Recombinant adenovirus of SEA and CD80 genes driven by MMRE and mouse TERT promoter induce effective antitumor immune responses against different types of tumor cells *in vitro* and *in vivo*

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Abstract. Staphylococcus enterotoxin A (SEA) is a powerful immunostimulant and can stimulate T cells bearing certain T-cell receptor β-chain variable regions when bound to major histocompatibility complex II molecules. SEA is widely used in research of antitumor therapy. The low affinity T-cell receptor (TCR) interaction with SEA in the absence of MHC class II antigens is sufficient for the induction of cytotoxicity but requires additional CD28/B7 signaling to result in proliferation of resting T cells. In this study, we constructed recombinant adenovirus (named as Ad-MMRE-mTERT-BIS) carrying membrane-expressing SEA (named as SEAtm) and CD80 driven by Myc-Max response elements (MMRE) and mouse telomerase reverse transcriptase (mTERT) promoter to reduce toxicity and to improve safety and efficiency. We demonstrated that Ad-MMRE-mTERT-BIS could make SEAtm and CD80 to co-express highly on the surface of Hepa1-6 and B16 cells, at low level on the surface of CT26 cells, but not in NIH3T3. Hepa1-6 and B16 cells infected by the recombinant adenovirus induced proliferation of CD4$^+$ and CD8$^+$ T cells and increased cytokine [interleukin (IL)-2, tumor necrosis factor (TNF)-α, interferon (IFN)-γ] production *in vitro*. Intratumoral injection of Ad-MMRE-mTERT-BIS in hepatoma and melanoma mouse models induced tumor-specific cytotoxic T cells in the spleen. Moreover, hepatoma and melanoma xenografts were suppressed by treatment with Ad-MMRE-mTERT-BIS and the survival time of treated mice was prolonged. These findings suggest that recombinant adenovirus of SEA and CD80 genes driven by mTERT promoter could induce effective antitumor immune responses against different kinds of tumor cells *in vitro* and *in vivo*.

Introduction

Superantigens (SAgs) derived from bacterial or viral products are known as potent activators, which bind to both MHC class II molecules and specific Vβ regions of T cell receptors as an unprocessed protein, resulting in the activation of more than 10-25% of the T-cell population (1). The activation of lymphocytes by superantigen resulted in cytokine production, proliferation and cytotoxicity, and could elicit systemic antitumor immunity *in vitro* and *in vivo* (2-7). This property of SAgs has been used in cancer immunotherapy. Antitumor strategies of superantigen included antibody-targeted super antigen (8-13), superantigen-transmembrane sequence fusion protein (14), and gene therapy (15). Staphylococcus enterotoxin A (SEA) is a kind of superantigen and was widely used in research of antitumor therapy (8-11,13,15). In our previous study (15), SEA was used for cancer gene therapy and induced efficient antitumor effects. The low affinity T-cell receptor (TCR) interaction of SEA in the absence of MHC class II antigens is sufficient for induction of cytotoxicity but requires additional CD28/B7 signaling to result in the proliferation of resting T cells (16). Previous studies have also shown that SEA, in combination with B7 costimulatory molecule, induced a stronger lymphocyte proliferation response and antitumor immunity than either SEA or B7 alone (17). In this study, SEA and B7 (CD80) genes were used to investigate their antitumor effects.

In the gene therapy of tumors, recombinant adenovirus is widely used for the transfer of foreign genes into tumor cells. However, the utilization of these vectors requires the specific and efficient expression of the transferred gene in tumor cells because infections of adenovirus to cells lack tissue specificity. One targeting strategy most often applied is the expression of

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the transgene controlled by a tumor-specific promoter, such as α-fetoprotein (AFP) promoter. However, true tumor-specific promoter is rare, and often these promoters are useful only for the particular types of cancers. Previously, we constructed recombinant adenovirus carrying SEAtm gene driven by AFP enhancer/promoter, which could only be used for hepatocellular carcinoma. *In vitro* and *in vivo* studies have demonstrated that the human telomerase reverse transcriptase TERT (hTERT) promoter is highly active in 80-90% human cancer cells but not in normal differentiated human cells. Therefore, hTERT promoter has been widely used for targeted gene therapy to many types of cancers (18). Similarly, telomerase activities were at high levels in approximately 90% of mouse cancers or tumor-derived cell lines through TERT transcriptional upregulation (19).

In our previous study, we found the proximal 333-bp fragment was the core promoter of the mTERT gene in the cancer cells. The proximal 333-bp fragment of mTERT promoter was used to drive SEA and CD80 gene in our constructed recombinant adenovirus in order to be applied in various types of cancers. Myc family protein, groups of the helix-loop-heilx/leucine zipper family, forms heterodimers with a partner protein, Max. This Myc-Max protein complex binds to the CACGTG sequence and activates transcription (20,21). Myc and Max protein expression increased in many kinds of cancers. Myc-Max response elements (MMRE) were used to increase the hTERT promoter activity (22,23).

In the present study, to extend the applicability of the gene therapy vectors for different kinds of tumors, we constructed a recombinant adenovirus carrying SEAtm and mouse CD80 gene driven by the proximal 333-bp fragment of mTERT promoter, upstream of which MMRE was used to increase the mTERT promoter activity. Then, antitumor effects of recombinant adenovirus were observed in hepatoma, colon cancer and melanoma *in vitro* and *in vivo*.

Materials and methods

Cell lines. Mouse hepatoma cell line Hepa1-6, melanoma cell line B16, colon cancer cell line CT26 and fibroblast cell line NIH3T3 were stored in our laboratory. All the cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 (Gibco-BRL) or high glucose Dulbecco's modified Eagle's medium (DMEM), which were supplemented with 10% fetal bovine serum and penicillin/streptomycin in an atmosphere of 5% CO_2 chamber at 37˚C.

Antibodies. Phycoerythrin (PE)-conjugated anti-mouse CD80 (eBioscience, San Diego, CA, USA) and CD4 (eBioscience), APC-conjugated anti-mouse CD8 (eBioscience), rabbit anti-SEA (Abcam, Cambridge, MA, USA), fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Biolegend) and anti-mouse CD3 (eBioscience) were used in this study.

Recombinant adenovirus preparation. We used the AdEasy vector system (Qbiogene Inc.), that is, human adenovirus serotype 5 and rendered replication defective by the deletion of the E1 and E3 gene. Preparations of the recombinant adenoviruses were performed as previously described (25). Proliferation, purification and titering of adenovirus Ad(empty),

Ad-MMRE-mTERT-CD80 (carrying mouse CD80 gene only), Ad-MMRE-mTERT-SEAtm (carrying SEAtm gene only) or Ad-MMRE-mTERT-BIS (carrying mouse CD80 and SEAtm genes) were performed as described in the manufacturer's protocol. None of the stocks of virus used in the experiments contained detectable replication-competent viruses as evaluated by PCR assay, which used two pairs of primers to detect adenoviral E1A DNA.

Adenovirus-mediated SEA and CD80 expression in different cell lines in vitro. Hepa1-6, B16, CT26 or NIH3T3 cells were plated at a density of $5x10^5$ cells/well in 6-well culture plates 24 h before the adenoviruses infection. Immediately before the infection with Ad(empty), Ad-MMRE-mTERT-CD80, Ad-MMRE-mTERT-SEAtm or Ad-MMRE-mTERT-BIS, culture medium was aspirated and the adenoviruses were distributed over the cell monolayer at multiplicity of infection (MOI) of 100. The ratio of the number of adenovirus per cell was expressed as MOI. After 48 h of culture, the cells were digested with 0.02% ethylenediaminetetraacetic acid, washed in PBS containing 2% calf serum, incubated with a rabbit anti-SEA polyclonal antibody and detected with FITC-labeled donkey anti-rabbit immunoglobulin G (IgG) and/or with PE-conjugated anti-mouse CD80. The analysis was performed by FCM.

The expression of SEA and CD80 on the surface of Hepa1-6 and B16 cells was also visualized by *in situ* immunofluorescent staining. Hepa1-6 or B16 cells were seeded at a density of $1x10^4$ cells/well onto glass coverslips and grown for 48 h after infection with Ad-MMRE-mTERT-BIS at MOI 100. Cells were stained with the Abs at 4˚C as described above and fixed with 4% paraformaldehyde in PBS for 30 min, and then washed twice in PBS buffer. Finally, the coverslips were mounted onto glass slides. Fluorescence distribution was analyzed using a laser confocal scanning microscope.

Proportion of $CD4^+$ and $CD8^+$ T lymphocytes and cytokine production in splenocytes induced by tumor cells infected with the recombinant adenoviruses in vitro. Mouse splenocytes (10^7 cells/well) were co-cultured with 10^6 inactivated Hepa1-6 or B16 cells non-infected or infected with Ad(empty), Ad-MMRE-mTERT-CD80, Ad-MMRE-mTERT-SEAtm or Ad-MMRE-mTERT-BIS in 24-well plate and incubated for 48 h in a humidified chamber at 37˚C, 5% CO_2. $CD4^+$ and $CD8^+$ T lymphocytes in splenocytes were stained with FITC-conjugated anti-mouse CD3, PE-conjugated anti-mouse CD4 and APC-conjugated anti-mouse CD8 and analyzed by FCM. IL-2, TNF-α, and IFN-γ in cell culture supernatant were measured using ELISA kits (eBioscience).

Mouse tumor preparation. Female C57BL/6 mice, 6-8 weeks of age, were obtained from the Experimental Animal Center (Health Science Center, Peking University, Beijing, China) under strictly controlled specific-pathogen-free conditions. Principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed, as well as the current version of the Chinese Law on the Protection of Animals. Mice were held in accordance with the permission of the responsible authority. Tumors were generated by subcutaneous injection of 10^6 Hepa1-6 or B16 cells per mouse in 100 µl of PBS on the

Table I. Recombinant adenovirus Ad-MMRE-mTERT-BIS -mediated expression of SEA and CD80 on the tumor cells and NIH3T3.

Cell lines	CD80-positive cells (%)	SEAtm-positive cells (%)	CD80- and SEA-positive cells (%)
NIH3T3	1.3±0.6	0.9±0.3	1.1±0.5
CT26	5.6±0.9	5.7±1.1	5.3±0.8
B16	41.2±1.9	39.3±2.1	39.1±1.5
Hepa1-6	89.7±2.9	88.6±2.3	87.2±3.1

right hind limb of C57BL/6 mice. Visible tumors developed at 7-9 days after tumor cell inoculation.

Tumor treatment in vivo. When the largest diameter of tumor exceeded 0.5 cm, the Hepa1-6 hepatoma-bearing mice and B16 melanoma-bearing mice were, respectively, randomized into the following groups, and ten mice were included in each group: 1, B16-PBS (blank control); 2, B16-Ad(empty); 3, B16-CD80; 4, B16-SEAtm; 5, B16-BIS; 6, Hepa1-6-PBS (blank control); 7, Hepa1-6-Ad(empty); 8, Hepa1-6-CD80; 9, Hepa1-6-SEAtm; and 10, Hepa1-6-BIS. The mice in the control group were intratumorally injected with 100 μl PBS per mouse. Mice in Ad(empty), CD80, SEAtm and BIS group were, respectively, injected intratumorally with 1x10^9 PFU of Ad(empty), Ad-MMRE-mTERT-CD80, Ad-MMRE-mTERT-SEAtm or Ad-MMRE-mTERT-BIS in 100 μl PBS per mouse. The mice were injected twice a week for 2 weeks. Five mice in each group were sacrificed on day 14 after the last injection, and splenocytes were isolated for CTL activity and IFN-γ-producing cell assay. The other 5 mice in each group were monitored for survival ($\leq$60 days). Tumor sizes were measured before adenovirus injection and subsequently twice a week. The final tumor volume was measured on day 30 after tumor cell inoculation (before any deaths occurred) to ensure inclusion of the data from all the mice. Linear calipers were used to measure the longest diameter (a) and width (b). The tumor volume was calculated using the formula: (ab^2/2) and was plotted as the mean tumor volume of the group ($\pm$ standard error, SE) versus days post-tumor challenge.

Systemic antitumor cytotoxicity assays of CTLs in vivo. The CytoTox 96 non-radioactive cytotoxicity assay (Promega, Madison, WI, USA) was performed to measure the cytotoxic activity of the splenocytes in mice bearing tumors injected with PBS, Ad(empty), Ad-MMRE-mTERT-CD80, Ad-MMRE-mTERT-SEAtm or Ad-MMRE-mTERT-BIS, according to the manufacturer's protocol. Briefly, splenocytes of mice in each group were prepared 14 days after the last injection. Hepa1-6 or B16 cells in RPMI-1640 medium with 10% FBS were used as the targets for the CTL assays. Targets (2x10^5 cells/well) were mixed with splenocytes at effector: target (E:T) ratios of 50:1, 25:1, and 12.5:1, and incubated for 4 h in a humidified incubator at 37˚C, 5% CO$_2$. Lysis solution was added to a portion of the target cells, prior to centrifugation, as a maximum LDH release control. Supernatant (50 μl) was transferred to the enzymatic assay plate after centrifugation, 50 μl of the substrate mix was added to each well; the plate was covered to protect it from light, and incubated for 30 min at room temperature. Stop solution (50 μl) was

added to each well and the absorbance was recorded at 490 nm. The percentage of specific lysis was determined according to the following formula: [A (experimental)-A (effector spontaneous)-A (target spontaneous)x100)/[A (target maximum)-A (target spontaneous)].

IFN-γ producing cell frequencies in vivo. Mouse IFN-γ ELISpot assay was performed in PVDF bottomed 96-well plates by using a murine IFN-γ ELISpot kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. Briefly, plates were coated overnight at 4˚C with anti-IFN-γ capture antibody and washed three times with PBST (PBS+0.05% Tween-20). Plates were blocked for 2 h with 2% skimmed dry milk/PBS. Splenocytes (1x10^6 cells/well) of mice after treatment were then co-cultured with the MMC inactivated Hepa1-6 cells or B16 (5x10^4/well) and incubated for 24 h at 37˚C. Only splenocytes were added into wells as negative control. Cells were then removed and a biotinylated IFN-γ detection antibody was added and incubated for 1 h. Following extensive washing with PBST and PBS, the plates were incubated with streptavidin-alkaline phosphatase for 1 h at 37˚C. Spots were visualized by the addition of the alkaline phosphatase substrate BCIP/NBT. The number of dots in each well was counted by two separate investigators in a blinded manner using a dissecting microscope.

Statistical analysis. One-way analysis of variance (ANOVA) was performed to determine differences of immune response among the various treatment groups. Newman-Keuls tests were performed as post-hoc analysis for one-way ANOVA. The antitumor effects were considered statistically significant when the P-value was <0.05.

Results

Recombinant adenovirus-mediated expression of SEA and CD80 on the tumor cells. Hepa1-6, B16, CT26 and NIH3T3 cells were infected with the recombinant adenovirus Ad-MMRE-mTERT-BIS. Flow cytometric analysis showed that SEAtm and CD80 proteins were efficiently co-expressed on the surface of the infected Hepa1-6 and B16 cells, the expression of SEAtm and CD80 proteins in Hepa1-6 was stronger than that in B16. A few infected CT26 cells and none of infected NIH3T3 cells expressed SEAtm and CD80 proteins (Table I). There were no significant differences between SEAtm and CD80 expression among the infected Hepa1-6 cells or B16 cells. To determine the distribution of CD80 and SEAtm protein on the infected cells, the cell images were visualized by laser confocal microscopy (Fig. 1).
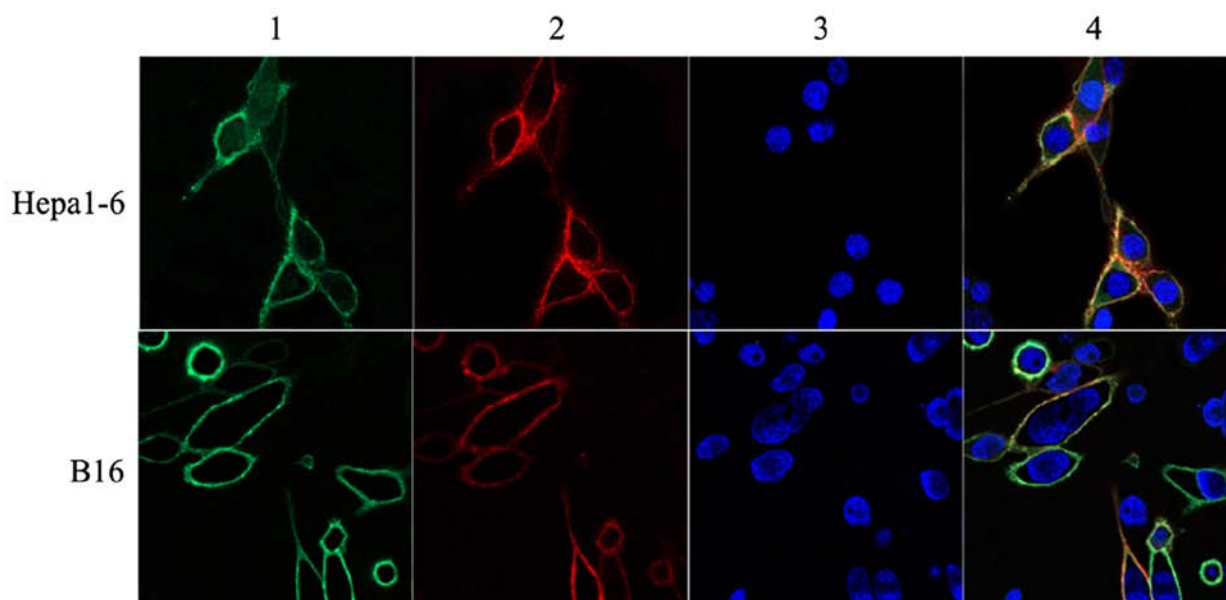
Figure 1. Laser confocal microscopic analysis of the Hepa1-6 and B16 cells infected with the recombinant adenovirus Ad-MMRE-mTERT-BIS. (1) The SEA protein expression (FITC labeled) is presented in green. (2) CD80 protein expression (PE labeled) is presented in red. (3) Nucleus (DAPI staining) is presented in blue. (4) Membrane of Hepa1-6 and B16 cells co-expressing SEA and CD80 in the merged image is presented in yellow.

Since a few infected CT26 cells expressed CD80 and SEAtm protein, we only observed CD80 and SEAtm protein expression on the surface of B16 and Hepa1-6 cells under laser confocal microscopy.

T lymphocyte sub-population proliferation induced by tumor cells infected the recombinant adenoviruses in vitro. The biological activity of CD80 and/or SEA expressed on the surface of Hepa1-6 and B16 cells *in vitro* was determined by the influence on the proportion of lymphocyte sub-population. The results are shown in Table II. The numbers of CD3+, CD3+ CD4+, and CD3+ CD8+ T lymphocytes in cultures induced by tumor cells infected with Ad-MMRE-mTERT-BIS, Ad-MMRE-mTERT-CD80 or Ad-MMRE-mTERT-SEAtm increased as compared with those induced by non-infected tumor cells or tumor cells infected with Ad(empty) (P<0.05). The numbers of CD3+ and CD3+ CD4+ T lymphocytes induced by tumor cells infected with Ad-MMRE-mTERT-BIS were the highest among all groups (P<0.05). The numbers of CD3+ and CD3+ CD4+ T lymphocytes in cultures induced by tumor cells infected with Ad-MMRE-mTERT-BIS or Ad-MMRE-mTERT-SEAtm increased compared to that in cultures induced by tumor cells infected with Ad-MMRE-mTERT-CD80 (P<0.05). There were no statistical differences in the number of CD3+ CD8+ T lymphocytes among cultures with tumor cells infected with Ad-MMRE-mTERT-BIS, Ad-MMRE-mTERT-SEAtm and Ad-MMRE-mTERT-CD80 (P>0.05).

Cytokine production of splenocytes induced by tumor cells infected by the recombinant adenoviruses in vitro. Splenocytes were isolated from a mouse and stimulated with inactivated Hepa1-6 or B16 cells non-infected or infected with Ad(empty), Ad-MMRE-mTERT-CD80, Ad-MMRE-mTERT-SEAtm or Ad-MMRE-mTERT-BIS and cultured for 48 h. The

cytokines IL-2, IFN-γ and TNF-α in cell culture supernatant were detected. The results are shown in Table III. The levels of the three cytokines in splenocytes induced by the tumor cells infected with Ad-MMRE-mTERT-BIS were the highest in all the groups. The levels of the three cytokines in splenocytes induced by tumor cells infected with Ad-MMRE-mTERT-SEAtm, the levels of the three cytokines in splenocytes induced by Hepa1-6 cells or IL-2 and TNF-α in splenocytes induced by B16 cells after infection with Ad-MMRE-mTERT-CD80 increased as compared with those induced by non-infected tumor cells or infected with Ad(empty). The levels of IFN-γ induced by Hepa1-6 cells and TNF-α induced by B16 cells infected with Ad-MMRE-mTERT-SEAtm were higher than those induced by the same cells infected with Ad-MMRE-mTERT-CD80.

IFN-γ-producing cell frequencies of splenic lymphocytes from the mice. Spleen lymphocytes were isolated from the mice (in triplicate) 14 days after the last injection, co-cultured with inactivated Hepa1-6 or B16 cells (treated with MMC, 100 µg/ml at 37˚C for 1 h) for 24 h. Cells were removed and IFN-γ-producing cell frequency was determined for each group of mice with different treatments. As shown in Fig. 2, IFN-γ-producing cell frequencies of lymphocytes in Hepa1-6 hepatoma-bearing mice or B16 myeloma-bearing mice injected with Ad-MMRE-mTERT-BIS was the highest among all groups. The IFN-γ-producing cell frequencies in the mice injected with Ad-MMRE-mTERT-CD80 and Ad-MMRE-mTERT-SEAtm were much higher than those in mice injected with Ad(empty) and PBS (P<0.05).

CTL activity of splenocytes of the mice. Splenocytes isolated from mice in different groups 14 days after the last injection and used as CTL effector cells, and tested against Hepa1-6 or B16 cells as target cells, CTL activities were determined at

Table II. The proportion of T lymphocyte sub-populations in splenocytes co-cultured with tumor cells infected with the recombinant adenoviruses *in vitro*.

Group	CD3+ T cells (%)		CD3+ CD4+ T cells (%)		CD3+ CD8+ T cells (%)	
	Hepa1-6	B16	Hepa1-6	B16	Hepa1-6	B16
Non-infection	77.87±0.88	67.87±0.78	52.79±0.47	45.34±0.57	26.36±0.53	21.36±0.59
Ad(empty)	78.28±0.91	67.74±0.82	52.26±0.91	45.58±0.95	26.02±0.08	22.16±0.78
Ad-MMRE-mTERT-CD80	87.13±0.90[a,b]	78.00±0.79[a,b]	53.27±0.93	46.23±0.85	33.86±1.72[a,b]	31.77±0.79[a,b]
Ad-MMRE-mTERT-SEAtm	90.41±1.20[a,b,c]	87.93±0.95[a,b,c]	57.27±0.77[a,b,c]	57.17±0.97[a,b,c]	33.14±1.14[a,b]	30.76±0.96[a,b]
Ad-MMRE-mTERT-BIS	94.53±0.50[a,b,c,d]	91.75± 0.92[a,b,c,d]	61.86±0.99[a,b,c,d]	60.46±0.89[a,b,c,d]	32.67± 1.48[a,b]	31.29±1.08[a,b]

[a,b,c,d]P<0.05 as compared with non-infected tumor cells, tumor cells infected Ad(empty), Ad-MMRE-mTERT-CD80 or Ad-MMRE-mTERT-SEAtm, respectively. The numerical values were the mean ± SD in each group (in triplicate).

Table III. Cytokine production of splenocytes induced by tumor cells infected by the recombinant adenoviruses *in vitro*.

Group	IL-2 (pg/ml)		IFN-γ (pg/ml)		TNF-α (pg/ml)	
	Hepa1-6	B16	Hepa1-6	B16	Hepa1-6	B16
Non-infection	7.9±0.7	7.3±1.1	37.6±6.7	59.7±11.6	28.3±1.5	35.9±0.9
Ad(empty)	8.2±1.5	7.3±0.7	34.6±13.9	60.8±7.1	28.2±2.5	35.6±1.8
Ad-MMRE-mTERT-CD80	22.6±4.2[a,b]	12.6±1.0[a,b]	206.5±51.8[a,b]	65.8±12.9	83.5±5.9[a,b]	69.9±3.5[a,b]
Ad-MMRE-mTERT-SEAtm	19.8±6.7[a,b]	13.3±0.9[a,b]	352.4±26.7[a,b,c]	74.8±7.0[a,b]	91.6±6.6[a,b]	89.1±6.8[a,b,c]
Ad-MMRE-mTERT-BIS	162.6±5.9[a,b,c,d]	70.7±3.9[a,b,c,d]	444.0±36.2[a,b,c,d]	349.3±24.5[a,b,c,d]	107.4±15.6[a,b,c,d]	159.0±7.0[a,b,c,d]

[a,b,c,d]P<0.05 as compared with non-infected tumor cells, tumor cells infected Ad(empty), Ad-MMRE-mTERT-CD80 or Ad-MMRE-mTERT-SEAtm, respectively. The concentrations of cytokines were the mean ± SD in each group (in triplicate).
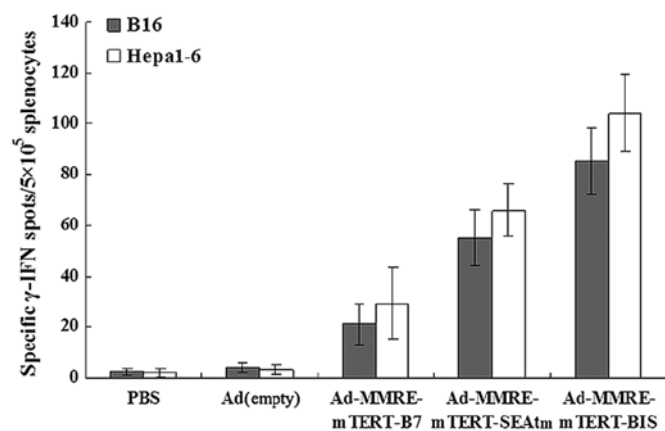

Figure 2. IFN-γ-producing cell frequency in the tumor-bearing mice treated with different vaccines was determined by an ELISpot assay. Splenocytes were isolated from the treated mice 14 days after the last injection. The splenocytes were co-cultured with inactivated tumor cells for 24 h. The number of IFN-γ-producing tumor-specific T cell precursors was determined by using the ELISpot assay. The spot numbers are the means ± SE in each group.

effector:target (E:T) ratios of 12.5:1, 25:1, and 50:1 by a standard CytoTox 96 non-radioactive cytotoxicity assay. As shown in Fig. 3A and B, lymphocytes derived from the mice treated with the Ad-MMRE-mTERT-BIS showed the highest CTL activities in all the groups. The CTL activities of the mice treated with Ad-MMRE-mTERT-CD80 and Ad-MMRE-mTERT-SEAtm were much higher than those of mice treated with Ad(empty) and PBS (P<0.05).

Antitumor effects of the recombinant adenoviruses in vivo. The results in Fig. 4 showed that tumor growth in mice treated with recombinant adenoviruses Ad-MMRE-mTERT-CD80, Ad-MMRE-mTERT-SEA or Ad-MMRE-mTERT-BIS was markedly inhibited as compared with that in mice treated with PBS or Ad(empty) from day 16 in Hepa1-6 tumor-bearing mice (Fig. 4A) and from day 19 in B16 tumor-bearing mice (Fig. 4B; P<0.05), the inhibition of the dual-gene therapy was significantly stronger than that of single gene therapy (P<0.05), there were no significant differences in tumor growth inhibition between the groups treated with Ad-MMRE-mTERT-CD80 and Ad-MMRE-mTERT-SEA (P>0.05). Five tumor-bearing mice in each group were monitored for their survival period. The results in Fig. 5 show that Hepa1-6 (Fig. 5A) or B16 (Fig. 5B) tumor-bearing mice treated with Ad-MMRE-mTERT-CD80, Ad-MMRE-mTERT-SEA or Ad-MMRE-mTERT-BIS survived longer than mice treated with PBS and Ad(empty) (P<0.05), tumor-bearing mice treated with Ad-MMRE-mTERT-BIS survived the longest of all the groups, there was no significant difference in the survival period between the mice treated with Ad-MMRE-mTERT-CD80 or Ad-MMRE-mTERT-SEA (P>0.05), two
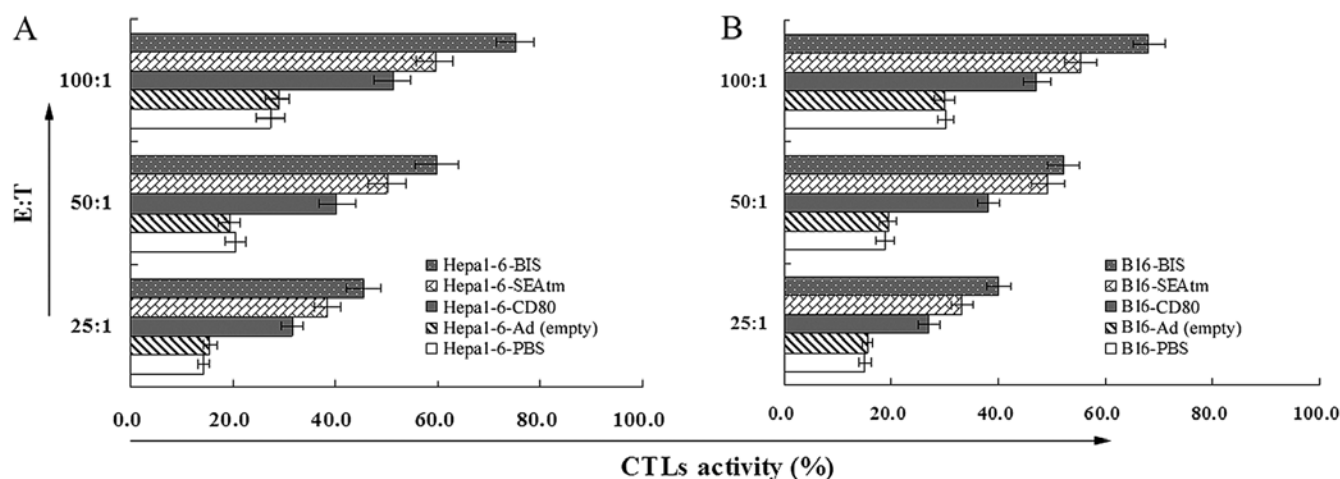
Figure 3. CTL cell activity in Hepa1-6 hepatoma (A) and B16 melanoma (B) -bearing mice treated with different adenovirus was determined by a standard CytoTox 96 non-radioactive cytotoxicity assay. Data are presented as mean specific lysis of triplicate values ± standard deviation. Splenocytes were isolated from the treated mice 14 days after the last injection and used as CTL effector cells. The Hepa1-6 and B16 cells were used as target cells. The CTL activity was determined at effector:target (E:T) ratios of 12.5:1, 25:1, and 50:1.
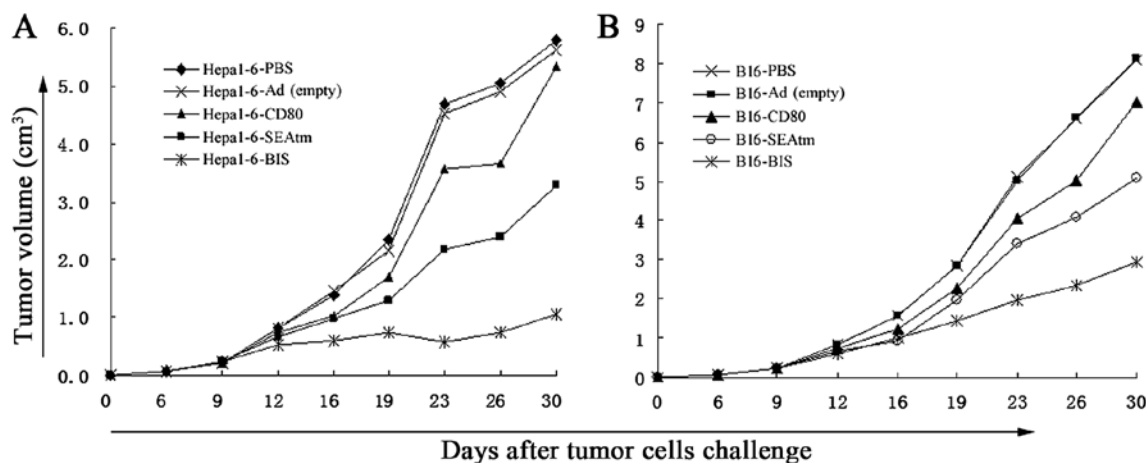

Figure 4. Ad-MMRE-mTERT-BIS decrease tumor growth of tumor-bearing mice. Mice subcutaneously inoculated with 1×10^6 Hepa1-6 (A) or B16 (B) tumor cells were intratumorally injected with PBS, Ad(empty), Ad-MMRE-mTERT-CD80, Ad-MMRE-mTERT-SEA or Ad-MMRE-mTERT-BIS. Tumor sizes are expressed as the mean diameter of the longest x the shortest diameter^2/2 as measured by a digital caliper twice a week for 30 days after tumor cell inoculation. Tumor growth was significantly decreased in mice treated with Ad-MMRE-mTERT-BIS compared to PBS, Ad(empty), Ad-MMRE-mTERT-CD80 or Ad-MMRE-mTERT-SEA from day 16, $P<0.05$.

mice in Hepa1-6-BIS group and one mouse in B16-BIS group had a survival period exceeding 60 days.

Discussion

As a SAg, SEA is a powerful immunostimulant. Previous studies have demonstrated that SEA anchoring onto MHC-II-negative tumor cells through antibodies directs T cell-mediated cytotoxicity against these tumors with reduced toxicity against normal MHC-II$^+$ cells (24-27). In addition, genetically engineered fusion protein of SEA with the transmembrane region sequence of c-erb-B2 could anchor on the surface of tumor and was capable of eliciting systemic antitumor immunity without any measured toxicity (28). These results indicated that the SAg anchoring on MHC-II-negative tumor cells assumes T cell stimulation, but it circumvents conventionally defined MHC 'presentation'. Furthermore, the anchored SAg showed a

greater reduction in MHC class II binding compared to native forms and could elicit MHC-II-independent T cell stimulation *in vitro* as long as co-stimulatory signals were provided (29,30). To decrease systemic activation as a consequence of SAg-MHC class II interaction with monocytes and B cells, and to localize the cytotoxic capabilities of SAg-activated T cells to tumor sites, the membrane-expressing SEA (31) was used in our study. To efficiently stimulate T cells, CD80 was simultaneously transduced into tumor cells. CD80 binds CD28 of T cells and provides the second signal for activation of T cells.

Pericuesta *et al* (32) generated the construct mTERT-GFP using the mTER gene promoter of 1, 2 or 5 kb upstream of the first ATG of the open reading frame of mTERT gene to promote the expression of EGFP. In their transgenic model, no fluorescent expression of the mTERT-EGFP construct could be identified in adult tissues. This suggests that although telomerase activity exists in colon, liver, ovary, and testis, there
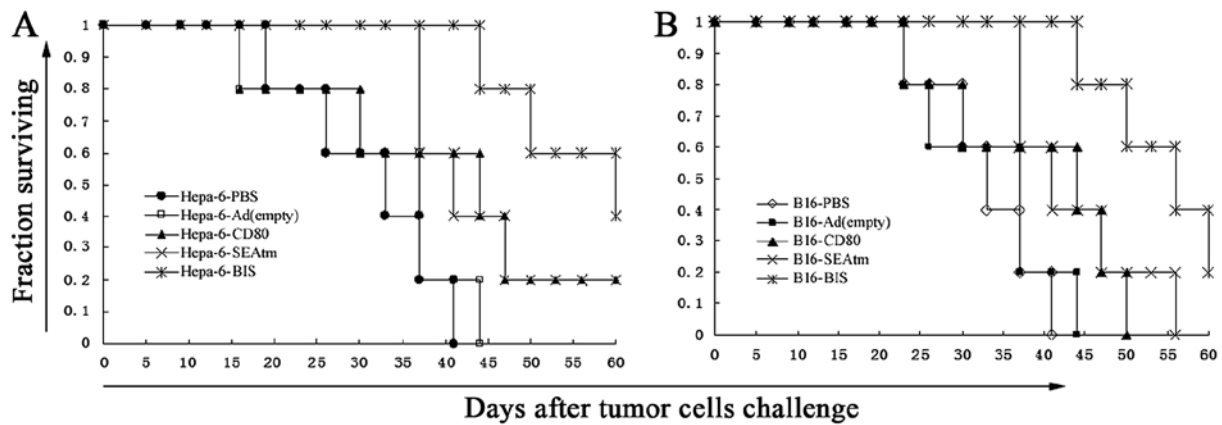
Figure 5. Ad-MMRE-mTERT-BIS enhance the survival of tumor-bearing mice. Mice subcutaneously inoculated with 1×10^6 Hepa1-6 (A) or B16 (B) tumor cells were treated with PBS, Ad(empty), Ad-MMRE-mTERT-CD80, Ad-MMRE-mTERT-SEA or Ad-MMRE-mTERT-BIS. Five mice in each group were observed for survival duration (for ≤60 days). Survival of tumor-bearing mice was significantly enhanced by the treatment with Ad-MMRE-mTERT-BIS compared to that of mice treated with PBS, Ad(empty), Ad-MMRE-mTERT-CD80 or Ad-MMRE-mTERT-SEA, P<0.05.

is a tight repression system of mTERT gene promoter in adult mouse tissues in physiological conditions. In our previous study (33), we found the proximal 333-bp fragment was the core promoter of the mTERT gene in the cancer cells. The proximal 333-bp fragment was able to make SEA express on the surface of hepatoma cell line Hepa1-6, melanoma cell line B16, colon cancer cell line CT26, but not in the fibroblast NIH3T3 cells. In our study, SEAtm and CD80 gene driven by mTERT promoter in the recombinant adenovirus were constructed. The results showed that SEAtm and CD80 were co-expressed in 87.2% of Hepa1-6 cells, 39.1% of B16 cells and 5.3% of CT26 cells, but not in fibroblast cell line NIH3T3 cells after infection with the recombinant adenovirus Ad-MMRE-mTERT-BIS. The positive rates of SEA and CD80 in Hepa1-6 cells, B16 cells and CT26 cells were different. The possible reasons are that the infection efficiency and expression efficiency of the same recombinant adenovirus Ad-MMRE-mTERT-BIS are different in different tumor cells. Ad-MMRE-mTERT-BIS was not used to treat CT26 colon cancer because the positive rate of SEA and CD80 in CT26 cells was very low after infection with Ad-MMRE-mTERT-BIS.

SEA are known as potent activators of T lymphocytes and effcient inducers of cytokine production (34,35). Various cytokines, such as interleukin, interferon and tumor necrosis factor, can destroy vascular endothelial cells of the tumors, promote thrombosis and reduce blood supply to the tumor tissues, resulting in tumor cell necrosis and apoptosis. Cytokines also stimulate proliferation and differentiation of T cells, which will in return produce more cytokines, so as to form endogenously circulating biological effects and speed up the apoptosis of cancer cells. Cytokines can suppress tumor growth both directly and synergistically (35). In addition, cytokines can induce LAK activity, activate natural killer cells and macrophages (10,36,37), facilitate penetration of high molecular weight proteins and upregulate cell adhesion molecules and MHC molecule expression on tumor cells. In our results, SEA expressed on the surface of Hepa1-6 and B16 cells was biologically active *in vitro*, as shown by the ability of Hepa1-6 and B16 cells infected by Ad-MMRE-mTERT-BIS to elicit proliferation and cytokine production of

splenocytes. After stimulation with Hepa1-6 and B16 infected by Ad-MMRE-mTERT-BIS, Ad-MMRE-mTERT-SEAtm or Ad-MMRE-mTERT-CD80, the proportion of CD3+, CD8+ or/and CD4+ T cells in splenocytes and the level of IL-2, IFN-γ and TNF-α produced by splenocytes increased. SEA and CD80 co-expressed tumor cells could more effectively activate splenocytes than SEA or CD80 expressed tumor cells.

Endogenously produced IFN-γ protects the host from not only growing transplanted tumors, but also the formation of primary chemically-induced and spontaneous tumors (38-42) and plays a crucial role for the eradication of tumors *in vivo* (43). Injection of neutralizing mAbs for IFN-γ into mice bearing transplanted, established Meth A tumors blocked LPS-induced tumor rejection (38). In addition, transplanted fibrosarcomas grew faster and more efficiently in mice treated with IFN-γ specific mAbs. Moreover, IFN-γ was shown to be involved in the antitumor effects of antibody-targeted superantigens (44). ELISpot is a sensitive functional assay used to measure INF-γ production at the single cell level. The ELISpot showed there were much more tumor-specific INF-γ-producing cells in the mice bearing hepa1-6 hepatoma and B16 melanoma treated with Ad-MMRE-mTERT-BIS compared with those in other groups. CD8+ CTLs are one of the most crucial components among antitumor effectors (45). In our study, the results showed higher tumor-specific CTL activity was induced in the mice bearing hepa1-6 hepatoma and B16 melanoma treated with the recombinant adenoviruses compared with that in other groups. The ELISpot and cytotoxicity assays indicate Ad-MMRE-mTERT-BIS could induce stronger systemic antitumor immunity than either Ad-MMRE-mTERT-SEAtm or Ad-MMRE-mTERT-CD80. The survival period of the mice bearing hepa1-6 hepatoma or B16 melanoma treated with Ad-MMRE-mTERT-BIS was significantly longer and their tumors grew more slowly than those of mice in other groups. The regression of tumor indicates local antitumor immunity induced by Ad-MMRE-mTERT-BIS treatment.

In summary, our findings show that tumor cells infected with the recombinant adenovirus bearing SEA and CD80 genes can generate stronger antitumor immunity than the recombinant adenovirus bearing a single gene *in vitro* and

in vivo, indicating that SEAtm and CD80 are able to stimulate antitumor immune responses synergistically. The same recombinant adenovirus bearing foreign gene controlled by TERT promoter may be used for targeting gene therapy of different kinds of tumors. The results provided experimental evidence that supports the feasibility and effectiveness of this novel approach in cancer immunotherapy. Additional underlying mechanisms need to be further studied.

Acknowledgements

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