Cancer-Targeted BikDD Gene Therapy Elicits Protective Anti-Tumor Immunity Against Lung Cancer

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Abbreviations: Bik, BCL-2 intereacting killer; BikDD, mutant form of Bik; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; IAP, inhibitor of apoptosis; IFN-γ, interferon gamma; Luc, luciferase; SV, Survivin VISA; VISA, VP16-GAL4-WPRE integrated systemic amplifier

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ABSTRACT

Targeted cancer-specific gene therapy is a promising strategy for treating metastatic lung cancer, which is a leading cause of lung cancer-related deaths. Previously, we developed a cancer-targeted gene therapy expression system with high tumor specificity and strong activity that selectively induced lung cancer cell killing without affecting normal cells in immunocompromised mice. Here, we found this cancer-targeted gene therapy, SV-BikDD composed of the survivin promoter in the VISA system to drive the apoptotic gene BikDD not only caused cytotoxic effects in cancer cells but also elicited a cancer-specific cytotoxic T lymphocyte response to synergistically increase the therapeutic effect and further develop an effective systemic antitumoral immunity against re-challenges of tumorigenic dose of parental tumor cells inoculated at distant sites in immunocompetent mice. In addition, this cancer targeted gene therapy does not elicit an immune response against normal tissues, but CMV-BikDD treatment does. The therapeutic vector could also induce proinflammatory cytokines to activate innate immunity and provide some benefits in anti-tumor gene therapy. Thus, the current study provides a promising strategy with benefit of antitumoral immune response worthy of in clinical trials for treating lung cancer via cancer-targeted gene therapy.

INTRODUCTION

Lung cancer is the most common cause of cancer deaths worldwide, and the metastatic form of the disease is a major cause leading to mortality (1). Targeted gene therapy is considered as a novel strategy for treating metastasized cancer or therapy refractory tumors owing to the benefit of tumor-specific expression of therapeutic genes including an apoptosis-inducing gene to kill the tumor cells by a cancer-specific promoter (2). The success of this strategy also depends on suitable vectors that can efficiently deliver a therapeutic gene into target cells with minimal toxicity. However, most of the identified cancer-specific promoters that do exhibit a high level of cancer specificity are still much weaker than the non-specific strong cytomegalovirus (CMV) promoter (3). To overcome this issue, a promoter amplification system called VP16-GAL4-WPRE integrated systemic amplifier (VISA) was developed to amplify the cancer-specific promoter activity and the activity in some cancer cell lines are even able to reach a level comparable to that of CMV in cancer cells yet remains inactive in normal tissues (4).

Previously, we demonstrated that a lung cancer-specific promoter, survivin, is highly active in lung cancer cells but not in other cell types, including immortalized normal cell lines (5). Briefly, survivin is a member of the inhibitor of apoptosis (IAP) gene family that mediates several anti-apoptotic functions (6, 7), and its expression is associated with disease progression and poor prognosis in many cancer types (8). Moreover, survivin has also been

implicated in the development of resistance to apoptosis-inducing agents (9) and promotion of metastasis (10). We integrated the survivin promoter into the VISA system to selectively enhance the expression of a therapeutic gene, BikDD, a mutant form of the pro-apoptotic Bcl2 interacting killer (Bik), to generate survivin-VISA-BikDD (SV-BikDD) and demonstrated that systemic treatment with SV-BikDD DNA in liposome complexes controlled tumor growth and significantly prolonged survival in immunocompromised mice with metastatic human lung cancer and produced virtually no toxicity compared with the CMV promoter vector (5).

Since immune response is also considered critical for determining the success or failure of gene therapy clinical trials, we further examined the role of immune response elicited under SV-BikDD gene therapy in immunocompetent mice with lung cancer. Here, we show that SV-BikDD cancer-targeted gene therapy not only stimulated innate immunity to provide advantages in anti-tumor treatment but also elicited a cytotoxic T lymphocyte (CTL) response to increase the therapeutic effect and protect against highly aggressive syngeneic TC-1 lung cancer when challenged again in animals, suggesting that SV-BikDD gene therapy can elicit cancer-specific immunity and confer protection against lung cancer. Thus, the current study provides a promising cancer-targeted gene therapy worthy of further development in clinical trials for treating lung cancer, especially the metastatic form of the disease.

MATERIALS AND METHODS

Cell Line. The mouse lung cancer cell line TC-1, which was obtained by cotransformation of HPV-16 E6/E7 and activated ras oncogene to primary mouse lung epithelial cells of C57BL/6 was kindly provided by T. C. Wu (11) in 1997 and established a working cell bank for tumor model in 2008. Experiments were performed within 6 months of receiving the cell lines from the established cell bank. The cancer cells were authenticated by their ability to form lung cancer tumors in C57BL/6 mice. The cells were tested for mycoplasma-free by PCR methods before this study. TC-1 cells were grown in RPMI medium 1640 supplemented with 10% FBS and 0.4 μg/ml G418.

Evaluation of Promoter Activity and Cytotoxicity. All the plasmids used in this study were constructed as previously described (5). To test promoter activity, we used dual-luciferase reporter assay (Promega, Madison, WI) to normalize transfection efficiency according to the manufacturer's instructions. Transfection of the promoter reporter plasmid was carried out with pRL-TK as an internal control for transfection efficiency. The dual-luciferase ratio was presented as the luciferase activity of the tested plasmids divided by the luciferase activity of pRL-TK. For determining cytotoxicity, pRL-TK was also used as a reporter gene in all experiments.

Lung Cancer Animal Model. Female C57BL/6 mice purchased from BioLASCO (Taiwan), at 6-8 weeks of age, were used for syngeneic lung cancer animal model. Mice were maintained in a specific pathogen-free environment in compliance with institutional policy and approved by the Laboratory Animal Care and Use Committee of the China Medical University (Protocol # 98-199-N). Each group has over 5 mice and each experiment was repeated. TC-1 murine lung cancer cells (1×10^6) were inoculated in C57BL/6 mice through intravenous injection to establish an experimental metastatic lung cancer animal model (12). Therapeutic plasmids were purified with an Endo-free Mega Prep Kit (Qiagen, Valencia, CA) according to the commercial protocol. Plasmid DNA was dissolved in endotoxin-free TE buffer, and the amount of endotoxin was determined to be less than 10 endotoxin units/mg of DNA by a chromogenic Limulus amoebocyte clotting assay (QCL-1000 kit, BioWhittaker, Walkersville, MD). HLDC (Hung Lab-modified DOTAP:Cholesterol) was produced in our laboratory according to Dr. Nancy Templeton's protocol (13). Plasmid DNA/HLDC complexes were prepared as previously described (3). Briefly, HLDC (20 mM) stock solution and stock DNA solution were diluted in 5% dextrose in water (D5W) and mixed in equal volumes to make a final solution of 4 mM HLDC and 50 μg DNA in 100 μl of solution (liposome:DNA ratio 1:2.6). We used this concentration of DNA/HLDC complexes for detection of promoter specificity and acute toxicity in animals. The DNA/HLDC complexes were diluted in D5W to prepare 100 μl of DNA:HLDC complexes containing 25 μg of DNA in 2 mM HLDC for each

treatment in a mouse. We began treating on day 7 after TC-1 cancer cells inoculation via intravenous injection of 100 μl of DNA:HLDC complexes containing 25 μg of DNA which were administered to mice twice per week for a total six treatments in the experimental metastatic lung cancer animal model. The lungs of mice were fixed in Bouin's solution and the lung tumor nodules were counted under stereomicroscope (14). For the *ex vivo* experiment, TC1 cells were transiently transfected with indicated plasmids and two days later, equal number of transfected cells was inoculated subcutaneously into 6-week old C57BL/6 mice. Tumor volume was monitored after inoculation of tumor cells. Subcutaneous TC1 cells and normal lung tissue were dissected and total protein lysate were extracted. Survivin protein was detected with anti-human survivin antibody (R&D System, Minneapolis, MN), and EF1α was used as total protein control with anti-EF1α antibody (Millipore, Temecula, CA) by Western blot analysis.

Bioluminescent Imaging and Quantification. To detect promoter specificity, promoter constructs were intravenously delivered to mice by liposome, and the luciferase activity was imaged by IVIS Imaging system (Xenogen, Alameda, CA) two days after injection. Mice were anesthetized with a mixture of oxygen and isoflurane and intraperitoneally injected with 100 μl of D-luciferin (Xenogen; 30 mg/ml in PBS). Mice were imaged 10 min after luciferin injection, and signals were analyzed using the Living Image software.

Immunohistochemical Staining. The lung tumor and other tissues dissected from mice were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Five-micrometer sections were cut and stained with H&E. Immunostaining for firefly luciferase was performed as previously described (4) by using the goat anti-firefly luciferase antibody (Abcam, Cambridge, MA), horseradish peroxidase-conjugated avidin biotin complex (ABC) from Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA), and AEC chromogen (Vector Laboratories). The sections were counterstained with hematoxylin and mounted. The intensity score was judged by a pathologist.

Cytometric Bead Array Immunoassay Analysis. The proinflammatory cytokines in sera were quantified using a mouse inflammation cytometric bead array (CBA) kit (BD Biosciences, San Jose, CA) following the manufacturer's instructions. In brief, serum samples were diluted to 50 μl with Assay Diluents and added into a mixture of six different antibody-coated beads. After addition of 50 μl of a mixture of PE-conjugated antibodies against the cytokines, the mixture was incubated for 2 hr at the room temperature. The mixture was then washed with Wash Buffer, transferred to a 96-well microtiter plate and analyzed by BD FACSArray Bioanalyzer (BD Biosciences). The concentration of IL-6, IL-10, MCP-1, INF-γ, TNF-α and IL-12p70 was determined using FACSArray software.

ELISpot Assay. The mouse IFN-γ ELISpot assay was performed as previously described (15). In brief, 2 x 10^5 splenocytes were incubated with 1 x 10^5 of irradiated (30 Gy) TC-1 cells or synthetic HPV16 E7 peptide (49–57, RAHYNIVTF)(16) or synthetic survivin peptide (mouse survivin 57-64, CFFCFKEL) (17) in anti-IFN-γ coated PVDF plate for 48 hr. After incubation, the cells were removed, and biotinylated anti-IFN-γ antibody (eBioscience, San Diego, CA) was then added to each well. The plates were again incubated at 37^{o}C for 2 hr. After 1-hr incubation at room temperature with an avidin-HRP complex reagent (eBioscience), the plates were washed, and then a 100 μl aliquot of 3-amine-9-ethyl carbazole (ACE; Sigma-Aldrich, St Louis, MO) staining solution was added to each well to develop the spots. The reaction was stopped after 4-6 min by placing the plate under tap water. The spots were then counted using an ELISpot reader (Cellular Technology, Shaker Heights, OH).

Cytotoxic T lymphocyte Assay. Cytolytic activity was measured by standard 4-h chromium release assay as previously described (15). In brief, every two weeks after the last treatment, erythrocyte-depleted splenocytes (1 x 10^6 cells/mL) were cultured *in vitro* with irradiated TC-1 (30 Gy) (1 x 10^5) and 10 U/mL of recombinant human IL-2 in 24-well plates for 5 days as effector cells. The TC-1 cells (5 x 10^5) were then pulsed with 100 μCi of ^{51}Cr ($Na_2{}^{51}CrO_4$; PerkinElmer, Waltham, MA, USA) at 37^{o}C for 1 hr to serve as target cells. The irradiated

TC-1 cells-stimulated splenocytes were mixed with labeled target cells at various effector-to-target ratios as indicated. After 4-hr incubation at 37^{o}C, the radioactivity of the supernatant was measured using a gamma counter (PerkinElmer). The percentage of specific lysis was calculated using the following equation: 100 x [(experimental release – spontaneous release) / (maximal release – spontaneous release)].

Preparation of normal lung cells from mouse. Isolation of normal lung cells from mice was performed as previously described (18, 19). Lung tissue was separated and flushed with 10 mL of 1 % P/S in PBS. The separated lung tissues were cut into 1-mm^3 pieces and incubated with 0.2 % of trypsin, 2 mg/mL of collagenase (Sigma) in pretreated medium (PM) for 30 min with 100 rpm rotation. The tissues were then placed in PM with 1000 U DNase I at room temperature for 5 min. The isolated cells were then filtered through 70 μm and 40 μm mesh (BD Bioscience). The filtrates were centrifuged and the pellet was re-suspended in RBC lysis buffer for 2 min. The cell pellet was added into anti-CD45 (45 μg/dish) and anti-CD16/32 (16.5 μg/dish) coated-dish at 37 ^{o}C for 2 hrs. The non-attached cells were collected and suspended with culture medium to 5 x 10^5 cells/well (6-well plate) for 2 days. These cells were irradiated with 2.5 Gy as a single dose from an RS2000 x-ray irradiator (Rad Source Technologies, Suwanee, GA). The irradiated normal lung cells were used to stimulate splenocytes from mice that has been treated with gene therapy.

Distant Site Tumor Challenge in Treated Animals. One week after the final treatment course, animals in all treatment groups were challenged with tumorigenic dose of the TC-1 parental tumor cells by injecting 5 x 10^5 cells subcutaneously at a single site on the right flank of the animals. The presence of subcutaneous tumors in animals was observed and calculated by using the formula V (mm^3) = a x $b^2/2$, where a is the largest dimension and b is the perpendicular diameter. The results were analyzed statistically by the Fisher exact test.

RESULTS

Cancer-specific targeting of survivin promoter to TC-1 cells *in vitro* and *in vivo*

To assess whether the host immune system plays some roles in our cancer-targeted gene therapy, a mouse lung cancer cell line, TC-1, was used to establish syngeneic lung cancer animal model in immunocompetent mice (11). Using a series of expression vectors that were previously constructed (5) to drive expression of luciferase (Fig. 1a; top), we found that survivin combined with VISA system consistently enhanced the survivin promoter activity in TC-1 lung cancer cells. Specifically, survivin-VISA exhibited a 17-fold increase in promoter activity compared with the survivin promoter alone, but the increase in activity was only about half of the CMV promoter (Fig.1a; bottom). To further examine whether the activity and specificity of survivin-VISA (SV) is still retained *in vivo*, luciferase expression constructs

driven by CMV and SV (Fig. 1a) complexed with liposomes were administered to tumor-free (without cancer cell inoculation) and tumor-bearing (with cancer cell inoculation) C57BL/6 mice via intravenous injection (Fig. 1b). Bioluminescent imaging revealed a strong signal in the thoracic area of mice treated with CMV-Luc construct in both tumor-free and tumor-bearing mice, but a low signal was observed in mice treated with SV-Luc construct (Fig. 1b, upper panel). There was no signal observed from other areas of the body of the mice. To precisely monitor the source of signal, these mice were sacrificed immediately after imaging, and their organs were dissected for *ex vivo* imaging (Fig. 1b, lower panel). Quantified photon signal showed high values in CMV-Luc- but not SV-Luc- treated groups in the lungs of tumor-free and tumor-bearing mice. Consistent with *ex vivo* imaging, immunohistochemical analysis also showed strong luciferase expression in both normal lung and tumor tissues in the CMV-Luc-injected group with an intensity score of 1+ and 3+, respectively, while in the SV-Luc-injected group the signal was only detectable in tumor tissues with intensity score 2+, but with intensity score 0 in normal lung tissue by a pathologist's judgment (Fig. 1c). The IHC results demonstrate a positive signal in both normal and tumor tissue in the CMV-Luc-injected group (Fig. 1c), thus it caused both strong bioluminescence imaging in tumor-free and tumor-bearing mice (Fig. 1b). However, there was only a strong signal in tumor tissue and no signal in normal tissue in SV-Luc-injected group (Fig. 1c). Since the luciferase signal was only located in the tumor nodules that were in

a small part of the lungs and not all, the overall bioluminescence imaging in lungs reflect slightly higher in SV-Luc injected tumor bearing mouse compared to tumor-free mouse, but is incomparable to CMV-Luc-injected group. Together, consistent with our previous study (5), the *in vivo* data here also demonstrated that survivin-VISA-vector-driven transgene expression is more lung cancer specific than CMV vector and appears to be suitable for gene therapy in syngeneic lung cancer animal model in immunocompetent mice.

SV-BikDD treatment inhibited mouse lung cancer cells growth *in vitro* and *ex vivo*

As mentioned above, we have previously designed a therapeutic vector that expresses a potent inducer of apoptosis, BikDD, for lung cancer. Bik mutants, BikDD in which threonine 33 and/or serine 35 were changed to aspartic acid to mimic the phosphorylation at these two residues, enhanced their binding affinity with the antiapoptotic proteins Bcl-XL and Bcl-2 and were more potent than wild-type Bik in inducing apoptosis and inhibiting cell proliferation in various human cancer cells (20). BikDD expression increased the protein level of cleaved caspase-3, which is one of the key executioners of apoptosis by Western Blot (Supplementary Figure 1). To determine the cytotoxicity effect of the therapeutic plasmids in mouse lung cancer cells, three treatments, SV (vector control without the BikDD gene), CMV-BikDD, and SV-BikDD were used to determine therapeutic effect *in vitro* and *ex vivo*. We first co-transfected TC-1 cells transiently with these plasmids plus pRL-TK (internal

control) and measured the relative luciferase activity with the control vector set at 100%. *In vitro* cytotoxicity assay showed that SV-BikDD nearly killed more than 80% of TC-1 cells as indicated by the loss of luciferase activity compared with the control (Fig. 2a). We then subcutaneously inoculated TC-1 cells that were transiently transfected with therapeutic plasmids *in vitro* and measured the tumor volume in C57BL/6 mice (Fig. 2b). Tumors removed from mice in each group on day 33 clearly demonstrated that pretreatment of TC-1 cells with SV-BikDD significantly reduced tumor growth in mice compared with untreated cells (Fig. 2b, lower panel). Interestingly, although *in vitro* cytotoxicity assay (Fig. 2a) showed that CMV-BikDD exhibited better cell killing effect than SV-BikDD, the reverse was observed in the *ex vivo* experiment in which pretreatment of SV-BikDD showed stronger tumor growth suppression than CMV-BikDD. In addition, mice that received cells pretreated with SV also showed significant initial tumor suppression with smaller tumor size than untreated group before day 25 post tumor inoculation ($P < 0.01$), suggesting that our VISA cassette might play a role in stimulating host immune response which in turn inhibits tumor growth and may partially explain the differential killing effect we observed between SV-BikDD and CMV-BikDD *in vitro* and *ex vivo*.

SV-BikDD inhibited tumor growth and prolonged mice survival time in an experimental metastatic lung cancer animal model

To evaluate the antitumor effect of SV-BikDD treatment in mouse suffering from metastatic lung cancer, TC-1 cells were intravenously injected to mimic metastatic circulating cancer cells in immunocompetent mice which were then given treatment by systemic delivery of liposome:DNA complexes one week later for a total of six treatments. On day 35 after tumor inoculation, fewer lung tumor nodules were found in mice from SV, CMV-BikDD, and SV-BikDD treatment groups compared with uncountable nodules in the lungs of PBS-treated mice ($P < 0.01$) (Fig. 3a). While SV, CMV-BikDD, and SV-BikDD treatment all prolonged survival time compared with PBS treatment ($P < 0.01$), mice from CMV-BikDD- and SV-BikDD-treated groups still had significantly longer survival time than SV group ($P < 0.05$) (Fig. 3b). Overall, mice that were administered SV-BikDD maintained a 20% survival rate over 17 months but not in the other treatment groups.

Targeted gene therapy elicits cancer-specific immune response

Next, we determined whether any cancer-specific immunity was elicited by our therapeutic plasmids treatments *in vivo*. After a total of six treatments, IFN-γ ELISpot assays were performed to detect T cell response against TC-1 cells in mice bearing circulating tumor cells. Two weeks after the final treatment, spleen cells were harvested and re-stimulated with irradiated TC-1 cells *in vitro* to detect the IFN-γ secretion. As shown in Fig. 4a, mice that were treated with SV, CMV-BikDD and SV-BikDD induced an increased number of

IFN-γ-secreting cells (111 ± 34, 205 ± 37, and 242 ± 13 spots per 1 x 10^6 cells, respectively) compared with the naive group (59 ± 10 spots per 1 x 10^6 cells), indicating all of the treated plasmids can stimulate T cell activation against TC-1 cells. Since the oncogenic E7 protein is overexpressed in TC-1 cancer cells, IFN-γ ELISPOT assays were performed to detect T cell response against E7 peptides (Fig. 4b). The results in T cell response against E7 peptides demonstrated similar pattern as against irradiated TC-1 cells. Next, we determined whether CTL response against TC-1 cells in the splenocytes obtained from mice with different treatments was elicited in two or four weeks after last treatment by chromium-51 (^{51}Cr) release assay (Fig. 4c). In the first detection point at two weeks after last treatment, we observed a significant increase in specific CTL activity directed against TC-1 cells from mice in the SV and SV-BikDD groups, but only a slight increase in the CMV-BikDD group compared with those from naive mice. However, in the second detection point at four weeks after last treatment, all of the treatment groups showed significantly increased CTL activity, suggesting that CTL activity against TC-1 cells in CMV-BikDD group was elicited more slowly than SV-BikDD group. These results indicate that both SV and SV-BikDD vectors induced tumor-specific cytotoxic activities faster than CMV-BikDD.

Since survivin protein is overexpressed in TC-1 tumor but not in normal lung tissue, it can serve as cancer-specific antigen (Fig. 4d, Western blot). A study recently identified a survivin-derived H-2K^b-restricted CTL epitopes that could inhibit tumor growth in mouse

model (17). We used the survivin-derived peptides as stimulator to activate survivin-specific T cell population in the splenocytes in ELISpot assay. The result showed that survivin peptides induced a higher number of IFN-γ secreting T cells in SV-BikDD-treated mice compared to naive mice (Fig. 4d). Together, the results from both IFN-γ ELISpot assay from TC-1 cells and survivin peptide stimulation demonstrated that SV-BikDD treatment could induce stronger cancer-specific T cell immunity than CMV-BikDD treatment.

SV-BikDD does not elicit T cell response against normal cells, but elicit anti-tumor immunity to protect secondary tumor challenge

Previous study indicated that CMV-BikDD treatment caused cytotoxicity in normal tissue such as lung and liver in BALB/c mice as measured by TUNEL assay, but not in SV-BikDD treatment (5). Here, we further performed an acute liver toxicity study in detection of serum level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and showed significantly increased cytotoxicity in C57BL6 mice treated with CMV-BikDD ($P = 0.03$ and 0.04, respectively), but not with SV-BikDD (Supplementary Figure 2). We next investigated whether normal tissue damage from CMV-BikDD treatment stimulates T cells response against normal tissues. To address this, two types of normal cells, splenocytes and normal lung cells, were used as stimulator to evaluate the normal cells-specific T cell responses in mice treated with the therapeutic plasmids. As shown in Fig.

5a, CMV-BikDD induced the highest number of IFN-γ secreting T cells against normal splenocytes compared with the naive, SV or SV-BikDD groups. In a parallel experiment using normal lung cells, we showed that CMV-BikDD also induced a substantial increase in IFN-γ secreting T cells against these normal cells (Fig. 5b). The results indicate that CMV-BikDD treatment induced stronger immunity against normal cells than other treatments.

The ability of the immune system to respond more strongly to the exposure of the same antigen is a hallmark of T cell-mediated immunity. To test whether the anti-tumoral immunity in the treated animals provides any protection against secondary tumor challenge, TC-1 cells were inoculated again subcutaneously after the treatment course in mice bearing circulating tumor cells on day 35 (Fig. 5c). All animals in the naive group, which never received any tumor injection prior to implantation of subcutaneous tumors at the challenge sites, demonstrated a tumorigenic dose of 5×10^5 TC-1 cells. In CMV-BikDD and SV-BikDD treatment groups, the percentage of tumor-free mice was over 50%, which was significantly higher than that of the naive group on day 50 after first cancer cells inoculation. Moreover, the tumor volume measured at the challenge site in mice was smaller in CMV-BikDD and SV-BikDD treatment groups compared with the naive and SV groups (Fig. 5d). These results indicate that mice gained protection against secondary distant site tumor challenge after administering cancer-targeted gene therapy.

Induction of innate immunity by gene therapy

To investigate whether the therapeutic plasmids containing CpG motifs stimulate innate immunity in host and mediate tumor remission, we analyzed the levels of proinflammatory cytokines from the serum after intravenous administration of plasmids in immunocompetent mice. The frequency of CpG motifs was similar in SV, CMV-BikDD, and SV-BikDD with 19, 17, and 19 in each plasmid, respectively (Fig. 6a). The proinflammatory cytokines, IL-6, MCP1, IFN-γ, TNFα, and IL-12 were elicited quickly 8 hours after DNA inoculation for all three plasmids, but these cytokines decreased to basal level after 24 hours (Fig. 6b). In contrast, IL-10 level did not change significantly between 8 and 24 hours after injection. These results suggest that all three plasmids could elicit proinflammatory cytokines to activate innate immunity and provide some benefits in anti-tumor gene therapy.

DISCUSSION

Here, we report that the cancer-targeted SV-BikDD system, which selectively enhanced BikDD expression to induce apoptosis in lung cancer cells, also elicited host anti-tumor immunity to synergistically block tumor growth in a syngeneic lung cancer animal model. The results demonstrate that SV vector without apoptotic gene expression has therapeutic effect through eliciting anti-tumor immunity (Fig. 2b, 3 and 4). We proposed that the

expression of exogenous protein from the VISA vector such as Gal4-VP16 fusion protein might serve as an tumor antigen in cancer cells (21). Similar observations were noted in which cancer cell lines modified to express foreign protein such as EGFP and β-galactosidase elicited cellular anti-tumor immunity when transplanted into immunocompetent mice (22-24). In addition, the presence of CpGs within DNA sequences has also been linked to stimulation of the mammalian immune system. One study has shown that this form of anti-tumor immunity can be generated using null vector approaches through activation of the innate immune system (25). Moreover, a rapid elevation in the serum proinflammatory cytokines in mice after systemic delivery of liposome:DNA complexes within 8 hr, followed by a reduction (Figure 6) also indicated the innate immune response elicited post treatment (26). These observations partially explained why treatment even with just vector alone led to a certain reduction of tumor growth compared with the untreated group. Furthermore, our cancer-targeted gene therapy may provide a similar function as cancer vaccine by introducing a surrogate antigen, Gal4-VP16 protein, driven by a cancer-specific promoter in cancer cells to raise anti-tumor immunity. This strategy would also prevent injury to normal tissue in response to the exogenous antigen expression due to control by the cancer-specific promoter.

While previous reports have found that apoptotic cells alone were not sufficient to provide the inflammatory signals required to induce dendritic cells maturation for activation of T cells (27), recent studies have shown that apoptosis induced by pathogens does elicit

potent immune responses associated with induction of IFN-γ and the generation of specific CTLs (28, 29). Our results demonstrate that therapeutic effects of plasmids are different in three types of assay systems (*in vitro*, *ex vivo* and *in vivo*). It shows that SV-BikDD treatment has more anti-tumor ability in *ex vivo* and *in vivo* than CMV-BikDD treatment, while CMV-BikDD has better *in vitro* cytotoxicity than SV-BikDD. Although the promoter activity of SV is only half of the CMV promoter activity, the anti-tumor ability of SV-BikDD was stronger than CMV-BikDD in animal model, indicating stronger synergistic anti-tumor effects with immune system in SV-BikDD treatment. In our study, we demonstrated SV-BikDD elicits T cell response against TC1 cancer cells, especially in survivin-overexpressed TC-1 cancer cells (Fig. 4d), but not against normal tissues such as splenocytes and lung cells, suggesting SV-BikDD treatment induced more cancer-specific immunity to kill tumor cells than CMV-BikDD. However, while CMV-BikDD treatment also elicits strong T cell response against TC-1 cancer cells in a comparable level to SV-BikDD, it elicits some T cell response against normal cells as well. Because normal cells and TC-1 cancer cells might present a similar cellular antigen, some active T cells against TC-1 cancer cells might be from the T cells against normal cells and cause the value to be over-estimated in CMV-BikDD treatment. Therefore, based on our result, the therapeutic effect in SV-BikDD treatment was better than CMV-BikDD treatment *in vivo* and this specific anti-tumoral immunity in mice also provided protection against secondary tumor challenge. Whether

non-specific tissue damage such as CMV-BikDD treatment may decrease the specific anti-tumor immunity still need further investigation. Collectively, our results showed that SV-BikDD vector not only induced apoptosis but also elicited an innate immune response and specific anti-tumor immunity, supporting it as an excellent candidate for alternative cancer treatment option in future clinical trials.

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FIGURES LEGENDS

Figure 1. Survivin-VISA vector is active *in vitro* and *in vivo* in mouse lung cancer. (**a**) Schematic diagram of engineered promoter driving luciferase constructs. The VISA system enhances transgene expression driven by the survivin promoter in a mouse lung cancer cell lines, TC-1. The transcriptional activity of the survivin, survivin-VISA and CMV promoters was measured in a mouse lung cancer cell line by cotransfected with indicated plasmid DNA and pRL-TK in dual luciferase assay. The relative luciferase activity shown here represents the dual luciferase activity ratio (firefly versus renilla luciferase) by setting CMV activity as 100%. (**b**) Survivin-VISA vector selectively expressed luciferase in the lung tumor tissue of syngeneic lung cancer animal model while CMV promoter strongly expressed luciferase in both of lung and lung tumor tissue of tumor-bearing mice. HLDC liposome:DNA complexes containing 50 μg plasmid were administered into mice by tail vein injection and luciferase activity was detected using a noninvasive imaging system (IVIS imaging system, Xenogen) after 48 hr. The promoter specificity of CMV and survivin-VISA (SV) was detected by driving luciferase expression in tumor-free and tumor-bearing mice (upper panel). The organs from mice were then dissected for *ex vivo* imaging (lower panel). The quantified signal from lungs and livers is shown in the lower panels. (**c**) Lungs from tumor-bearing mice were fixed and processed for immunohistochemical analysis for firefly luciferase expression by using anti-Luc antibody. The luciferase protein was stained in red and nucleus in blue. The intensity

score was judged by a pathologist and shown in the bottom of each picture.

Figure 2. The cytotoxic effects of BikDD expression in mouse lung cancer cells. (**a**) *In vitro* cell killing effect of BikDD. Indicated therapeutic plasmid and pRL-TK were co-transfected into cells by Lipofetamine 2000. The renilla luciferase activity was detected 48 hours post transfection, and relative cell viability was measured by setting control as 100%. (**b**) Expression of BikDD driven by survivin-VISA vector inhibits tumor growth more strongly than by CMV promoter in mouse TC1 lung cancer *ex vivo* syngeneic models. Mouse lung cancer cells, TC-1 transfected with indicated plasmids treatment, were harvested and inoculated subcutaneously into 6-week old C57BL/6 mice. Tumor volume was monitored after inoculation of tumor cells. The tumors from mice were then dissected on day 33 after tumor inoculation (lower panel). * p-value < 0.05; ** p-value < 0.01.

Figure 3. The therapeutic gene, BikDD driven by survivin-VISA promoter inhibited tumor growth and prolonged survival in syngeneic metastatic lung cancer animal model. (**a**) C57BL/6 mice that received intravenous injection of 1 x 10^6 TC-1 lung cancer cells were treated with 25 μg of liposome:DNA complexes by intravenous injection for a total six times within 3 weeks. Lung tumor nodules were counted under stereomicroscope on day 35 after tumor inoculation. The tumor nodules in PBS treatment group were uncountable, and thus

were set at 300 (maximum) for counting convenience. Each group contained 5 mice. (**b**) Kaplan-Meier survival analysis. The mean survival time is 1.04±0.04, 2.85±0.25, 5.27± 1.07,and 6.24±1.72 (limited to 17) months in PBS, SV, CMV-BikDD, and SV-BikDD groups, respectively. Each group has 10 mice. * p-value < 0.05; ** p-value < 0.01.

Figure 4. Targeted gene therapy elicited anti-tumor immunity in mice. (**a**) IFN-γ ELISpot assay were performed to detect T cell activation against TC-1 cancer cell in mice with different treatments. Two weeks after the last treatment, spleen cells were harvested and analyzed for IFN-γ by an ELISpot assay. Irradiated TC-1 cells were used as stimulators. Each group has 5 mice. (**b**) Synthetic HPV16 E7 peptides (49–57, RAHYNIVTF) were used as stimulator to activate E7-recognized T cells population. (**c**) Every two weeks after the last treatment, effector cells (splenocytes) from treated mice were cocultured with irradiated TC-1 cells for 5 days and subjected to a standard ^{51}Cr-release assay with TC-1 cells as targets. CTL assays were performed with 3 mice per group and observed with Effector/Target (E/T) ratios of 11 and 33. The data shown represent one of two separate experiments with similar results. (**d**) Western blot analysis showed that survivin protein is highly expressed in TC-1 tumor but not in normal lung tissue. EF1α protein was detected as total protein control. Synthetic survivin peptides (mouse survivin 57-64, CFFCFKEL) were used as stimulator to activate survivin-recognized T cells population. Each group has 6 mice. The bars represent mean

values for each group. In this figure, error bars indicate SEM, * p-value < 0.05 and ** p-value < 0.01.

Figure 5. SV-BikDD did not elicit T cell response against normal cells, but elicited protective anti-tumor immunity. (**a**) IFN-γ ELISpot assay was performed by using irradiated splenocytes as stimulators to detect the T cell response against normal cells. Each group has 5 mice. (**b**) Using irradiated normal lung cells as stimulators to detect the T cell response against normal cells. Each group has 5 mice. (**c**) Systemic antitumoral immunity against parental tumor cells challenged at distal sites. 5 x 10^5 cancer cells were inoculated subcutaneously (re-challenged) one week after the last treatment in C57BL/6 mice with metastatic lung cancer cells. Naïve group is a control group to demonstrate the tumorigenic dose of cancer cells for inoculation of 5 x 10^5 cancer cells. Subcutaneous tumor size was measured on day 50 post first tumor inoculation. The number shown above each column indicates the subcutaneous tumor-free events over total mice number. (**d**) The subcutaneous tumor volume was measured on day 50 after first tumor inoculation. Data spots for individual animal in each treatment group were plotted. The bars represent mean values for each group. In this figure, error bars indicate SEM, * p-value < 0.05 and ** p-value < 0.01.

Figure 6. Induction of proinflammation cytokines in gene therapy. (**a**) The CpG content of

therapeutic plasmids. (**b**) Profile of the proinflammatory cytokines induced by SV, CMV-BikDD and SV-BikDD. The proinflammatory cytokines in sera were quantified using a mouse inflammation cytometric bead array (CBA) kits.