petzke MM:
15. Li Z, Zhang C, Zhou Z et al: Small intestinal intraepithelial lymphocytes
14. Guo Q, Lan P, Yu X et al: Immunotherapy for hepatoma using a dual-func
12. Alter G, Suscovich TJ, Teigen N et al: Single-stranded RNA derived from HIV-
10. Heil F, Hemmi H, Hochrein H et al: Species-specific recognition of single-
9. Kawai T, Akira S: The role of pattern-recognition receptors in innate immu
8. Cavallo F, De Giovanni C, Nanni P et al: 2011: The immune hallmarks of
7. Fu YX: New immune therapy targets tumor-associated environment: from
6. Hanahan D, Weinberg RA: Hallmarks of cancer: The next generation. Cell,
5. Bui JD, Schreiber RD: Cancer immunosurveillance, immunoediting and in
3. Cervantes A, Roda D, Tarazona N et al: Current questions for the treatment
2. Jemal A, Bray F, Center MM et al: Global cancer statistics. Cancer J Clin,
1. Guggenheim DE, Shah MA: Gastric cancer epidemiology and risk factors. J

References:

1. Guggenheim DE, Shah MA: Gastric cancer epidemiology and risk factors. J Surg Oncol, 2013; 107: 230–36
2. Jemal A, Bray F, Center MM et al: Global cancer statistics. Cancer J Clin, 2011; 61: 69–90
3. Cervantes A, Roda D, Tarazona N et al: Current questions for the treatment of advanced gastric cancer. Cancer Treat Rev, 2013; 39: 60–67
4. Jin X, Shi YI: Isobavachalcone induces the apoptosis of gastric cancer cells via inhibition of the Akt and Erk pathways. Exp Th Med, 2016; 11: 403–4
5. Bui JD, Schreiber RD: Cancer immunosurveillance, immuneediting and inflammation: Independent or interdependent processes? Curr Opin Immunol, 2007; 19: 203–8
6. Hanahan D, Weinberg RA: Hallmarks of cancer: The next generation. Cell, 2011; 144: 646–74
7. Fu YX: New immune therapy targets tumor-associated environment: from bone marrow to tumor site. Cell Mol Immunol, 2012; 9: 1–2
8. Cavallo F, De Giovanni C, Nanni P et al: The immune hallmarks of cancer. Cancer Immunol Immunother, 2011; 60: 319–26
9. Kawai T, Akira S: The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors. Nat Immunol, 2010; 11: 373–84
10. Heil F, Hemmi H, Hochrein H et al: Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science, 2004; 303: 1526–29
11. Cervantes I, Weinerman B, Basole C, Salazar JC: TLR8: The forgotten relative reinvindicated. Cell Mol Immunol, 2012; 9: 434–38
12. Alter G, Suscovich T, Teigen N et al: Single-stranded RNA derived from HIV-1 serves as a potent activator of NK cells. J Immunol, 2007; 178: 7658–66
13. Scholch S, Rauber C, Tietz A et al: Radiotherapy combined with TLR7/8 activation induces strong immune responses against gastrointestinal tumors. Oncotarget, 2015; 6: 4663–76
14. Guo Q, Lan P, Yu X et al: Immunotheraphy for hepatoma using a dual-function vector with both immunostimulatory and pim-3-silencing effects. Mol Cancer Ther, 2014; 13: 1503–13
15. Li Z, Zhang C, Zhou Z et al: Small intestinal intraepithelial lymphocytes express TLR7 and T cell receptor gammaddelta are involved in bacterial clearance during Salmonella enterica serovar Typhimurium infection. Infect Immun, 2012; 80: 565–74
16. Xu Q, Fan L, Zhong T et al: The nematocyst venom of Chrysaora helvola Brandt leads to apoptosis-like cell death accompanied by uncoupling of oxidative phosphorylation. Toxicol, 2016; 110: 74–78
17. Barrat FT, Meeker T, Gregorio J et al: Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. J Exp Med, 2005; 202: 1131–39
18. Love AC, Schwartz I, Petzke MM: Barrella burgdorferi RNA induces type I and III interferons via Toll-like receptor 7 and contributes to production of NF-kappaB-dependent cytokines. Infect Immun, 2014; 82: 2405–16
19. Smits EL, Ponsaerts P, Bermenon NZ, Van Tendeloo VF: The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy. Oncologist, 2008; 13: 859–75
20. Liu M, Fang B, Shi Y et al: Prolatmine nanoparticles for improving shRNA-mediated anti-cancer effects. Nanoscale Res Lett, 2015; 10: 134
21. Yokota J: Tumor progression and metastasis. Carcinogenesis, 2000; 21: 497–503
22. Mutlu P, Ural AU, Gunduz U: Differential oncogene-related gene expressions in myeloma cells resistant to prednisone and vincristine. Biomed Pharmacother, 2012; 66: 506–11
23. Ma Y, Gai Y, Yan J et al: Puerarin attenuates anoxia/reoxygenation injury through enhancing Bcl-2 associated athanogene 3 expression, a modulator of apoptosis and autophagy. Med Sci Monit, 2016; 22: 977–83
24. Lin B, Huang D, Yu C et al: MicroRNA-184 regulates doxorubicin resistance in osteosarcoma cells by targeting BCL2L1. Med Sci Monit, 2016; 22: 1761–65
25. Zhou Z, Yu X, Zhang J et al: TLR7/8 agonists promote NK-DC cross-talk to enhance NK cell anti-tumor effects in hepatocellular carcinoma. Cancer Lett, 2015; 369: 298–306
26. Diebold SS, Kaisho T, Hemmi H et al: Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science, 2004; 303: 1529–31
27. Swartz MA, Iida N, Roberts EW et al: TLR8: Emerging roles in cancer therapy. Cancer Res, 2012; 72: 2473–80
28. Haque A, Rahman MA, Fuchs JR et al: FLLL12 induces apoptosis in lung cancer cells through a p53/p73-independent but death receptor 5-dependent pathway. Cancer Lett, 2015; 363: 166–75
29. Sun B, Xu M: Matrine inhibits the migratory and invasive properties of nasopharyngeal carcinoma cells. Mol Med Rep, 2015; 11: 4158–64
30. Bosowski A, Czarocka B, Bardadin K et al: Expression of Bcl-2 family proteins in thyrocytes from young patients with immune and nonimmune thyroid diseases. Horm Res, 2008; 70: 155–64
31. Zhang H, Li Y, Huang Q et al: MIR-144a promotes apoptosis by targeting Bcl-2 in colorectal cancer. Cell Death Differ, 2011; 18: 1702–10
32. Jagni HV, Josyula VR, Hariparupu RC et al: Nanoformulation of siRNA silencing Bcl-2 gene and its implication in cancer therapy. Arzneimittelforschung, 2011; 61: 577–86
33. Li H, Li X, Bai M et al: Matrine inhibited proliferation and increased apoptosis in human breast cancer MCF-7 cells via upregulation of Bax and down-regulation of Bcl-2. J Clin Exp Pathol, 2015; 8: 14793–99
34. Bi D, Yang M, Zhao X, Huang S: Effect of cridium lactone on serum mutant p53 and BCL-2/BAX expression in human prostate cancer cells PC-3 tumor-bearing BALB/C nude mouse model. Med Sci Monit, 2015; 21: 2421–27
35. Yu DF, Wu FR, Liu Y et al: Bcl-2 gene silence enhances the sensitivity to 5-Flourouracil in gastric adenocarcinoma cells. Biomed Pharmacother, 2015; 67: 615–19
36. Wei W, Wang Y, Yu X et al: Expression of TP53, BCL-2, and VEGFA genes in esophageus carcinoma and its biological significance. Med Sci Monit, 2015; 21: 3016–22
37. Anz D, Koelzer VH, Moder S et al: Immunostimulatory RNA blocks suppression by regulatory T cells. J Immunol, 2010; 184: 939–46
38. Bourquin C, Schmidt L, Lanz AL et al: Immunostimulatory RNA oligonucleotides induce an effective antitumoral NK cell response through the TLR7. J Immunol, 2009; 183: 6078–86
39. Lin KJ, Lin TM, Wang CH et al: Down-regulation of Toll-like receptor 7 expression in hepatitis-virus-related human hepatocellular carcinoma. Hum Pathol, 2013; 44: 334–41
40. Khairdin N, Gantier MP, Blake SJ et al: siRNA-induced immunostimulation through TLR7 promotes antitumoral activity against HPV-driven tumors in vivo. Hum Cell Biol, 2012; 90: 187–96
41. Berger M, Abrasser A, Kim S et al: TLR8-driven IL-12-dependent reciprocal and synergistic activation of NK cells and monocytes by immunostimulatory RNA. J Immunother, 2009; 32: 262–71
42. Tsukada K, Kitazawa T, Fukushima A et al: Macrophage tolerance induced venting TLR tolerance. Cancer Res, 2011; 71: 5125–33