Biochemical and Biophysical Research Communications 440 (2013) 222–228

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A DNA vaccine against cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) prevents tumor growth

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ARTICLE INFO

Article history:
Received 26 August 2013
Available online 13 September 2013

Keywords:
Cytotoxic T-lymphocyte associated-antigen-4 (CTLA-4)
Antigen-presenting cells (APC)
Vaccine
Liposome

ABSTRACT

Co-stimulatory signaling pathway triggered by the binding of B7.1/B7.2 (CD80/86) of antigen-presenting cells (APCs) to CD28 of T cells is required for optimal T-cell activation. Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is a negative regulator of T cell activation, which competes with CD28 for B7.1/B7.2 binding with a greater affinity. Ipilimumab, a monoclonal antibody against CTLA-4, has shown positive efficacy in a pivotal clinical trial for the treatment of metastatic melanoma and was approved by FDA. However, the cost of monoclonal antibody-based therapeutics might limit the number of patients treated. To develop a novel therapeutics specifically targeting CTLA-4, we constructed a DNA vaccine by cloning the sequence of CTLA-4 fused with a transmembrane domain sequence of placental alkaline phosphatase (PLAP) into a mammalian expression plasmid, pVAC-1. Immunization with the resulting construct, pVAC-1-hCTLA-4, elicited antibody specific to human CTLA-4 with cross reactivity to murine CTLA-4, which was sufficient for inhibiting B16F10 tumor growth in c57BL/6 mice in the absence of measurable toxicity. Coupling liposome with pVAC-1-mCTLA-4 could break tolerance to self-antigen in BALB/c mice and induce potent immunity against murine CTLA-4, and suppress growth of subcutaneous renal cell carcinoma (Renca).

1. Introduction

The immune system exerts "tumor surveillance" by identifying and eradicating cancer cells before they grow into tremendous amounts. However, tumor cells are capable of switching off anticancer immunity via a process called "immunoediting", which selects tumor variants nonimmunogenic to immune system, thereby escaping from tumor surveillance [1]. Additionally, tumor cells may adopt the strategy of "immunesubversion" through active inhibition of anticancer immunity. It is well known that effective adaptive immunity needs the interaction of major histocompatibility complex (MHC) and tumor-associated antigen (TAA) peptide complex on antigen-presenting cells (APC) with the T-cell receptor. A later elucidated costimulatory signal regarding the binding of B7.1/B7.2 (CD80/86) of APC to CD28 of T cell is required for optimal T-cell activation [2].

Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) is a subsequently characterized player of this signaling pathway, and turns out to be an essential negative regulator. CTLA-4 is up-regulated after the activation of T cells and competes with CD28 in binding to B7.1/B7.2 with a greater affinity. Therefore, the immune response is impeded and unwanted nonspecific responses to self-antigens could be avoided [3,4]. However, while CTLA-4 protects individuals from autoimmune disease, it could also suppress anticancer immunity. Several approaches manipulating T-cell costimulating pathway are being explored to enhance anticancer immune response. An antibody targeting CTLA-4, ipilimumab, has demonstrated clinical benefit in pivotal trial for metastatic melanoma and was subsequently granted FDA approval. Moreover, the anti-CTLA-4 antibodies are being actively investigated in other indications, including non-small cell lung, prostate, pancreatic, liver and colorectal cancers, etc. [5,6].

Promising anticancer results of anti-CTLA-4 antibodies have been increasingly demonstrated by numerous preclinical and clinical studies. The approval of ipilimumab was hailed as a great breakthrough of cancer therapy [7]. Additionally, when it was combined with Nivolumab, an antibody against another immune-modulating protein PD-1, even more promising results were evidenced by rapid tumor response in a significant portion of trial patients [8]. However, the staggering price may dissuade numerous cancer

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.09.031

patients to be treated with this innovative therapeutics [9]. In this study, we aim to develop a vaccine specifically targeting CTLA-4 by cloning the coding sequence of either murine or human CTLA-4 fused with a transmembrane domain sequence of placental alkaline phosphatase (PLAP) into a mammalian expression plasmid, pVAC-1. We examined the immunogenicity of the resultant CTLA-4 DNA vaccine and observed enhanced humoral response against CTLA-4 in immunized mice. Immunization of pVAC-1-hCTLA-4 induced human CTLA-4 specific antibody with cross reactivity to murine CTLA-4 and suppressed B16F10 tumor growth *in vivo*. Moreover, cationic liposome-coupled pVAC-1-mCTLA-4 was able to overcome tolerance to murine CTLA-4 self-antigen in mice and suppressed growth of subcutaneously inoculated Renca. Our findings may offer an alternative anti-CTLA-4 therapy rather than antibody for the treatment of cancer.

2. Materials and methods

2.1. Cell lines and reagents

Murine melanoma cancer cell line, B16, and renal cell carcinoma cell line, RENCA, were purchased from American Type Culture Collection. B7.1 and B7.2 stable CHO cells were kind gifts from Dr. Mi-Hua Tao at Academia Sinica, Taiwan. Blasticidin was purchased from Sigma Aldrich (St. Louis, MO).

2.2. Construction of CTLA-4 DNA vaccines

The DNA sequences encoding murine and human CTLA-4 were PCR amplified using a cDNA library obtained respectively from leukocyte of mouse and human as template. The forward and reverse primers for amplifying human CTLA-4 were 5′-TGCCAGGATCCTGGCAATGCACGTGGCCCA-3′ and 5′-GTGGTGAATTCAGAATCTGGGCACGGTTCTG-3′, respectively, whereas those for murine CTLA-4 were 5′-GGCCAGGATCCCTGAATTCATGGAAGCCATACAGGTGACCCA-3′ and 5′-GTGGTCAATTGGTCAGAATCCGGGCATGGTTC-3′. The resulting PCR products were cut with BamHI and EcoRI, introduced in PCR primers, ligated and fused with IL2 secretion signal sequence (IL2ss) and a transmembrane domain sequence of placental alkaline phosphatase (PLAP), at N- and C-terminus, respectively, into a mammalian expression plasmid, pVAC-1 (Invitrogen, Carlsbad, CA).

2.3. Immunization of mice with pVAC-1-mCTLA-4 and pVAC-1-hCTLA-4

c57BL/6 mice (n = 5/group) aged 6–7 weeks were vaccinated by intramuscular injection into quadriceps with 100 μg of either pVAC-1-hCTLA-4 or pVAC-1 control vector, as naked DNA. Vaccinations were conducted once per week for 4 weeks. 1 week after the last immunization, the mice were subcutaneously inoculated with B16-luc cells (1×10^5) in the left hind leg. Similarly, BALB/c mice (n = 5–6/group) aged 6–7 were immunized with either pVAC-1-hCTLA-4 or pVAC-1 control vector, as naked DNA or complexed with cationic liposome. 1 week after completion of vaccination, the mice were inoculated with RENCA cells (1×10^5) in the left hind leg. Tumor measurement was performed using calipers and the tumor volumes were calculated according to the formula of $(1/2)$ length (L) $\times$ width $(W)^2$.

2.4. Detection of serum antibodies against CTLA-4

*One week a*fter the last immunization of mice, the serum was collected and examined for specificity to human and murine CTLA-4. 96-well ELISA plates were prepared by coating with either human or murine CTLA-4 (R&D systems, Minneapolis, MN, USA) at 1 μg/ml in borate saline (BS) buffer, pH 8.4, for 4 h at room temperature, and then blocked with borate saline plus 1% (w/v) bovine serum albumin (BS–BSA). Serial twofold dilutions of mouse serum in BSA (1:50 to 1:800) were added to duplicate wells and incubated overnight at 4 °C. Plates were washed with PBS with 0.05% (v/v) Tween-20 and incubated with HRP conjugated goat anti-mouse IgG diluted 1:2000 in BS–BSA for 2 h at room temperature. HRP substrate 2,2′-azino-bis(ethylbenzthiazoline sulfonic acid) was added and incubated for 20 min at room temperature. Absorbance was measured at 405 nm on an ELISA reader.

2.5. Flow cytometry analysis

CHO-B7.1 and CHO-B7.2 cells (5×10^5) were incubated with 2 ng/ml of hexa-histidine tagged human CTLA (hCTLA-4-his$_6$) in the presence or absence of serum of mice immunized with the pVAC-1-hCTLA-4 or the control DNA vaccine at room temperature for 1 h. The bound hCTLA-4-his$_6$ protein was detected with FITC-labeled mouse anti-his$_6$ antibody at 1:200 and subjected to FACS analysis.

2.6. Cytotoxicity assay

*T*he B16F10-bearing mice immunized with either control vector, pVAC-1, or pVAC-1-hCTLA-4 were sacrificed and the splenocytes were collected for examination of cytotoxic effect on cancer cells. The splenocyte-mediated cytotoxicity was measured using the previously screened B16F10 cells stably expressing luciferase (B16F10-luc cells). The B16F10-luc cells (2000 cells/well) were added into 96-well in the presence of 100- or 30-fold (2×10^5 or 6×10^4 cells) of splenocytes. Three days after incubation, the cells were added with luciferase substrate, luciferin, and subjected to IVIS Imaging System for quantification of cell viability represented by photon counts per second.

2.7. Interferon-gamma measurement

The amounts of interferon-gamma produced by splenocytes encountering B16F10-luc in the cytotoxicity assay were using an ELISA kit (R&D system) according to the vendor's manual. In a separate experiment, the splenocytes obtained from the immunized mice were incubated with purified human CTLA4 antigen and the stimulated secretions of interferon-gamma were also quantitated.

2.8. Data analysis

Data are given as a means (±SEM) of at least two independent experiments. Data fitting and statistical analyses were computed using GraphPad Prism (GraphPad Software, San Diego, CA).

3. Results

3.1. Immunization of mice with hCTLA-4 DNA vaccine generates antibody against both human and murine CTLA-4

To develop a novel therapeutics specifically targeting CTLA-4, we constructed a DNA vaccine by cloning the sequence of either human or murine CTLA-4 fused with a transmembrane domain sequence of placental alkaline phosphatase (PLAP) into a mammalian expression plasmid, pVAC-1. After immunization of mice by intramuscular injection once per week for four consecutive weeks, the mice serum was collected and examined for specificity to both human and murine CTLA-4. Our data indicates that this DNA vaccine can induce antibody specific to human CTLA-4 and to a lesser degree to murine CTLA-4 (Fig. 1A and B).

3.2. Serum from mice immunized with pVAC-1-hCTLA-4 blocks association of hCTLA-4 with B7.1 and B7.2

Given that CTLA-4 exerts its function by binding to CD-80 (B7.1) and CD-86 (B7.2) on the surface of antigen-presenting cells, the serum of the immunized mice was examined for the inhibitory effect on the interaction between purified hexa-histidine (his_6) tagged CTLA-4 protein and the B7.1/B7.2 stably expressed on Chinese hamster ovarian (CHO) cells. The cells (5×10^5) were incubated with 2 μg/ml of his_6-tagged human CTLA in the presence or absence of mice serum immunized with the pVAC-1-hCTLA-4 DNA vaccine or the control DNA vaccine. The bound proteins were detected with FITC-labeled mouse anti-his_6 antibody at 1:200 and subjected to FACS analysis. The results were shown in (Fig. 1C and D). It was indicated that the interaction between His_6-human CTLA-4 (2 ng/ml) and B7.1/B7.2 expressed on stably transfected CHO cells (5×10^5 cells/assay) was blocked by the serum obtained from the mice immunized with the pVAC-1-hCTLA-4 DNA vaccine, whereas no sign of inhibition was found in those from the control group at 1:25 dilution (Fig. 1C and D).

3.3. Suppressed growth of B16 melanoma in hCTLA-4 DNA immunized mice

To examine the protective effect of immunity against CTLA-4, we subcutaneously inoculated the immunized c57BL/6 mice with

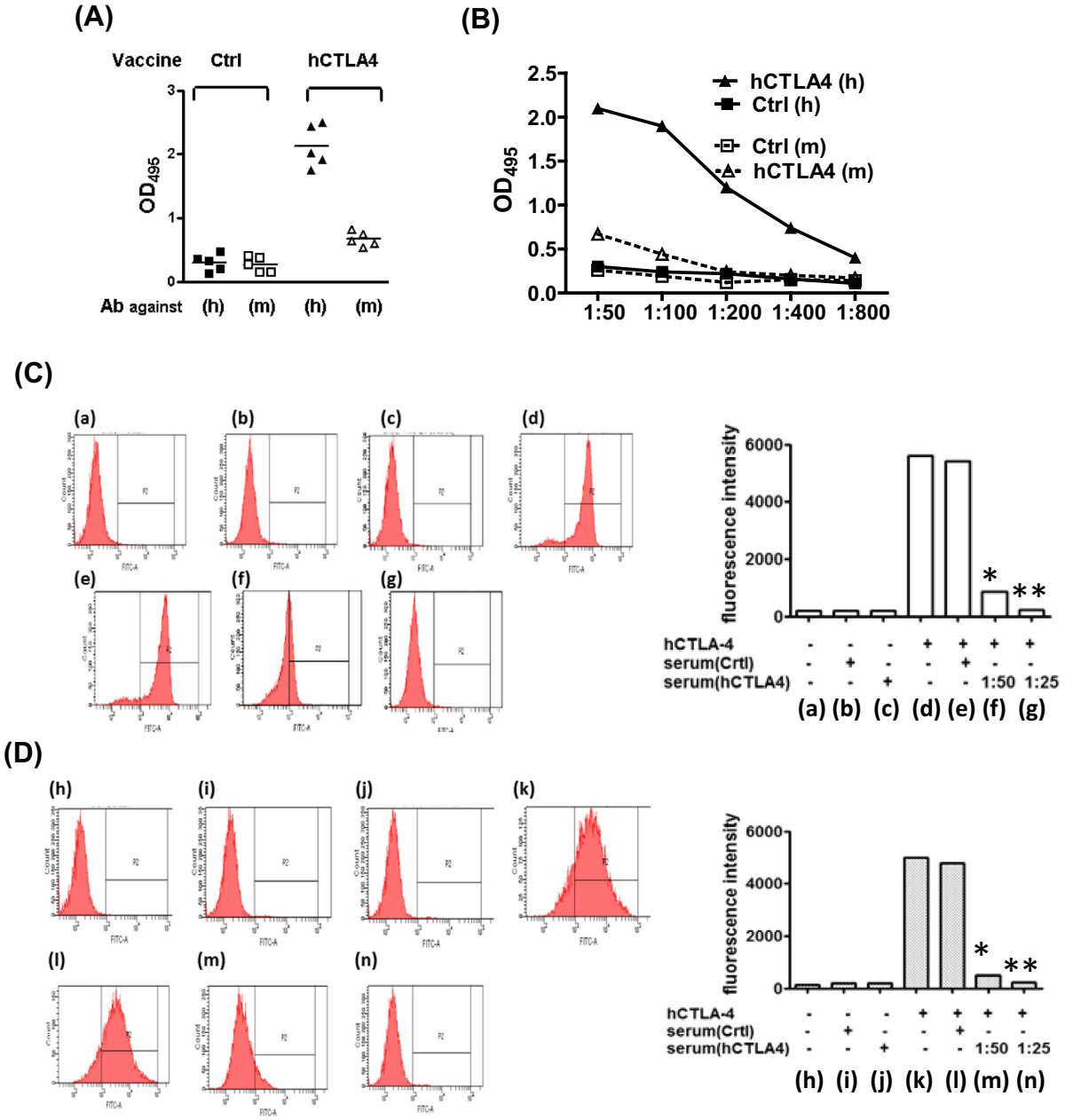

Fig. 1. Production of anti-hCTLA-4 antibodies in mice vaccinated with the pVAC-1-hCTLA-4. c57BL/6 mice (5 mice/group) were injected intramuscularly with naked DNA vaccine or control plasmid. 1 week after the last vaccination, sera from the immunized mice demonstrated hCTLA-4-specific antibody titers in (A) 50-fold as well as (B) serial dilutions of sera, compared with the control groups ($p < 0.005$). Cross-reactivity to mCTLA-4 was also detected in pVAC-1-hCTLA-4 immunized mice as compared with control group ($p < 0.05$). The binding of hCTLA-4-his_6 protein to CHO cell surface (C) B7.1 and (D) B7.2 was determined as mean fluorescent intensity (MFI) using an anti-his_6-tag antibody labeled with FITC. In the presence of hCTLA-4-his_6 protein, MFI was greatly enhanced (bar-d) and unaffected by serum from mice treated with control vector (bar-e), whereas 25-fold and 50-fold dilutions of serum of pVAC-1-hCTLA-4 immunized mice significantly suppressed the association (single and double asterisk indicate $p < 0.05$ and <0.001, respectively). Representative data from two independent experiments are shown.

B16F10 melanoma cells. The B16F10 tumor growth rate in the pVAC-hCTLA-1 vaccinated mice was significantly slower than that in the mice treated with control plasmid, pVAC-1 (Fig. 2A). Although the pVAC-hCTLA-1 vaccinated mice obtained immune response against CTLA-4 sufficient for inhibition of tumor growth, they did not show any sign of diarrhea indicating colitis, which is the most common side effect in cancer patients enrolled in anti-CTLA-4 monoclonal antibody trial [10]. The mice did not show discernible difference in body weight gains during a period of 5 weeks while being treated with either pVAC-1-hCTLA-4 or pVAC-1 (Fig. 2B).

3.4. Enhanced cytotoxicity of splenocytes from mice immunized with hCTLA-4 DNA vaccine

To characterize the mechanisms of the anticancer effect of pVAC-1-CTLA-4 vaccine, the mice were sacrificed and the splenocytes were examined for cytotoxic effect on cancer cells. We measured the splenocyte-mediated cytotoxicity by using previously screened B16F10 stably expressing luciferase, B16F10-luc, of which the cell numbers demonstrated good correlation with luciferase activity in photon counts per second (Fig. 3A). The B16F10-luc cells (2000 cells/well) were added into 96-well in the presence of 100- or 30-fold (2×10^5 or 6×10^4 cells) of splenocytes. 3 days after incubation, the cells were added with luciferase substrate, luciferin, and subjected to IVIS Imaging System for quantification of cell viability in photon counts per second. The splenocytes from mice immunized with pVAC-1-hCTLA-4 demonstrated superior effects on cell viability of B16F10 (Fig. 3B). Additionally, to characterize the immune response of splenocytes encountering B16F10-luc, we measured the amount of interferon-gamma produced by splenocytes incubated with B16F10-luc using ELISA kit specific for murine interferon-gamma. The splenocytes obtained from CTLA-4 DNA immunized mice produced higher amount of interferon-gamma compared to those from pVAC-1 vaccinated control mice (Fig. 3C).

3.5. Cationic liposome-coupled pVAC-1-mCTLA-4 breaks tolerance to self-antigen

We immunized the mice with pVAC-1-mCTLA-4 encoding murine CTLA-4, in an attempt to induce antibody in mice more specific to autogenic antigen. In the absence of coupling cationic liposome, the "naked" pVAC-1-mCTLA-4 DNA vaccine failed to arouse significant antibody titers targeting mCTLA-4 (Fig. 4A). Given that cationic liposome coupling to DNA can increase transfection efficiency as well as enhance immune response, we investigated mCTLA-4-liposome complex for the induction of immune response against murine CTLA-4. This approach substantially elicited antibody titer against murine CTLA-4 and to a lesser degree against human CTLA-4 (Fig. 4B and C). The mice immunized with cationic liposome-coupled pVAC-1-mCTLA-4 also demonstrated inhibited growth of renal cell carcinoma (RENCA) compared to those immunized with control vector, pVAC-1 with or without liposome, or naked pVAC-1-mCTLA-4 (Fig. 4D).

4. Discussion

Upon activation of T-lymphocyte through interaction with APC, CTLA-4 is up-regulated and efficiently competed with CD28 for B7.1/B7.2 coupling. This process suppresses undesired autoimmunity against self-antigens as well as anticancer effect of immune system [3]. CTLA-4 has been validated as a critical target for anticancer therapy evidenced by the positive results of a pivotal trial of anti-CTLA-4 antibody, ipilimumab, in metastatic melanoma patients [11] as well as by encouraging data from currently ongoing trials for various cancers [12,13]. The only CTLA-4 targeting therapies under clinical development are two monoclonal antibodies, ipilimumab and tremelimumab. In this study, we describe a DNA vaccine as an alternative to suppress CTLA-4-mediated negative effect on anticancer immunity.

DNA vaccines have been shown to elicit both humoral immunity and cytoimmunity. Vaccination using DNA are being enthusiastically developed and investigated in numerous preclinical and clinical studies against various diseases ranging from infection, neural degeneration to cancers [14,15]. DNA vaccination may be adopted to target the tumor-associated molecules, thereby preventing tumor growth and metastasis [14,16]. Furthermore, DNA vaccine can be produced, purified in large quantities, and stored at a relatively low cost compare with biological drugs [17,18]. It is becoming a potent vaccine strategy in cancer immunotherapy.

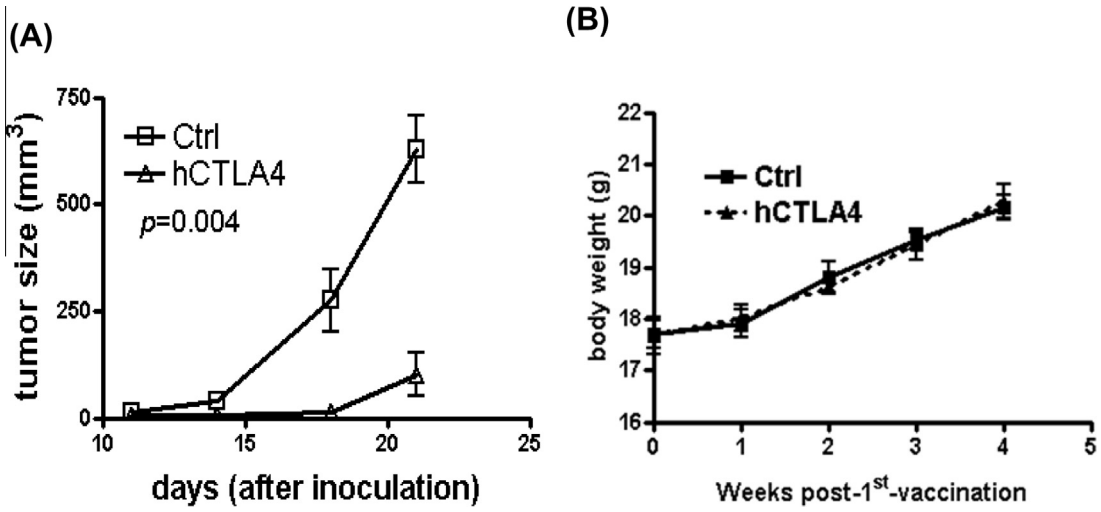

Fig. 2. Effect of the pVAC-1-hCTLA-4 DNA vaccine on tumor growth and body weight. (A) 1 week after the last vaccination, c57BL/6 mice were challenged with B16F10 melanoma cells (1×10^5). The average tumor growth of 5 mice per group is displayed. Suppressed tumor growth was exhibited in pVAC-1-hCTLA-4 immunized mice as compared with the control group ($p = 0.004$). (B) Body weights for mice vaccinated with pVAC-1-hCTLA-4 or control vector. There is no significant difference between individual groups.

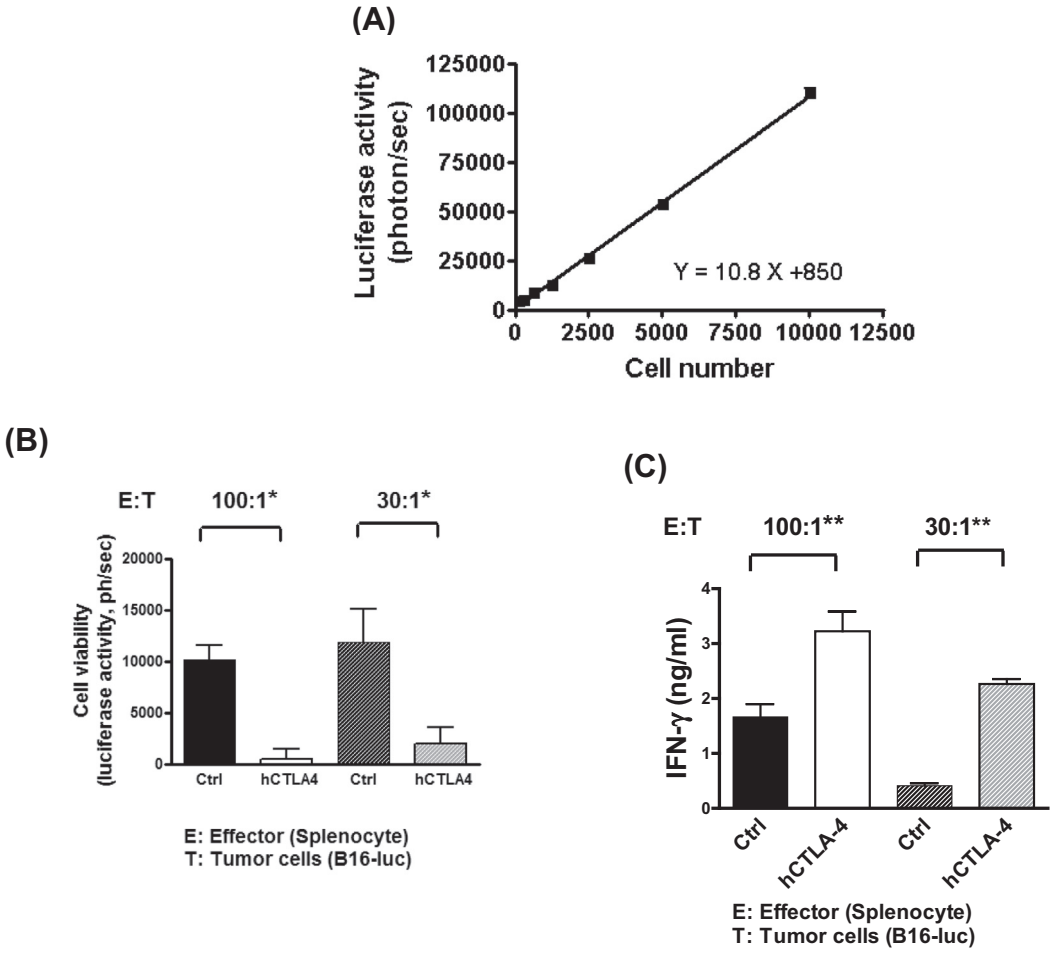

Fig. 3. Mice immunized with pVAC-1-hCTLA-4 DNA vaccine exhibits enhanced cellular immunity against inoculated B16 cells. (A) Matching bioluminescence to the cell numbers of B16F10-Luc, the calculated number of photons emitted from a single cell per second is 10.8. (B) Cytotoxic T-lymphocyte (CTL) activity of splenocytes against B16F10-Luc was assessed by detecting bioluminescent intensity resulted from luciferase activity of viable B16F10-Luc cells. The splenocytes from mice immunized with pVAC-1-hCTLA-4 demonstrated more significant cytotoxic effect on B16F10-Luc (asterisk indicates $p < 0.05$). (C) The culture medium from splenocyte mixed with B16F10-Luc was subjected to the measurement of interferon-gamma. Stimulation of interferon-gamma secretion by splenocytes is more significant in the group of mice immunized with pVAC-1-hCTLA-4 (double asterisk indicates $p < 0.005$).

In addition to vaccine efficacy concerning gene expression and immunogenicity, vaccine toxicity and safety are critical issues in clinical use [19]. Common toxicity observed in clinical development of anti-CTLA-4 monoclonal antibody includes hepatitis, colitis, dermatitis, uveitis, and nephritis, etc. [20]. Fortunately, these immune-related adverse effects were mostly self-limited and responded to steroids and immunosuppressants [21]. Our results demonstrated that pVAC-1-hCTLA-4 vaccine stimulates antibody specific for human CTLA-4 with cross reactivity to murine CTLA-4 adequate for suppressing B16F10 tumor growth in c57BL/6 mice in the absence of discernible toxicity, including loose stool and body weight loss. Reasonable explanation for the negligible gastrointestinal toxicity may be that the pharmacokinetic profile and amount of the antibody induced by DNA vaccination are different from those of ipilimumab and tremelimumab. In spite of the lack of apparent toxicity in mice, CTLA-4 DNA vaccination efficacy against tumor growth is retained. Nevertheless, autoimmunity resulting from DNA vaccination remains a concern, especially when immune modulating protein like CTLA-4 is targeted as an antigen. Therefore, immune profile surveillance for unwanted immune response is mandatory during anti-CTLA-4 DNA vaccination.

Anti-CTLA-4 antibody is poised to play a significant role in future anticancer treatment not only for approved indication of metastatic melanoma but also for various types of advanced tumor of which the clinical trials are showing promising results [22]. On top of the encouraging therapeutic window, the anti-CTLA-4 DNA vaccine in this study has another advantage considering the cost. Although the recently approved ipilimumab was praised as a major advance in cancer therapy, its substantial price will inevitably limit the number of patients who may benefit from this innovative treatment. Generalized use of immunotherapy by cost-down strategy may save the total budget expended on these patients.

$CD4^+CD25^{high}Foxp3^+$ regulatory T-cell (Treg) has the ability to attenuate the activation of other immune cells, thereby, averting autoimmune diseases. However, Treg also suppresses the immune response to pathogens and cancer cells [23]. CTLA-4 is constitutively expressed in Treg through Foxp3 controlled transcription [24]. Wing et al. has shown that the specific deficiency of CTLA-4 in Tregs leads to spontaneous development of systemic Lymphoproliferation, autoimmune disease, as well as potent tumor immunity [25]. Strategies to reduce the expansion of Tregs have been exploited for cancer treatment [26]. It is worth evaluating the effect of anti-CTAL-4 DNA vaccine on the viability of Treg in the near future to unravel the mechanism of this vaccine to augment T-cell cytotoxicity and subsequent inhibition of tumor growth.

Tolerance to self-antigen is a critical issue to be addressed when developing anticancer vaccine [27]. In this study, naked human CTLA-4 encoding gene was able to induce antibody titer against

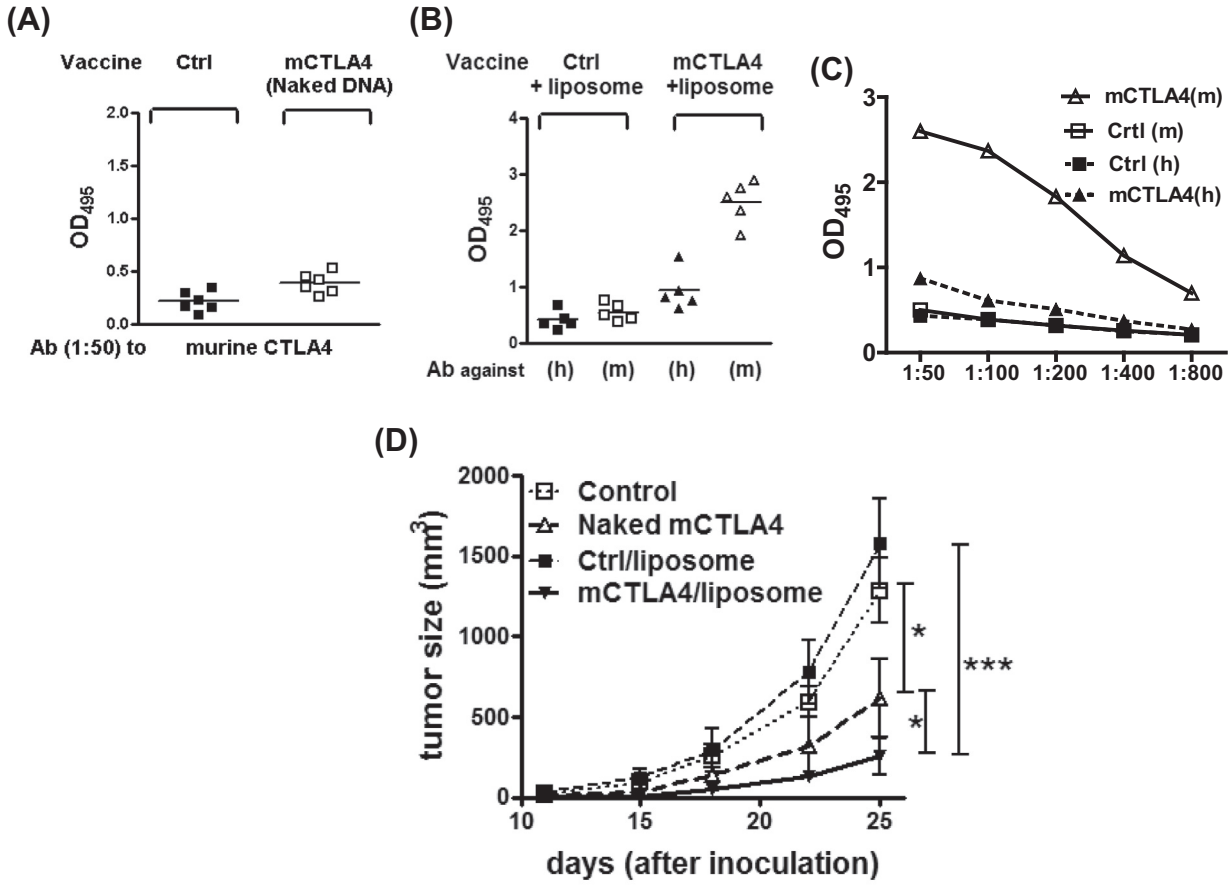

Fig. 4. Immunization with pVAC-1-mCTLA-4 as naked DNA or complexed with liposome. (A) Vaccination of mice with pVAC-1-mCTLA-4 as naked DNA did not induce significant antibody titers against murine CTLA-4. (B and C) When pVAC-1-mCTLA-4 was coupled with cationic liposome, immunized mice generate antibody against both human and murine CTLA-4. (D) BALB/c mice (n = 5–6/group) immunized with pVAC-1-mCTLA-4 or control plasmid in the presence or absence of coupling liposome were challenged with 1×10^5 RENCA cells. Vaccination with pVAC-1-mCTLA-4, especially complexed with liposome, significantly suppressed tumor growth. Single and triple asterisk indicate $p < 0.05$ and $p < 0.001$, respectively.

human CTLA-4, whereas similar approach using murine CTLA-4 DNA vaccine failed to elicit significant amount of antibody against murine CTLA-4. In light of the adjuvant property of cationic liposome, its coupling with pVAC-1-mCTLA-4 was shown to prevent tolerance to self-antigen in BALB/c mice evidenced by the potent immunity against murine CTLA-4. Vaccination-induced antibody targeting murine CTLA-4 suppresses Renca renal cell carcinoma tumor growth in BALB/c mice. Tumor size of the mice immunized with liposome coupling pVAC-1-mCTLA-4 is about one third of that of naked DNA treated mice.

In conclusion, serum from pVAC-1-hCTLA-4 immunized mice suppressed binding of human CTLA-4 to B7 and exhibited significant anti-tumor effect *in vivo* with no measurable toxicity in a B16F10 tumor model. On the other hand, when naked pVAC-1-mCTLA-4 DNA was administered to the experimental mice, it failed to elicit significant increase the titer of antibody specific for murine CTLA-4. This tolerance to self-antigen, murine CTLA-4, was broken when pVAC-1-mCTLA-4 was complexed with a cationic liposome. These results offer evidence that support immunotherapy targeting CTLA-4 by breaking immune tolerance to a self-antigen, which may be explored for cancer therapy.

Declaration of competing interests

Lan, K.L. and Shih, Y.S. are inventors on the following US patent applications: US20120328693.

Acknowledgments

This work was supported by Grants from the National Science Council of Taiwan (NSC 99-2314-B-075 -001 -MY3) and the Taipei Veterans General Hospital (V101C-075).

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