

Preventive DNA vaccination against CEA-expressing tumors with anti-idiotypic scFv6.C4 DNA in CEA-expressing transgenic mice

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Abstract Carcinoembryonic antigen (CEA) is expressed during embryonic life and in low level during adult life. Consequently, the CEA is recognized by the immune system as a self-antigen and thus CEA-expressing tumors are tolerated. Previously, we constructed a single chain variable fragment using the 6.C4 (scFv6.C4) hybridoma cell line, which gave rise to antibodies able to recognize CEA when C57/B16 mice were immunized. Here, the scFv6.C4 ability to prevent the CEA-expressing tumor growth was assessed in CEA-expressing transgenic mice CEA2682. CEA2682 mice immunized with the scFv6.C4 expressing plasmid vector (uP/PS-scFv6.C4) by electroporation gave rise to the CEA-specific AB3 antibody after the third immunization. Sera from immunized mice reacted with CEA-expressing human colorectal cell lines CO112, HCT-8, and LISP-1, as well as with murine melanoma B16F10 cells expressing CEA (B16F10-CEA). Cytotoxic T lymphocytes (CTL) from uP/PS-scFv6.C4 immunized mice lysed B16F10-CEA (56.7%) and B16F10 expressing scFv6.

C4 (B16F10-scFv6.C4) (46.7%) cells, against CTL from uP-immunized mice (10%). After the last immunization, 5×10^5 B16F10-CEA cells were injected into the left flank. All mice immunized with the uP empty vector died within 40 days, but uP/PS-scFv6.C4 vaccinated mice (40%) remained free of tumor for more than 100 days. Splenocytes obtained from uP/PS-scFv6.C4 vaccinated mice showed higher T-cell proliferative activity than those from uP vaccinated mice. Collectively, DNA vaccination with the uP-PS/scFv6.C4 plasmid vector was able to give rise to specific humoral and cellular responses, which were sufficient to retard growth and/or eliminate the injected B16F10-CEA cells.

Keywords DNA vaccine · Single-chain fragment variable · Anti-idiotypic antibody · Immunological tolerance · Carcinoembryonic antigen · Colorectal cancer

Abbreviations

B16F10-CEA	Murine melanoma cell line B16F10 expressing CEA
B16F10-scFv6.C4	Murine melanoma cell line B16F10 expressing scFv6.C4
CEA	Carcinoembryonic antigen
CEA2682	CEA-expressing transgenic mice
CFSE	Carboxyfluorescein diacetate succinimidyl ester
FAPESP	Sao Paulo research foundation
RPMIc	RPMI 1640 medium with supplements
SB	Sleeping beauty
scFv	Single-chain variable fragment
scFv6.C4	Single-chain variable fragment 6.C4
SD	Standard deviation
uP/PS-scFv6.C4	scFv6.C4 expressing plasmid vector

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Introduction

Human gastrointestinal tract neoplasms as well as non-small-cell lung and breast carcinomas produce elevated levels of the tumor-associated antigen (TAA) carcinoembryonic antigen (CEA), which is a densely glycosylated 180-kDa membrane-bound protein [1, 2]. The CEA is produced at high levels in the fetal colon, but its production in the normal adult colonic epithelium is reduced to trace levels [1]. As CEA is expressed during the fetal development, it is considered a self-antigen. However, significant levels of autoantibodies against CEA were detected in the sera from some patients with CEA-producing tumors [3–7], and this is an important evidence that even the CEA self-antigen can activate the immune system in some conditions giving rise to humoral and cellular responses.

One way to combat CEA-expressing tumors is stimulating the immune system with a CEA epitope mimicking molecules. Such mimics may be created by immunizing animals with the anti-CEA monoclonal antibodies (mAb) to generate anti-idiotypic (Id) antibodies, which may have similar three-dimensional structures [8–11].

In many animal models, the specific and efficient protective immunity against CEA-producing tumors was observed after vaccination with anti-Id antibodies [12–16]. Chatterjee and co-workers advanced their anti-Id-based vaccines to treat resected colon-cancer patients with an anti-Id monoclonal antibody in the protein form. In all patients (32) followed in this study, a consistent anti-CEA humoral and cellular immune response was observed [17]. Similar protective immunity against other tumor models had also been observed in both preclinical studies and clinical trials [18–21].

We have described an anti-Id monoclonal antibody, designated mAb 6.C4, which was able to mimic CEA functionally. The anti-Id mAb 6.C4 was shown to elicit antibodies that recognized CEA *in vitro* and *in vivo* [11, 22]. Using the mAb 6.C4 producer hybridoma, the variable heavy and light chain (scFv) sequences were isolated to construct the scFv6.C4 [13]. C57BL/6 mice immunized with a plasmid vector expressing scFv6.C4 were able to elicit antibodies that recognize CEA.

In this study, we immunized CEA-expressing transgenic mice, CEA2682, [23] with scFv6.C4 to assess its protective activity against CEA-expressing tumor cells.

Materials and methods

Research Ethics Committee approval

All animal work described here was performed in full compliance with the institutional guidelines and was approved by the Research Ethics Committee of the Universidade

Federal de São Paulo, Brazil (<http://www.unifesp.br/reitoria/ceua>; Approval number: CEP 2129/08).

Construction of vectors

To construct the uP/PS-scFv6.C4 plasmid vector, pcDNA3-PS/scFv6.C4 [13] was digested with *Hind III* and *Xba I* to release the scFv6.C4, which was inserted into the uP vector [24], previously treated with the same enzymes.

To construct the Sleeping Beauty (SB)-based integrative vector pT2-CAGGS-CEA, the CEA fragment was obtained by digesting pRC/CMV-CEA ([25], which was kindly provided by Dr. Wolfgang Zimmermann, University of Munich, Munich, Germany) with *Hind III* and *Xba I* and treated with Klenow polymerase. The CEA fragment was inserted into the pT2-IDUA vector [26], which was previously digested with *Ava I* (to remove the IDUA fragment) and then treated with Klenow polymerase to make the pT2-CAGGS-CEA vector.

Cell culture, transfection and characterization

Human colorectal carcinoma cell lines CO112 [27], HCT-8 [28] and LISP-1 [29] and murine melanoma cell line B16F10 [30] were maintained (37 °C; 5% CO₂ humidified atmosphere) in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Grand Island, NY, USA), L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml) and buffered with sodium bicarbonate (24 mM) plus 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer (10 mM). The supplemented medium was named RPMIc.

To promote permanent expression of CEA or green fluorescent protein (GFP) in B16F10 cells, 5×10^5 cells in Nucleofector solution (100 µl) containing pT2-CAGGS-CEA (4 µg) and pCMV-SB100 (4 µg), or pT2-HB-AMAXA-GFP (4 µg) and pCMV-SB100 (4 µg) were transferred into a cuvette and submitted to the U008 (Amata Nucleofector II Device; Lonza, Basel, Switzerland) program. The transformed cells were named with basis on the transferred genes B16F10-CEA or B16F10-GFP. The pRC/CMV-CEA and pEGFP-N3 (Clontech; Mountain View, CA, USA) vectors were used as transient controls, being nucleofected as described above. The nucleofected cells were maintained in RPMIc and incubated at 37 °C in a humidified incubator with 5% CO₂. Gene expression was analyzed at different times after transfection.

Vector integration analysis by polymerase chain reaction (PCR)

CEA cDNA integration into the cell genome was assessed by PCR using the following primers (final concentration:

0.4 mM): 5'-CATTGCAACAGCTACAGTC-3' and 5'-AGTGCAGTGGTATCAGAAAC-3'. The thermocycler was programmed to 94 °C (1 min) and 30 cycles of 94 °C (1 min), 58 °C (1 min) and 72 °C (1 min). After the final cycle, the reaction continued (72 °C; 7 min) and the reaction product was then maintained at 4 °C. As an internal control of reaction, the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene was used with the following primers: 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. The PCR products were analyzed by electrophoresis (1% agarose gel with ethidium bromide).

Immunocytochemistry of HCT-8, CO112, LISP-1 and B16F10-CEA cells

Cells were seeded on the coverslips placed in a 24-well plate and incubated in a humidified chamber with 5% CO₂ for 24 h. The media were aspirated, and the cells were fixed in paraformaldehyde (4%; 1 h). After washing with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄; pH 7.2), NH₄Cl (50 mM; pH 8.0) was added and the plate was left for 15 min. Cells were washed again with PBS, before permeabilization (10 min) with PBS containing 0.5% Triton X-100 (PBS-Triton). After new washings, the cells were blocked (30 min) with PBS containing bovine serum albumin (BSA; 10%) and non-fat milk (8%). Cells were incubated (4 °C; humidified chamber; overnight) with diluted (1:50) sera from uP-PS/scFv6.C4 immunized mice. Then, the coverslips were washed with PBS containing Tween-20 (0.25%; PBS-Tween) and incubated for 1 h with diluted biotinylated secondary antibody rabbit anti-mouse IgG (Dako; 1:100).

All coverslips were washed with PBS-Tween. The coverslips coated with HCT-8, CO112, or LISP-1 cells were incubated for 1 h with diluted Alexa 594-streptavidin (Invitrogen; 1:1000). After washings, cell nuclei were labeled during 15 min with diluted 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen; 1:1000). In the case of coverslips coated with B16F10-CEA cells, the coverslips were incubated for 1 h with diluted HRP-streptavidin (Dako; 1:500), and the reaction was developed in a dark chamber at room temperature for 10 min with AEC solution (0.5 mg/ml; Sigma) and H₂O₂ (0.01%) in acetate buffer (0.05 M sodium acetate; pH 5.5). In both cases, at the end, coverslips were rinsed with distilled water and mounted with Fluoromount aid (Sigma).

Quantification of CEA-specific antibody response

Sera from vaccinated mice were evaluated for anti-CEA antibodies (AB3 = AB1') by ELISA. For this purpose, one hundred microliters of a solution containing 0.5 µg/ml

of CEA (Sigma–Aldrich, Germany) in PBS was added to each well of 96-well plates and maintained at 37 °C for 1 h. Plates were blocked with 1% (w/v) BSA (Sigma–Aldrich) in PBS (PBS-BSA) at room temperature for 1 h. The mouse sera diluted in PBS-BSA 0.1% (1:100) were added and incubated at 37 °C for 2 h. The wells were washed three times with PBS containing 0.05% Tween-20 (PBS-Tween), and anti-IgG murine antibody conjugated with biotin (Sigma–Aldrich) diluted in PBS-BSA 0.1% (1:5000) was added and incubated for 2 h at 37 °C. After new washings, horseradish peroxidase-streptavidin (Dako, Carpinteria, CA, EUA) diluted in PBS-Tween (1:1000) was added and incubated for 30 min at room temperature in a dark chamber. The wells were washed as described before adding the ortho-phenylenediamine (3 mg/ml) in 50 mM citrate–phosphate buffer (51.4 mM Na₂HPO₄, 24.3 mM acetic acid, pH 5.0) containing 0.03% H₂O₂. The reactions were stopped with 2 N H₂SO₄. The optical density at 492 nm was read in an ELISA reader (Spectra Max M2^e, Molecular Devices, EUA). Triplicates of each sample were performed.

Mouse tumor model

Human CEA-transfected B16F10 cells (5×10^3 – 5×10^5 cells per mouse and 3 mice per group) were injected (left flank; subcutaneous route) into CEA-expressing transgenic mice (CEA2682; kindly donated by Wolfgang Zimmermann; University of Munich; Munich, Germany) [23], and the tumor size was periodically measured with a caliper during 40 days. If a tumor reaches 1000 mm³, this mouse is euthanized.

Preventive DNA vaccination and tumor cell challenge

CEA2682 mice were immunized by injection of 50 µg of uP-PS/scFv6.C4 or uP plasmid DNA in 50 µl PBS at each quadriceps muscle. Six electric pulses (100 V, 40 ms duration per pulse, 1 s interval) were applied through needle electrodes (Electroporator T820—BTX Genetronics, San Diego, CA, EUA) that were placed around DNA injection site. Four subsequent immunizations were performed with 2-week intervals. Blood samples were collected 7 days after each immunization to quantify anti-anti-idiotypic (anti-anti-Id or AB3) antibody production. Fifteen days after the last immunization, some mice were euthanized to obtain spleens for assessment of cellular response by T-cell proliferation and cytotoxicity assays. To assess the protective effect of the vaccination, mice were challenged with s.c. injection of 5×10^5 B16F10-CEA cells in the left flank, 10 days after the last immunization. Immediately after challenge, mice received a new boost with the same immunization dose. All animals were euthanized 15 days after tumor cell injection. Tumor volume was estimated

according to the following formula: $V = A \times B \times C$ (A : length, B : width, C : height) and expressed in mm^3 .

In vitro T-cell proliferation assay

Fifteen days after the B16F10-CEA injection, splenocytes were collected and treated with the ammonium chloride potassium buffer (0.15 M NH_4Cl ; 10 mM KHCO_3 ; 0.1 mM disodium, EDTA; pH 7.4) to lyse the red blood cells. For the proliferation assay, 7×10^6 splenocytes suspended in PBS (140 μl) were labeled at 37 °C for 15 min with 2.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Cell-Trace™; CFSE Cell Proliferation Kit; Invitrogen; Carlsbad, CA, USA). Cells were centrifuged at $1500 \times g$ for 5 min and washed three times with RPMI containing 10% FBS. In a 96-well plate (U-bottom shape), 4×10^5 cells in 200 μl RPMIc were plated per well. The cells were stimulated with 2.0 $\mu\text{g}/\text{ml}$ CEA (Sigma–Aldrich) or scFv6.C4, obtained from the supernatant of HEK293 cells transfected with uP-PS/scFv6.C4 by the calcium phosphate method, or 2.5 $\mu\text{g}/\text{ml}$ concanavalin A (ConA; Sigma–Aldrich). Non-stimulated cells were used as a negative control. All experiments were carried out in triplicate. After 6 days of incubation at 37 °C in a CO_2 chamber, the cells were washed with MAC buffer (PBS containing 2 mM EDTA and 0.5% BSA; pH 7.2) and labeled in a dark chamber at 4 °C for 45 min with anti-CD4-PercP (1:200), anti-CD8-APC (1:200) or anti-CD45R/B220-PE (1:200) antibodies (BD Bioscience; San Jose, CA, USA). The labeled cells were washed three times and suspended in 200 μl MAC buffer. The samples were analyzed by flow cytometry (BD FACS Canto; Mississauga, ON, USA), using the BD FACSDiva (BD Bioscience; Sao Paulo, SP, Brazil) and Flow Jo (Three Star; Ashland, OR, USA) softwares.

Cytotoxicity assay

The B16F10-CEA and B16F10-scFv6.C4 cells were used as targets. Initially, 5×10^3 cells of each condition were seeded per well in 96-well plates containing RPMIc. After 24 h, 5×10^5 splenocytes were added per well in the presence of 2.0 $\mu\text{g}/\text{ml}$ CEA for effector stimulation. After 5 days, 10 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; Invitrogen) were added per well and incubated for 3 h at room temperature. The supernatant was discarded and 100 μl dimethyl sulfoxide (DMSO) were added, and the solution was left under slow agitation in a dark chamber for 15 min. Optical densities ($\lambda = 595$ nm) of samples were read using a Spectra Max (model M2^e; Orleans Drive Sunnyvale, CA, USA) spectrophotometer, and the lysis percentage was determined using the following formula:

$$\% \text{ cell lysis} = 100 - [(100 - \text{CVE}) / \text{RCV}]$$

CVE: $\text{OD}_{595\text{nm}}$ with target cells RCV: $\text{OD}_{595\text{nm}}$ without target cells

Statistical analysis

Statistical analyses were performed using the GraphPad (v. 5.0; La Jolla, CA, USA) software. Data were analyzed using analysis of variance (ANOVA) followed by the Bonferroni test. The data were presented as mean \pm standard deviation (SD). A value of $P < 0.05$ was considered to be significant. The survival analysis was performed using the Mantel–Cox method.

Results

In vivo tumor model with B16F10 cells expressing CEA

To establish an animal model and evaluate scFv6.C4 vaccination, first we focused on creating a mouse tumor cell line expressing human CEA permanently. The murine melanoma cell line B16F10 [30], which is a well-known cell line in tumor biology due to its high capacity of tumorigenesis in vivo, was nucleofected. Initially, the GFP expressing vectors pEGFP-N3 and pT2-HB-AMAXA-GFP, which are non-integrative and integrative vectors, respectively, were used to evaluate transfection efficiency and transgene expression duration in B16F10 cells. Transfection efficiency, which was assessed 2 days after nucleofection by counting GFP positive cells, was about 85–90% in both vectors (not shown). GFP gene expression in pEGFP-N3 transfected cells could be observed until day 7, but no GFP positive cell was observed at day 15 (Fig. 1a). On the other hand, about 50% were GFP positive cells in the plate of the pT2-HB-AMAXA-GFP transfected cells on day 30, indicating long-term GFP gene expression and high nucleofection efficiency.

Based on these results, B16F10 cells were transfected with the CEA-expressing vectors, pRC/CMV-CEA or pT2-CAGGS-CEA, which are non-integrative and integrative systems, respectively. By immunocytochemistry analyses, CEA expression was observed only in the cells transfected with pT2-CAGGS-CEA and the naturally expressing cell line CO112 (Fig. 1b). The estimated transfection efficiency was about 90% (not shown). Integration of the CEA expression cassette was confirmed by PCR, only in the pT2-CAGGS-CEA-transfected B16F10 cells (Fig. 1c).

To establish the conditions of the in vivo experiments, several concentrations of B16F10-CEA cells were injected into the left flank of CEA2682 transgenic mice. With the highest concentration (5×10^5 cells), a visible tumor was observed around day 10 post-injection, reaching a volume of 800- mm^3 15 days after injection (Fig. 1c). Injection of

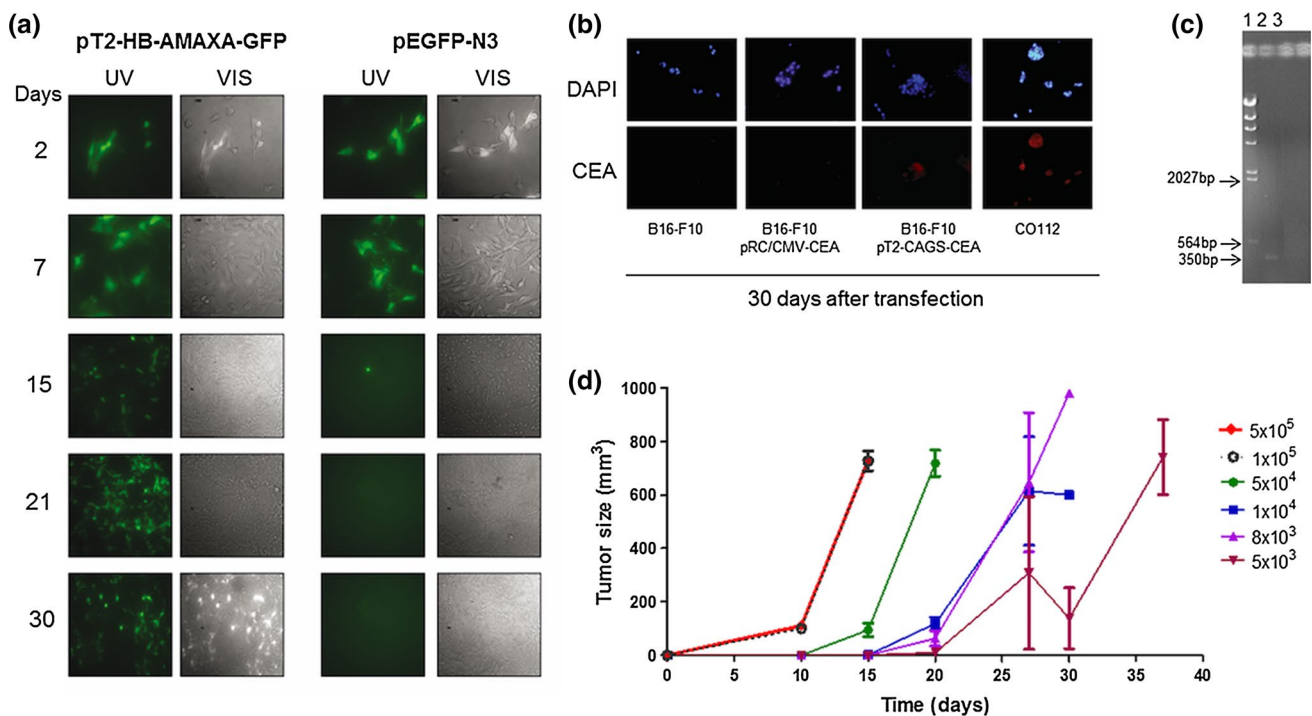


Fig. 1 Establishment of a tumor model with B16F10-CEA. **a** B16F10 cells were nucleofected with pT2-HB-AMAXA-GFP and pCMV-SB100 or pEGFP-N3, and GFP expression was monitored during 30 days by fluorescence microscopy. **b** B16F10 cells were nucleofected with pT2-CAGGS-CEA and pCMV-SB100 (B16F10-CEA) or pRC/CMV-CEA, and CEA expression was detected by immunocytochemistry after 30 days. The CO112 and B16F10 cells

were used as positive and negative controls, respectively. **c** Integration of CEA in the genome was analyzed by PCR 4 weeks after nucleofection. 1: λ Hind III ladder. 2: B16F10-CEA. 3: B16F10. The expected amplicon is 350 bp (base pair). **d** Tumor growth rate was determined after s.c. injection of B16F10 cell suspension in the left flank. Each *time point* corresponds to 3 mice per group. Representative data of 2 biological replicate experiments are shown

lower numbers of cells postponed the onset of tumor formation, and also decreased the tumor growth rate, as can be noted by the decrease in the curve slope. Based on these observations, we used 5×10^5 B16F10-CEA cells in the following experiments.

Humoral and cellular immune responses induced by uP-PS/scFv6.C4 DNA vaccination

To evaluate the immune response induced after vaccination with the uP/PS-scFv6.C4 vector, the CEA2682 mice were immunized four times with 2-week intervals. The DNA vaccination with plasmid solution was performed by i.m. injection followed by electroporation based on our previous experience [31].

Blood samples were collected 1 week after each immunization to determine the AB3 antibody titers. A significant AB3 titer was detected after the third immunization, and the highest titer was achieved after the fourth immunization (Fig. 2a). However, additional immunization did not increase the antibody titers (not shown).

To assess CEA-specificity of the AB3 antibody, sera obtained after the last immunization were tested

by immunocytochemistry against the CEA-expressing human colorectal cell lines CO112, HCT-8, and LISP-1 and murine cell lines B16F10-CEA. Pre-immune sera or sera from mice immunized with the uP vector were used as negative controls (Fig. 2b). All the CEA-expressing cell lines were strongly marked with sera from uP-scFv6.C4-immunized mice, whereas pre-immune sera (Fig. 2b) or sera from mice vaccinated with the uP vector did not show any reaction. These results confirm the effectiveness of the scFv6.C4 recombinant protein to mimic CEA.

To evaluate the CTL immune response, two target cells, B16F10-CEA or B16F10-scFv6.C4, were initially constructed to express CEA and scFv6.C4, respectively.

About 10% of B16F10 cells transfected with the empty vector uP (which was used as a control) were lysed by splenocytes from mice immunized with uP/scFv6.C4 or uP. Such percentage was considered as nonspecific basal activity. Furthermore, splenocytes obtained from CEA2682 immunized with uP vector presented basal CTL activity upon B16F10-CEA and B16F10-scFv6.C4 cells. On the other hand, uP-PS/scFv6.C4-immunized CEA2682 mice showed a strong CTL activity, lysing B16F10-CEA (56.7%) and B16F10-scFv6.C4 (46.7%) cells (Fig. 2c). The

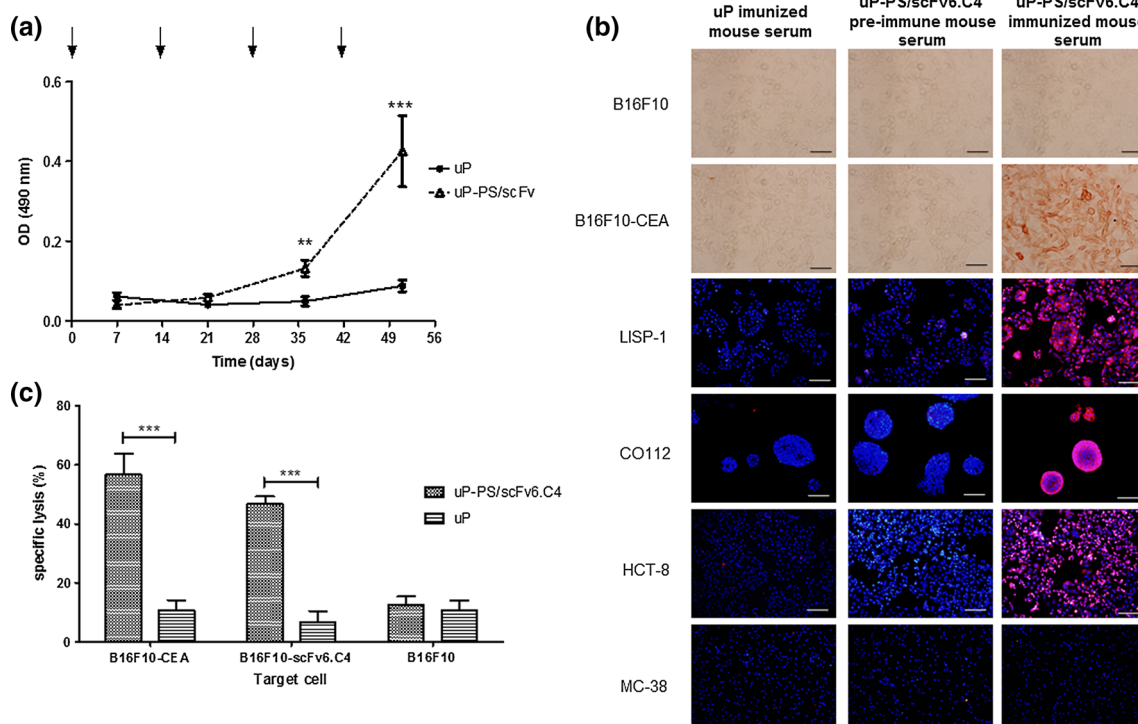


Fig. 2 Humoral and cellular responses raised after DNA vaccination with uP-PS/scFv6.C4. **a** CEA-specific AB3 antibodies from mice immunized with uP-PS/scFv6.C4 were detected by ELISA. Blood samples were collected 7 days after each immunization and diluted 1:100 for assays. Sera from mice immunized with uP vector were used as control. *Arrows* indicate day of immunization. Values for OD_{490nm} are expressed as mean \pm SD of each group ($n = 12$ per group). Representative data of 2 biological replicate experiments are shown. *** $p \leq 0.001$ and ** $p \leq 0.01$. **b** Immunocytochemistry of human colorectal adenocarcinoma cell lines, murine melanoma cell

lines (B16F10 and B16F10-CEA) and murine colon tumor cell line (MC38) using sera from uP-PS/scFv6.C4-immunized mice. Sera from uP-immunized mice and pre-immune sera from uP-PS/scFv6.C4-immunized mice were used as negative control. *Bar* 100 μm . **c** CEA-specific CTL activity was determined using splenocytes isolated 7 days after the last immunization. B16F10-CEA, B16F10-scFv6.C4, and B16F10 cells were used as targets (target:effector ratio: 1:50). Data are expressed as mean \pm SD of each group of mice (3 mice per group). Representative data of 2 biological replicate experiments are shown. *** $p \leq 0.001$

percentage of lysis in the CEA-transfected cells was higher than in the scFv6.C4-transfected ones. However, the difference was not statistically significant.

Protection by uP-PS/scFv6.C4 DNA vaccination against tumor challenge

To assess the effectiveness of the preventive DNA uP-PS/scFv6.C4 vaccine, 5×10^5 B16F10-CEA cells were injected into the left flank of mice 10 days after the last immunization, and the tumor growth was measured periodically. In mice vaccinated with the uP vector, tumor volumes were about 180 mm^3 on the 10th day, reaching about 800 mm^3 on the 15th day (Fig. 3). Soon after the last tumor measurement, these mice were euthanized due to tumor overgrowth. On the other hand, tumor volume in mice vaccinated with uP-PS/scFv6.C4 was only about 20 mm^3 on the 10th day, reaching 210 mm^3 on the 15th day. This difference indicates that this vaccination strongly prevented tumor growth in comparison to the control group. The

difference between these two groups was statistically significant ($p < 0.05$) (Fig. 3).

The preventive DNA vaccine study was also carried out challenging mice with a lower number of B16F10-CEA cells (8×10^3 cells) following the same immunization protocol as used above. In mice immunized with the uP vector, visible tumors appeared between the 16th and 30th days after injection, and the tumor volume reached 800 mm^3 in less than 10 days later. However, visible tumors were observed only on the 34th day after tumor injection in 3 out of 5 uP-PS/scFv6.C4 vaccinated mice, and no visible tumors were seen over 100 days of observation in the rest of the vaccinated mice (Fig. 3c). These results showed that the vaccine regimen with the uP-PS/scFv6.C4 vector is able to delay tumor growth either increasing animal survival or even preventing tumor growth completely.

To assess specific T- and B-cell proliferative activities in vitro, splenocytes from vaccinated and B16F10-CEA-challenged mice were harvested and stimulated with CEA

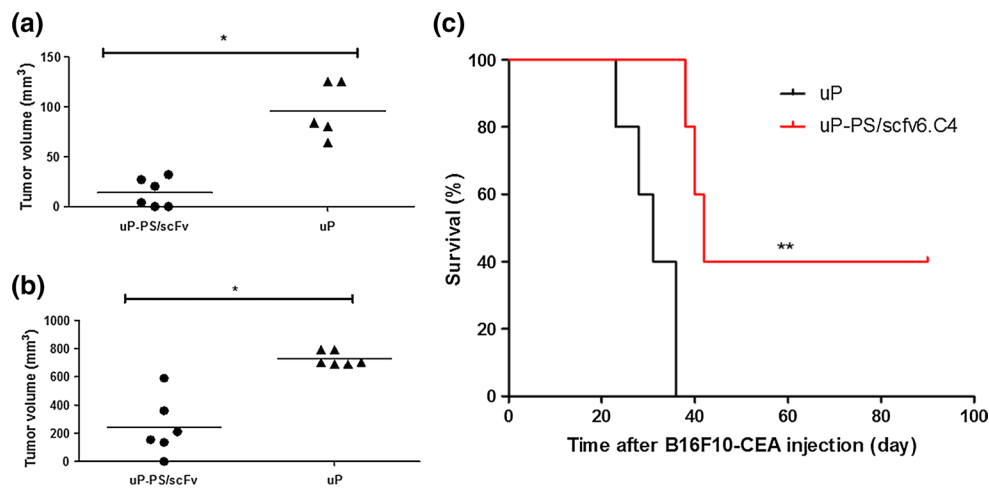


Fig. 3 Protection by uP-PS/scFv6.C4 DNA vaccination against B16F10-CEA tumor challenge. CEA-expressing CEA2682 mice were immunized four times with uP-PS/scFv6.C4 or uP, and ten days after the last immunization 5×10^5 B16F10-CEA cells were injected subcutaneously. Representative data of 2 biological replicate experiments are shown below. Tumor growth was measured with a caliper 10 days

(a) and 15 days (b) later ($n = 6$ per group). In other immunized mice, 8×10^3 B16F10-CEA cells were injected subcutaneously and tumor growth was followed for 100 days ($n = 5$ per group). c To estimate survival rate by the Mantel–Cox method, mice with tumors smaller than 500 mm^3 were considered as survivors. * $p \leq 0.01$, ** $p \leq 0.01$. $n = 5$ per group

or scFv6.C4. ConA (2.5 $\mu\text{g/ml}$) and no stimulator were used as positive and negative controls, respectively.

CD4⁺ and CD8⁺ T-cells from uP-immunized mice proliferated about 10% after stimulation with CEA or scFv6.C4; meanwhile, in the cells from the uP/PS-scFv6.C4-immunized mice, CEA stimulated $22.4 \pm 2.6\%$ CD4⁺ and $24.2 \pm 2.0\%$ CD8⁺ cells, and scFv6.C4 stimulated $22.5 \pm 1.0\%$ CD4⁺ and $32.2 \pm 0.6\%$ CD8⁺ cells (Fig. 4). As the unstimulated CD4⁺ and CD8⁺ cells had about 10% proliferation and ConA-stimulated cells about 50%, the main T-cells' proliferative activity from uP/PS-scFv6.C4-immunized mice should be specific to CEA and scFv6.C4.

The B-cell proliferation after stimulation with CEA or scFv6.C4 was less than 10%, irrespective of vaccinated groups. A similar B-cell proliferation rate was seen without stimulation (Fig. 4c), showing no specificity of B cells from uP/PS-scFv6.C4-immunized mice to CEA and scFv6.C4. These results indicate that the immune protection seen after vaccination is mostly due to T-cell activities.

Discussion

Active immunotherapy using a TAA epitope surrogate to stimulate immune system is an attractive strategy because, in principle, TAA-expressing tumors may be recognized by the patients' immune system as nonself, consequently, be pursued and destroyed. Based on this principle, some groups constructed CEA epitope surrogates and showed elicitation of humoral and cellular responses [8, 10, 13].

In our previous study, we described the construction of scFv6.C4, a CEA epitope surrogate, using RNA isolated from the hybridoma cell line 6.C4 and the expression vector pcDNA3-PS/scFv6.C4 [11, 13]. C57BL/6 wild-type mice immunized with pcDNA3-PS/scFv6.C4 raised a specific humoral immune response against CEA, and this response was shown by both immunocytochemistry using the human colon adenocarcinoma cell line CO112 [27] and immunohistochemistry using patient tumor biopsies [13]. However, scFv6.C4 ability to elicit immune response and provide protection against tumor challenge in CEA-expressing transgenic mice has not yet been demonstrated.

The vector delivery mode and transgene expression level are important variables that significantly affect the immune response of vaccinated animals [32, 33]. In this study, we used uP-PS/scFv6.C4 instead of pcDNA3-PS/scFv6.C4 vector because the uP vector was constructed with the strong CMV promoter and enhancer, without eukaryotic reporter or selection gene, which drives the transgene expression level much higher than pcDNA3 vector does [24]. In addition, electroporation instead of simple i.m. vector injection was adopted to transfer the uP-PS/scFv6.C4 vector into the skeletal muscle because in our previous study we observed a high level of gene transfer and a consequent high level of transgene expression [31]. Although electroporation appears to be very invasive, it has already been used in clinical studies for DNA vaccines and immunotherapies without significant side-effects or discomfort [34].

To test our DNA vaccine in vivo, we used the genetically modified tumor cell line B16F10 to permanently express human CEA gene with the SB system. This cell line was

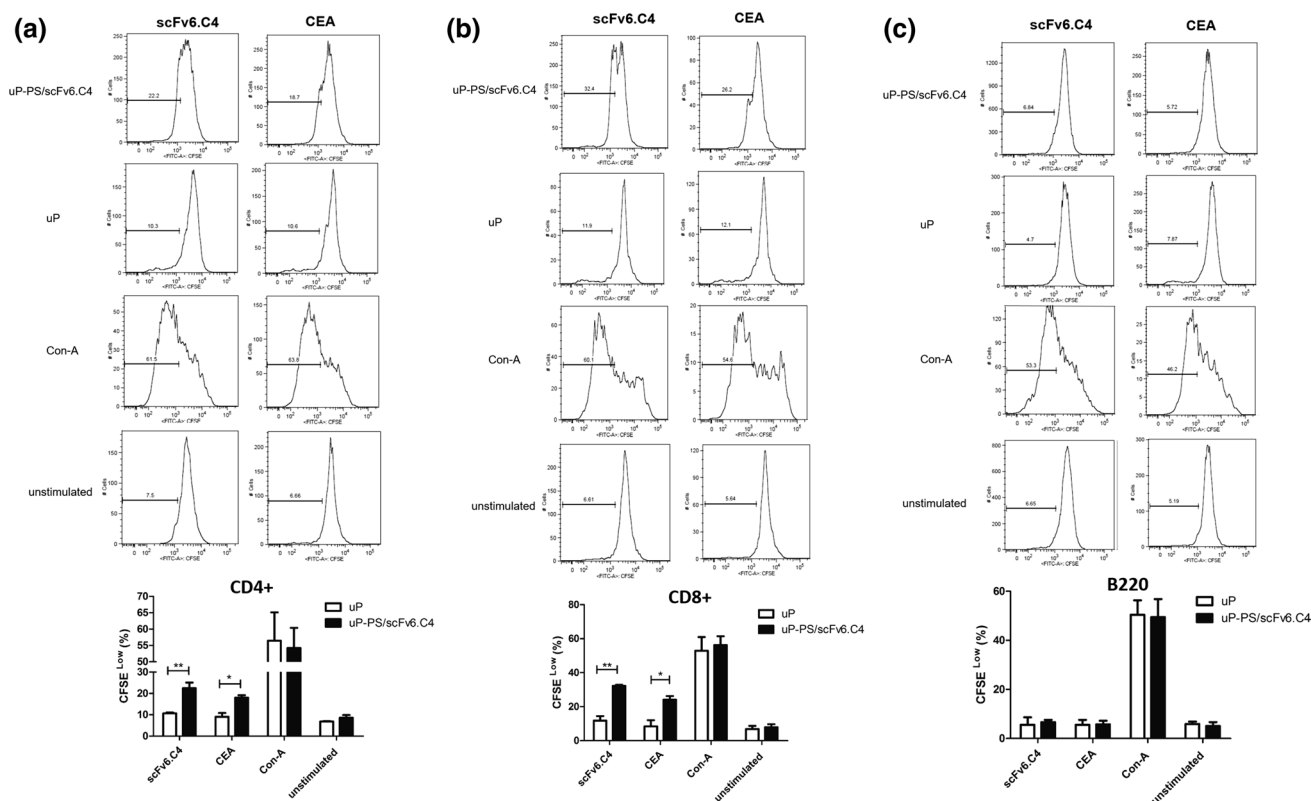


Fig. 4 Proliferation assay of splenocytes. CEA-expressing CEA2682 mice were immunized with uP-PS/scFv6.C4 or uP, as described in the legend of Fig. 3 and Materials and Methods section. Fifteen days later, these mice were euthanized to obtain splenocytes, which were labeled with 2.5 μ M CFSE and stimulated with CEA or scFv6.

C4 for 6 days. Frequency of CD4+ (a), CD8+ (b) and CD45R/B220 (c) CFSE-low cells were determined by flow cytometry. Data are expressed as mean \pm SD of each group of mice (4 per group). Representative data of 2 biological replicate experiments are shown. * $p \leq 0.05$, ** $p \leq 0.01$

chosen for our study because it has the same genetic background of the CEA2682 mouse cells [30] and high capacity to both grow in vivo when injected subcutaneously and form a well-localized solid tumor that can be easily measured with a caliper. In addition, this cell line can be used to create a metastatic model by simple injection in the tail vein [35], which is our next challenge to show the functioning of our vaccine in the final stage of cancer.

Figure 3 shows a clear evidence of the immune protection after vaccination with uP-PS/scFv6.C4 vector. However, this protection was not enough to eliminate the 5×10^5 B16F10-CEA injected cells, and all vaccinated mice had to be euthanized later due to tumor overgrowth. Classically, as efficiency of any therapeutics is dose dependent, we have hypothesized that the number of injected B16F10 cells was higher than the immunity level provided by vaccination. To test this hypothesis, we challenged the vaccinated mice injecting 8×10^3 B16F10-CEA cells, which delayed about 10 more days in comparison to the 5×10^5 cell injection to form a similar tumor size (Fig. 1c). After challenge with less B16F10-CEA cells, 40% of vaccinated mice remained free of tumor during all

period of experimentation. These results clearly show that this vaccination raised a specific immunity against CEA. However, the level of immune response, which depends on the immunization protocol, and the challenge with tumor cells, which depends on the type and number of injected cells, must be quantitatively evaluated by varying these parameters to estimate vaccination efficiency. Such parameters become more relevant when planning clinical trials, because individual physiological and pathological variabilities are higher than those of the experimental conditions used here by us and by other investigators who use isogenic animals and a tumor cell line for tumorigenesis. For example, in a phase-III study (343 colorectal patients) with the 3H1 anti-Id antibody, a CEA epitope surrogate [8], they showed strong (only about 12%), weak (31%), and no (30%) immune responses, with median survivals of 28.2, 15.8 and 8.3 months, respectively [36]. These data show that vaccination with the CEA epitope surrogate raised the immune response enough to combat the last-stage of tumor progression in these patients. However, the best outcomes depend on raising a strong immune response, which depends on the relationship between immunization

protocol and tumor progression state, beyond other clinical factors such as comorbidity and aging.

Cellular response assessed by a specific cell lysis assay showed a slightly higher activity on cells expressing CEA than scFv6.C4 (Fig. 2c), which was not statistically significant. On the other hand, the T-cell proliferation assay showed higher stimulation with scFv6.C4 than CEA, thus indicating an apparent higher affinity of target cells to scFv6.C4 than to CEA. Nevertheless, as we have used scFv6.C4 from the supernatant of HEK293 cells transfected with uP-PS/scFv6.C4 the actual scFv6.C4 concentration is unknown; therefore, these results allow us to only conclude that a cellular response occurred without, however, power for quantitative comparison. Production, purification and quantification of the recombinant protein scFv6.C4 are technical limitations we faced in this study.

Collectively, we showed that preventive DNA vaccination performed by electroporation of the uP-PS/scFv6.C4 vector raised humoral and cellular responses in CEA-expressing transgenic mice, which were sufficient to retard and/or eliminate injected B16F10-CEA cells.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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