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Tumor cells with B7.1 and transmembrane anchored staphylococcal enterotoxin A generate effective antitumor immunity

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Abstract

Staphylococcus enterotoxin A (SEA) stimulates T cells bearing certain TCR β-chain variable regions, when bound to MHC-II molecules, and is a potent inducer of CTL activity and cytokines production. To decrease toxicity of SEA to the normal MHC-II$^+$ cells and to localize the immune response induced by SEA to the tumor site, my colleague previously genetically fused SEA with B7.1 transmembrane region (named as SEAtm) to make SEA express on the surface of tumor cells and tumor cells modified with SEAtm could induce efficient antitumor immunity *in vitro*. The tumor cell vaccines modified with multiple immune activators frequently elicited stronger antitumor immune responses than single-modified vaccines. In this study, we modified the tumor cell vaccine with B7.1 and SEAtm to improve efficiency in the application of SEA. First, SEAtm gene was subcloned from recombinant plasmid pLXSNSEP by PCR and murine B7.1 gene was cloned from splenocytes derived from C57BL/6 mice by RT-PCR. Then, the eukaryotic co-expression vector of SEA and murine B7.1 gene was constructed and named as pcDNA-BIS. B16 cell lines stably expressing SEA and/or B7.1 were established by screening with G418 after transfection and inactivated for the preparation of tumor cell vaccines to treat mice bearing established B16 tumors. The results indicated that the dual-modified tumor cell vaccine B16/B7.1 + SEAtm (B16-BIS) elicited significantly stronger antitumor immune responses *in vivo* when compared with the single-modified tumor cell vaccines B16/B7.1 (B16-B7.1) and B16/SEAtm (B16-SEAtm), and supported the feasibility and effectiveness of the dual-modified tumor cell vaccine with superantigen and co-stimulatory molecule.
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Keywords: Tumor vaccine; Immunotherapy; Staphylococcus enterotoxin A; Murine B7.1

Among the various strategies used in cancer biotherapy, activation of the immune system by vector-mediated transfer of immunomodulatory genes is a promising approach because tumor cells lack or down-regulate stimulatory surface antigens to escape protective immune responses [1]. Introduction of immunostimulatory antigens such as MHC-I, MHC-II, or B7-1 onto surfaces of tumor cells can induce antitumor immunity as demonstrated by rejection of parental tumor *in vivo* [2,3]. Superantigens (SAgs) are potent immunomodulatory molecules, each of which stimulates T cells bearing certain TCR vβ elements and is capable of activating more than 10–25% of the T cell population when bound as an unprocessed protein outside the antigenic groove of MHC-II molecules [4]. SAg-activation of lymphocytes results in cytokine production, proliferation, and cytotoxicity, and can elicit systemic antitumor immunity [5–7]. Bacterial SAgs, such as staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), and toxic shock syndrome toxin-1 (TSST-1), are well known as very potent activators of T cells that can elicit strong immune responses both *in vitro* and *in vivo* [8,9]. To decrease toxicity of SEA to the normal MHC-II$^+$ cells and localize the immune response induced by SEA to the tumor site, transmembrane region of B7.1

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has been fused genetically with SEA (named as SEAtm) to make SEA express on the surface of tumor cells in our laboratory and SEAtm could induce stronger antitumor responses *in vitro* [10].

The escape of tumor cells from immune surveillance can involve several mechanisms, which indicated that a single immune activator might be less effective than multiple activators at inducing sufficient immune response against tumor cells. Several studies have shown that tumor cell vaccines created by multiple-transfection can induce much stronger antitumor immunity than those made by single-gene transfection. Therefore, in this study, the eukaryotic co-expression vector of SEAtm and murine B7.1 gene was constructed and transfected into B16 cells for the preparation of a dual-modified tumor cell vaccine to treat mice bearing B16-tumor. We investigated the feasibility of the approach for preparing a dual-anchored tumor cell vaccine, and the effectiveness of the tumor cell vaccine by this novel approach in an animal model.

Materials and methods

Vectors, bacterial strains, and cell line. The eukaryotic expression vectors pIRES2-EGFP from BD Biosciences Clontech (USA) and pcDNA3.1+ from Invitrogen Corporation (USA) and *Escherichia coli* DH5α were conserved in this laboratory. pMD18-T vector was purchased from TaKaRa corporation (Japan). DH5α was routinely cultured in LB (Luria–Bertani) medium. Antibiotics were added in the following concentration ampicillin (100 μg/ml) and kanamycin (50 μg/ml). Murine melanoma B16 cell line, derived from the C57Bl/6 mouse strain, was obtained from Chinese Academy of Science (Shanghai) and maintained in RPMI-1640 (Gibco-BRL), which were supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin in an atmosphere of 5% CO_2 chamber at 37 °C.

Mice. Female C57BL/6 mice, 6–8 weeks of age, were obtained from the Experimental Animal Center (FMMU, China) under strictly controlled specific-pathogen-free conditions at the Cancer Institute Animal Facility (Xi'an, China). Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) as well as the current version of the Animal Protection Law of China were followed. Mice were held in accordance with permission of the responsible authority. Mice were housed 5 per cage in a 12 h light/dark cycle at an ambient temperature of 22 ± 2 °C and humidity of $50 \pm 10\%$ with food and water *ad libitum*. Cages were changed twice a week to ensure hygienic conditions. The animals were allowed to acclimate to the facility for 2 weeks before randomization into different experimental groups.

Construction of the eukaryotic co-expression vector of SEA and murine B7.1 gene. The murine B7.1 gene was cloned by RT-PCR from the splenocytes derived from C57BL/6 mice with the primers (P1: 5′-gcg gtcgac agatct atggcttgcaattgtcagttg-3′ containing a *Sal*I and a *Bgl*II sites and P2: 5′-gcg ccgcgg ctaaaggaagacggtctgttc-3′ containing a *Sac*II site) into pIRES2-EGFP upstream of IRES sequence to construct pIRES-B7.1. The SEAtm gene was subcloned into pMD18-T vector from previously constructed pLXSNSEAtm [10] by PCR with primers (5′-gcg agatct ccatgg aaaaaacagcatttaca-3′ containing a *Bgl*II site and a *Nco*I site, and 5′-ttatacagggcgtacacttttc-3′). The resulting plasmid was named pMD18-SEAtm. Positive recombinant clones were confirmed by restriction endonuclease digestion and DNA sequencing. Then, B7.1 gene and IRES, sequence from pIRES-B7.1 digested by *Sal*I and *Nco*I were together subcloned into pMD18-SEAtm upstream of SEA gene to construct pMD18-BIS. B7.1, IRES and SEA segments from pMD18-BIS digested by *Bgl*II and *Xba*I were all together subcloned into pcDNA3.1+ vector with the restriction sites of *Bam*HI and *Xba*I to construct pcDNA3.1+-BIS. B7.1 and SEAtm gene were subcloned into pcDNA3.1+ from pIRES-B7.1 and pMD18-SEAtm to construct pcDNA3.1+-B7.1 and pcDNA3.1+-SEAtm, respectively.

Preparation of cell-based vaccines. B16 cells were seeded in 24-well plates with cell number of 5×10^4/well in 5% complete DMEM, allowed the cells to reach 70% confluence the next day. Then B16 cells were transfected with pcDNA3.1+-BIS, pcDNA3.1+-B7.1, pcDNA3.1+-SEAtm, or pcDNA3.1+ using lipofectamine 2000 (Invitrogen, USA). Transfected cells were screened in the presence of 800 μg/ml G418 (Sigma Chem. Co.). Then the G418-resistant cells were cloned in 96-well plates by a limited dilution method. The stable positive clones were selected by flow cytometric assay and confocal microscopy, and maintained at 37 °C in 5% CO_2 in RPMI-1640 containing 15% fetal bovine serum and 400 μg/ml G418. The screened cells were incubated with mitomycin C (MMC, 100 μg/ml) at 37 °C 5% CO2 incubator for 1 h, washed twice with PBS, and resuspended in PBS for using as tumor cell vaccine. The cells transfected with pcDNA3.1+-BIS were designated as B16-BIS vaccine, B16 transfected with pcDNA3.1+-B7.1 were designated as B16-B7.1 vaccine, B16 transfected with pcDNA3.1+-SEAtm were designated as B16-SEAtm vaccine, and B16 cells transfected with pcDNA3.1+ were designated as B16-pcDNA vaccine, B16 cells inactivated with MMC were used as the control (designated as B16 vaccine).

Antibodies. Biotin anti-mouse B7.1 (eBioscience, USA), rabbit anti-SEA (Toxin Technology, Sarasota, FL), fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and ExtraAvidin-Cy3 conjugate (Sigma, USA) were used.

Flow cytometry. Tumor cells (1×10^6) were washed once with PBS containing 2% fetal bovine serum and resuspended in 50 μl wash buffer containing Biotin anti-mouse B7.1 or rabbit anti-SEA antibody, incubated at 4 °C for 45 min, then washed twice with wash buffer, resuspended in wash buffer containing a fluorescein-labeled secondary antibody or ExtraAvidin-Cy3 conjugate, and incubated at 4 °C for 45 min. After washing twice with wash buffer, the cells were resuspended in 500 μl PBS for flow cytometric analysis (Beckman–Coulter, Hialeah, FL).

Confocal microscopic analysis. The expression of SEA and/or B7.1 on the surface of B16 cells after stable transfection was also visualized by *in situ* indirect immunofluorescent staining. Tumor cells were seeded at low density onto glass coverslips and grew for 24 h. Cells were stained with the Abs at 4 °C as described above and fixed with 2% paraformaldehyde in PBS for 30 min, then washed twice in PBS buffer. Then the coverslips were mounted onto glass slides. Fluorescence distribution was analyzed using a laser confocal scanning microscope.

Measurement of lymphocyte proliferation. Proliferation of lymphocytes was detected by ^{3}H-TDR assay. Splenocytes were derived from C57BL/6 mice and co-cultured with inactivated tumor cell vaccines at a ratio of 2:1 for 72 h in a 96-well plate. Lymphocytes were labeled with [^{3}H] thymidine (1 μCi/well) for 18 h before harvesting. Incorporation of [^{3}H] thymidine was determined using a liquid-scintillation counter.

Immunotherapy with the inactivated tumor cell vaccines. To establish the tumor model, B16 cells cultured for 3 days were harvested with 0.05% trypsin–EDTA and washed twice with PBS. 1×10^5 B16 cells in 100 μl PBS were subcutaneously injected in the right rear flank of each C57BL/6 mice. Tumor size was determined by measuring tumors at two dimensions using a digital caliper twice a week, beginning at 7 days after inoculation. Tumor size was calculated using the formula: length × width^2/2. To test the effectiveness of B16-TM-SEA vaccine, the tumor-bearing mice were randomized into the following groups on third day after inoculation, and ten animals were included in each group: (1) PBS (blank control), (2) B16 vaccine, (3) B16-pcDNA vaccine, (4) B16-SEAtm vaccine, (5) B16-B7.1 vaccine, and (6) B16-BIS vaccine. On seventh day after the tumor cell inoculation, all the tumor-bearing mice in the respective groups were injected in the left rear flank with 100 μl PBS or 1×10^6 cells in 100 μl PBS containing B16 vaccine, B16-pcDNA vaccine, B16-B7.1 vaccine, B16-SEAtm vaccine cells, and B16-BIS vaccine, respectively. The mice were injected three times totally with an interval of three days. Five mice in each group were killed on seventh day after last vaccination, and splenocytes were isolated for CTL activity and IFN-γ-producing cell assay. The other 5 mice in each group were monitored for survival (≤90 days). The final tumor volume was measured on day 28 after tumor cell inoculation

(before any deaths occurred) to ensure inclusion of the data from all the mice.

Cytotoxicity assays. To determine immune cell cytotoxicity, we used CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), which is based on the colorimetric detection of the released enzyme, lactate dehydrogenase (LDH) [17,18]. Briefly, splenic effector cells were isolated from five of ten mice in each group, which were sacrificed 7 days after the last injection. The erythrocytes were lysed with 0.83% ammonium chloride. Macrophages were removed by adherence on plastic plates for 2 h, and the remaining non-adherent cells (lymphocytes) were retained and pooled. A separate sample of splenic lymphocytes was co-cultured with inactivated B16 (treated with 100 μg/ml MMC for 1 h) for 7 days in the presence of 20 U/ml recombinant murine IL-2 (Sigma, St. Louis, MO) and then collected for testing of the CTL activity. B16 melanoma cells in RPMI 1640 medium with 10% FBS were used as the targets for the CTL assays. Targets (2×10^5 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 (10×) 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: 100 × (Experimental − Effector Spontaneous − Target Spontaneous)/(Target Maximum − Target Spontaneous).

IFN-γ-producing cell frequency. Mouse IFN-γ ELISpot assay was performed in PVDF-bottomed 96-well plates (Millipore, Bedford, MA, USA) by using a murine IFN-γ ELISpot kit (Diaclone, Besancone, France) according to the manufacturer's instructions with minor modifications. 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 dried milk. Splenocytes (1×10^6 cells/well) isolated from treated mice were then added into the plates and co-cultured with inactivated (treated with 100 μg/ml MMC for 1 h) Hepa1-6 cells (5×10^4/well, respectively). Only splenocytes were added into wells as negative control. After culture for 24 h at 37 °C, 5% CO_2, cells were removed and a biotinylated IFN-γ antibody was added and incubated for 2 h. Following extensive wash 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. The final dots number was calculated as that negative control dots number was subtracted from the total.

Statistical analysis. One-way 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 less than 0.05.

Results

Cloning and sequencing of SEAtm and B7.1, and construction of the eukaryotic co-expression vector containing SEA and murine b7.1 gene

The SEAtm and murine B7.1 gene were cloned, respectively, as described in Materials and methods. The sequences of B7.1 gene and the fusion gene SEAtm were consistent with the corresponding sequence in GenBank, and have 100% homogeneity. For constructing the co-expression vector containing SEAtm and murine B7.1 gene, the B7.1 gene and IRES sequence were first subcloned into pMD18-SEAtm, which are located upstream of SEAtm gene, to construct pMD18-BIS. Then, the B7.1 gene, IRES, and SEAtm gene digested from pMD18-BIS with *Bgl*II and *Xba*I were all together subcloned into pcDNA3.1+ with the multiple cloning sites of *Bam*HI and *Xba*I to construct pcDNA-BIS. Restriction endonuclease digestion analysis of pMD18-BIS and construction map of pcDNA-BIS are shown in Fig. 1. Because there was a *Kpn*I site in IRES sequence, pcDNA-BIS was digested with *Kpn*I and *Xba*I, and two segments were digested from pcDNA3.1+-BIS.

Lymphocyte proliferation

The authors tested whether B16 cells introduced with mB7.1 and/or SEAtm gene could stimulate lymphocyte proliferation in vitro. The results (in Fig. 2) showed that B16-B7.1 and B16-SEAtm vaccines could significantly

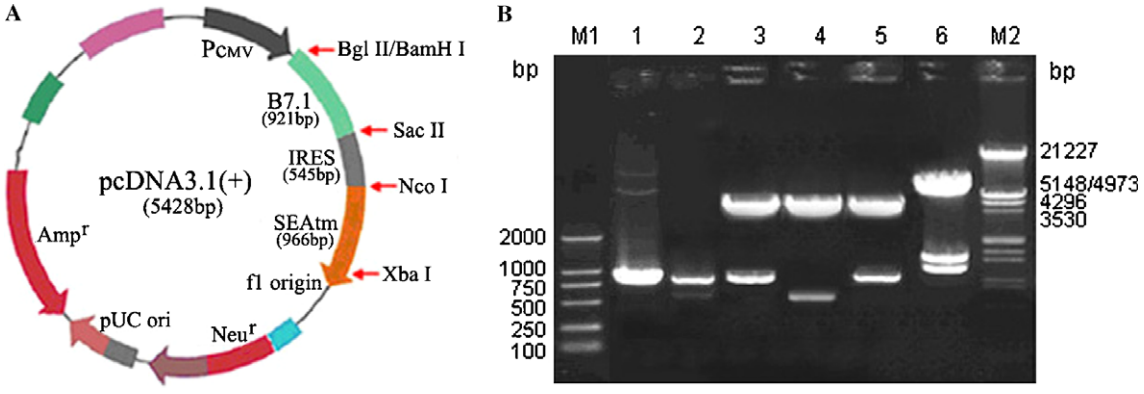

Fig. 1. (A) Schematic representation of the eukaryotic co-expression vector of SEA and murine B7.1 gene. (B) Electrophoresis of PCR product of SEAtm fusion gene and RT-PCR product of B7.1, and analysis of the recombinant plasmids digested with restriction endonucleases. SEAtm fusion gene or B7.1 gene were amplified by PCR or RT-PCR. The recombinant pcDNA3.1+-BIS plasmid was digested with *Kpn*I and *Xba*I, and the recombinant pMD18-BIS was digested, respectively, with *Sal*I + *Sac*II, *Sac*II + *Nco*I, and *Nco*I + *Xba*I. All the PCR, RT-PCR, and digested products were analyzed by electrophoresis in a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide. M1, DL2000 DNA marker; lane 1, SEAtm PCR product (986 bp); lane 2, B7.1 RT-PCR product (945 bp); lane 3, the recombinant pcDNA3.1+-BIS vector digested with *Kpn*I and *Xba*I; lane 4, the recombinant pMD18-T-BIS vector digested with *Sal*I and *Sac*II; lane 5, pMD18-T-BIS vector digested with *Sac*II and *Nco*I; lane 6, pMD18-T-BIS vector digested with *Nco*I and *Xba*I; M2, *Eco*RI/*Hin*dIII λ DNA ladder.

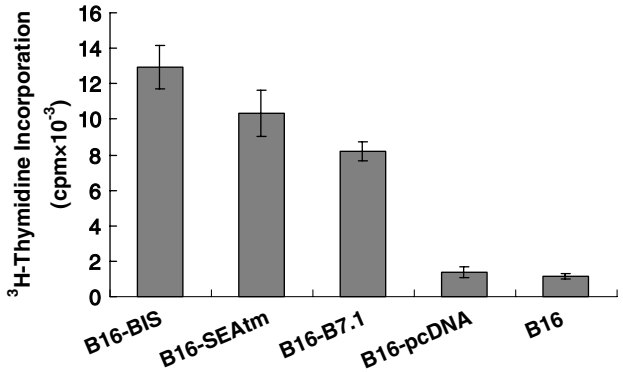

Fig. 2. Stimulating lymphocyte proliferation. Cell vaccines were added at a 1:2 tumor/lymphocyte (T:L) ratio to 2×10^5 lymphocytes/well in a 96-well plate. Values on the *y*-axis represent the average cpm ± SD of triplicate cultures. Background values of lymphocytes alone were subtracted.

stimulate lymphocyte proliferation compared with B16-pcDNA and B16 cells ($p < 0.05$), meanwhile, the B16-BIS vaccine exhibited a stronger stimulation of lymphocyte proliferation than that of B16-B7.1 and B16-SEAtm vaccines *in vitro* ($p < 0.05$). There was no difference between B16-pcDNA and B16 cells at stimulating lymphocyte proliferation.

Expression of SEA and/or murine B7.1 on the tumor cells

B16 cell was introduced with B7.1 and/or SEAtm gene. Flow cytometric analysis showed that B7.1 and/or SEA protein was expressed on the surface of the transfected cells. After screening with G418, 94.2 ± 2.3% of B16 cells transfected with pcDNA3.1+-BIS expressed B7.1 and 93.4 ± 3.1% of them expressed SEA, 95.5 ± 2.2% of B16 cells transfected with pcDNA3.1+-B7.1 expressed B7.1, and 94.5 ± 2.5% of B16 cells transfected with pcDNA3.1+-SEAtm expressed SEA (in triplicate). There were no significant differences in SEA and B7.1 expression percentage among the B16 cells transfected with pcDNA3.1+-BIS, pcDNA3.1+-B7.1, and pcDNA3.1+-SEAtm. To determine the distribution of B7.1 and/or SEAtm protein on the transfected cells, the cells images, were visualized by laser confocal microscopy (Fig. 3).

Antitumor effects of the tumor cell-based vaccine

Tumor growth in mice treated with the B16-B7.1, B16-SEAtm, or dual-anchored B16-BIS vaccines was markedly inhibited as compared with those treated with PBS, B16-pcDNA, or B16 vaccine controls (Fig. 4A). The antitumor effect of the dual-anchored B16-BIS vaccine was significantly stronger than that of the B16-B7.1, B16-SEAtm vaccine ($p < 0.05$). There were no significant differences in tumor growth inhibition between the groups treated with the B16-B7.1 and B16-SEAtm vaccines. Five tumor-bearing mice in each group were monitored for their survival period. The results in Fig. 4B showed that melanoma-bearing C57BL/6 mice treated with dual-anchored B16-BIS vaccine survived longer than those treated with the B16-B7.1 vaccine ($p < 0.05$), the B16-SEAtm vaccine ($p < 0.05$), and the PBS, B16, or B16-pcDNA vaccine controls ($p < 0.01$), but there was no significant difference in the survival period between the mice treated with the B16-B7.1 and B16-SEAtm vaccines ($p > 0.05$).

CTL activity of splenocytes from the mice

Splenocytes isolated from the vaccinated mice (in triplicate) 7 days after the last therapy were co-cultured with inactivated B16 cells (treated with mitomycin C, 100 μg/ml at 37 °C for 1 h) for 7 days in the presence of recombinant murine IL-2 (40 U/ml), collected as CTL effector cells, and tested against B16 cells as target cells, CTL activity was determined at 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. 5, lymphocytes derived from the mice treated with the B16-BIS vaccine showed the highest CTL activity compared with lymphocytes derived from all other groups. The CTL activity of the mice in the B16-B7.1 and B16-SEAtm vaccine groups was also much higher than that in the B16-pcDNA, B16 vaccine, and PBS groups ($p < 0.05$).

IFN-γ-producing cell frequency of splenic lymphocytes from the mice

Spleen lymphocytes were isolated from the vaccinated mice (in triplicate) 7 days after the last therapy, co-cultured with inactivated B16 cells (treated with mitomycin C, 100 μg/ml at 37 °C for 1 hour) 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. 6, IFN-γ-producing cell frequency of lymphocytes in the tumor-bearing mice treated with the B16-BIS vaccine was the highest among all other groups. The IFN-γ-producing cell frequency in the mice treated with B16-B7.1 and B16-SEAtm vaccines was also much higher than those with B16-pcDNA and B16 vaccine ($p < 0.05$).

Discussion

SAgs derived from bacterial or viral products are known as potent activators of T lymphocytes and efficient inducers of cytokine production. This property of SAgs has been used in cancer immunotherapy [8,9]. Treatment exploiting the therapeutic potential of SAgs requires modifications 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. To achieve these aims, one of the commonly applied strategies is that SAg was anchored on the tumor cells, including application in which the SAgs were ligated to tumor-specific monoclonal, anti-idiotypic, or bifunctional antibodies and fused with the transmembrane region

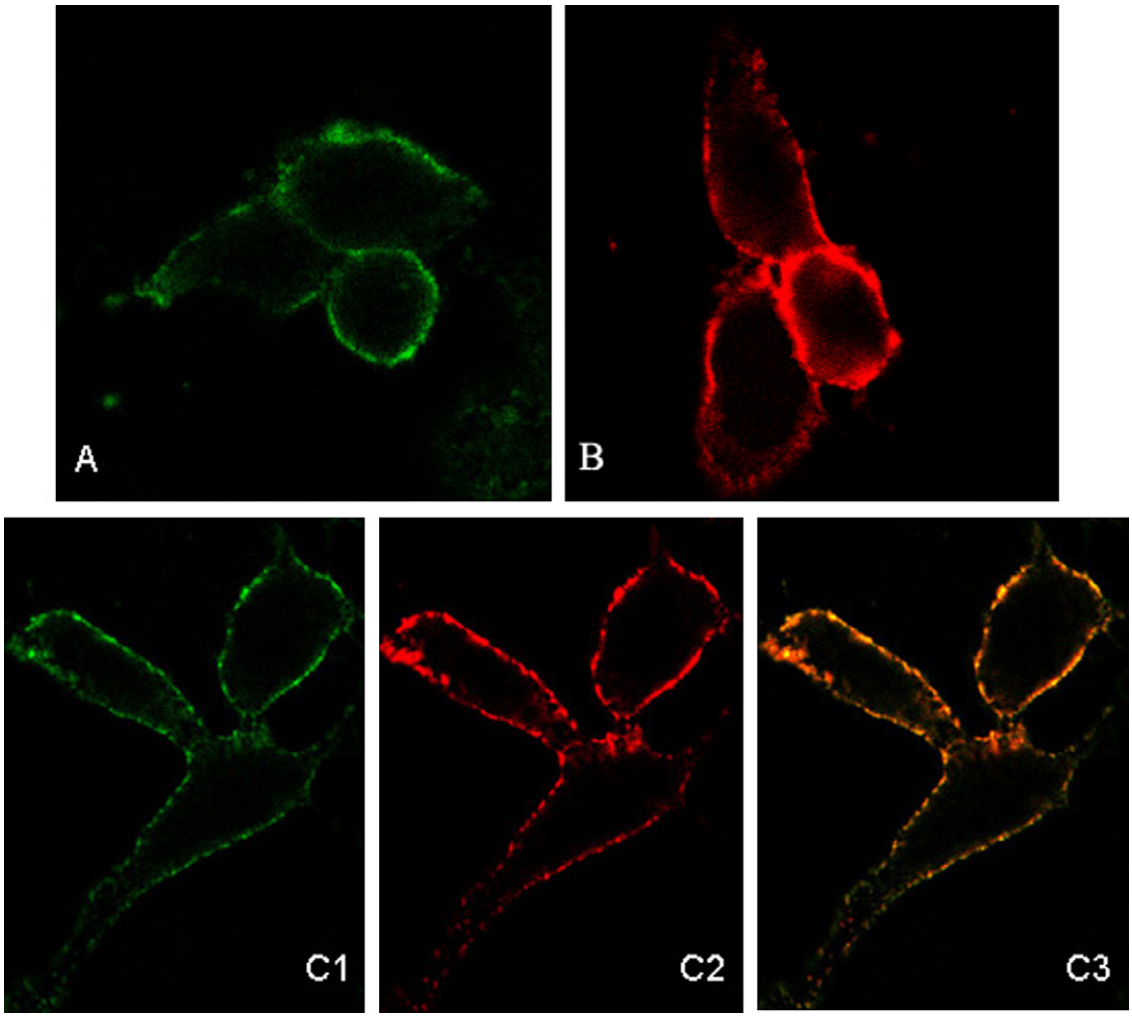

Fig. 3. Laser confocal microscopic analysis of the B16 cells introduced with murine B7.1 gene and/or transmembrane-anchored staphylococcal enterotoxin A (SEAtm) fusion gene. (A) The SEA antigen was expressed on the B16-SEAtm cells (Fluorescein isothiocyanate [FITC] labeled). (B) B7.1 molecule was expressed on the B16-B7.1 cells (Cy3 labeled). (C) mB7.1 and SEA antigens were expressed on the B16-BIS cells. (C1) SEA-FITC is presented in green. (C2) B7.1-Cy3 is presented in red. (C3) The merged image is presented in yellow. Biotinylated anti-mouse B7.1, ExtrAvidin-Cy3 conjugate, rabbit anti-SEA, and FITC-conjugated goat anti-rabbit IgG were used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

sequence of the protooncogene c-erb-B2 or GPI signal sequence [11–13]. 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 [14,15]. As SAg, SEA is a powerful immunostimulant. Previous studies have demonstrated 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 [16,17]. 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 [18]. To target immune response induced by SEA to the tumor site, SEA was previously genetically fused with B7.1 transmembrane region (SEAtm) in our laboratory and could be expressed on the surface of tumor cells [10].

Previous studies demonstrated that tumor cells lacking B7.1 were poorly immunogenic, and therefore they failed to initiate an appropriate immune response [19]. Introducing B7.1 onto the tumor cell surface by gene transfection may improve the immunogenicity of tumor cells and result in the rejection of parental tumors in animals [19,20]. Other studies have shown that SAg, in combination with B7 co-stimulatory molecule, induced a strong lymphocyte proliferation response [21,22]. In this study, to enhance the immunogenicity of tumor cell vaccine, we used the membrane-expressed SEAtm and B7.1 to modify tumor cells. The results showed that both SEA and B7.1 could be co-expressed on the surface of B16 cells, which were

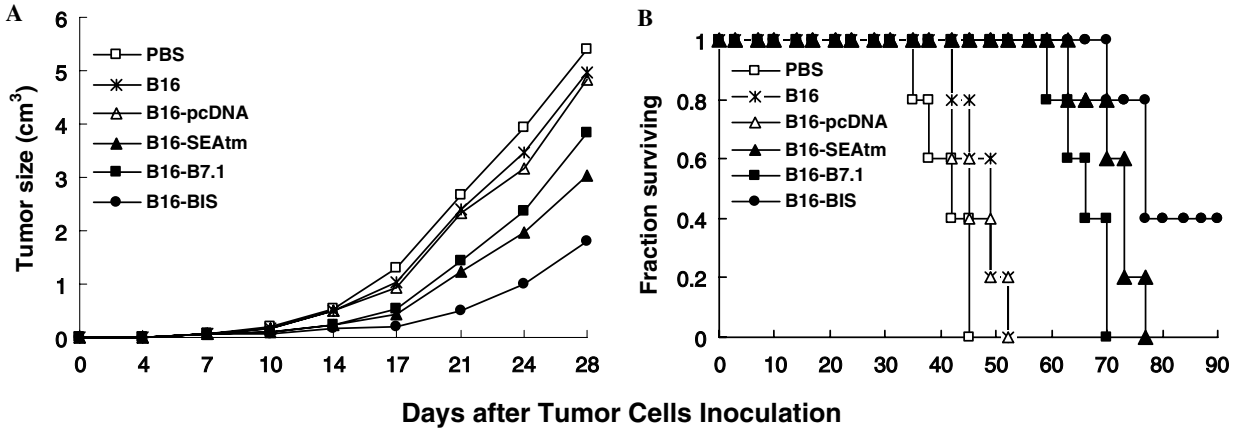

Fig. 4. Tumor size (A) and survival period (B) analysis of tumor-bearing mice after inoculation of phosphate-buffered saline (PBS), B16, B16-pcDNA, B16-B7.1, B16-SEA, and B16-BIS vaccine. C57BL/6 mice were inoculated subcutaneously with 1×10^5 B16 cells on day 0. The tumor-bearing C57BL/6 mice were divided into 6 groups (each group containing 10 mice) on the third day, and were injected with the following preparations on the seventh day: PBS, B16, B16-pcDNA, B16-B7.1, B16-SEA, and B16-BIS vaccine. Tumor sizes are expressed as the mean diameter of the longest × the shortest diameter^2/2 as measured by a digital caliper twice a week. Seven days after the last vaccination, five mice in each group were killed and splenic lymphocytes were isolated for CTL activity. The other 5 mice in each group were observed for survival duration (for $\leqslant$90 days).

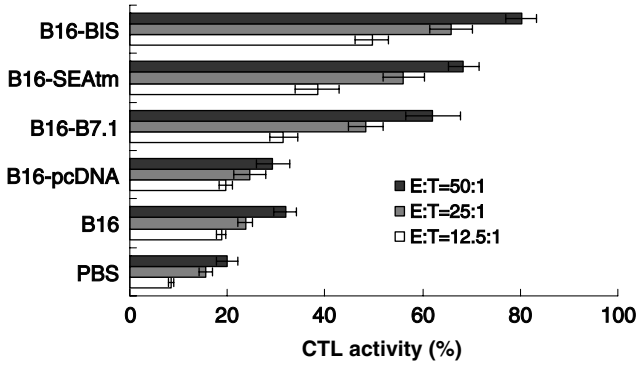

Fig. 5. CTL cell activity in the tumor-bearing mice treated with different vaccines was determined by a standard CytoTox 96 non-radioactive cytotoxicity assay (Promega, Madison, WI). Data are presented as mean specific lysis of triplicate values ± standard deviation. Splenocytes were isolated from the treated mice 7 days after the last injection. The splenocytes were co-cultured with inactivated B16 for 7 days in the presence of recombinant murine interleukin-2 (40 U/ml) and then collected as CTL effector cells. The 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.

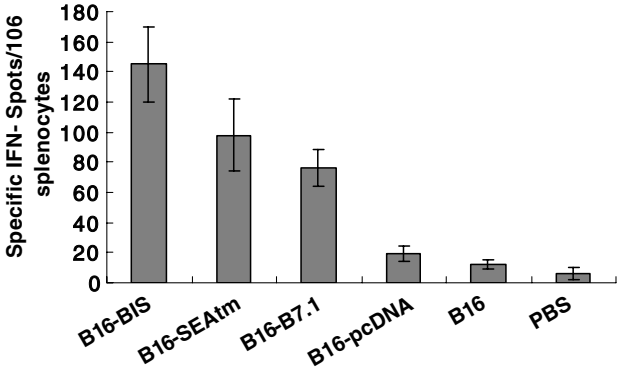

Fig. 6. IFN-γ-producing cell frequency in the tumor-bearing mice treated with different vaccines was determined by a ELISpot assay (Diaclone, Besancone, France). Splenocytes were isolated from the treated mice 7 days after the last therapy. The splenocytes were co-cultured with inactivated B16 for 24 h. The number of IFN-γ-producing tumor-specific T cell precursors was determined by using the ELISpot assay. The spot numbers were the means ± SE in each group.

transfected with pcDNA3.1+-BIS. B16-BIS vaccine exhibited a greater ability than the B16-SEAtm vaccine and B16-B7.1 vaccine to stimulate lymphocyte proliferation.

Endogenously produced IFN-γ not only protects the host from growing of transplanted tumors, but the formation of primary chemically induced and spontaneous tumors [23–27] and plays a crucial role for the eradication of tumors *in vivo* [28]. Injection of neutralizing mAbs for IFN-γ into mice bearing transplanted, established Meth A tumors blocked LPS-induced tumor rejection [23]. In addition, transplanted fibrosarcomas grew faster and more efficiently in mice treated with IFN-γ specific mAbs. Moreover, IFN-γ was involved in the antitumor effects of antibody-targeted superantigens [29]. 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 treated with B16-BIS vaccine compared with those in other groups. $CD8^+$ CTLs are one of the most crucial components among antitumor effectors [30]. To determine the tumor-specific CTL activity in splenocytes induced by immunotherapy with B16-BIS vaccine, cytotoxicity assays were performed. The results showed higher tumor-specific CTL activity was induced in the mice treated with B16-BIS vaccine compared with that in other groups. The ELISpot and cytotoxicity assays indicated B16-BIS vaccine induced stronger systemic antitumor immunity than B16-B7.1 and B16-SEAtm vaccine. The survival period of the mice treated with B16-BIS vaccine was significantly longer and their tumors grew more slowly than those of mice treated with other vaccine. The regression of tumor indicated local antitumor immunity induced by B16-BIS vaccine.

In summary, our findings show that tumor vaccines modified with transmembrane-anchored SEA and B7.1 by transfection can generate stronger local and systemic antitumor immunity than a single-modified tumor vaccine in vivo, indicating that SEAtm and B7.1 were able to stimulate antitumor immune responses synergistically. The results provided experimental evidence that supports the feasibility and effectiveness of this novel approach in cancer immunotherapy. The underlying mechanisms need to be further studied.

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