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ORIGINAL ARTICLE

CEA Plasmid as Therapeutic DNA Vaccination against Colorectal Cancer

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ABSTRACT

Background: Human colorectal cancer cells overexpress carcinoembryonic antigen (CEA). CEA is a glycoprotein which has shown to be a promising vaccine target for immunotherapy against colorectal cancer. **Objective:** To design a DNA vaccine harboring CEA antigen and evaluate its effect on inducing immunity against colorectal cancer cells in tumor bearing mice. **Methods:** In the first step the coding sequence of the CEA was cloned into the pcDNA3.1 vector. The mice were injected with the vaccine construct and the immune responses were monitored during the experiment period. The specific IgG anti-CEA, IFN-γ, IL-2 and IL-4 were measured by ELISA and levels of IFN-γ was detected by ELISpot assay. The lymphocyte proliferation was assessed using a 5-bromo-2-deoxyuridine (BrdU) cell proliferation assay kit. **Results:** Immunization of the mice with the CEA plasmid resulted in stimulation of CEA-specific T cell and antibody responses. The serum level of specific IgG antibodies against CEA was increased in immunized mice. Moreover, the injection of CEA plasmid led to the stimulation of T-helper-1 by increase in the secretion of IFN-γ, IL-2 and lymphocyte proliferation response. **Conclusion:** As the CEA DNA vaccine displayed encouraging antitumor effects, therefore, we suggest that it can be a potential therapeutic modality for colorectal cancer and is worthy of further investigation.

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Keywords: Carcinoembryonic Antigen, Colorectal Cancer, DNA Vaccine

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INTRODUCTION

Colorectal cancer (CRC) is the second most common cancer in females and the third most common cancer in males and is one of the most leading causes of cancer-related death worldwide (1,2). In more than 20% of CRC patients at the time of diagnosis, tumor cells metastasize to other organs and the survival rate reduces to approximately 12% (3,4). Despite noticeable advances in chemotherapy, surgical management and biological therapies, the average survival time of patients with advanced CRC is 30 months and metastatic CRC is still the fourth most common cancer-related cause of death (5). The main obstacles in reaching tumor-specific immune responses are: 1- Tolerance of peripheral T cells against tumor self-antigens (Ags), and 2- the inability of the immune system to induce effective CD8+ cytotoxic T-cell responses (CTLs) for the eradication of metastases and maintaining immune memory responses for long time tumor relapse prevention (6,7). Therapeutic vaccines for the stimulation of the immune system against tumor antigens and subsequent elimination of tumor cells have been designed. There are a variety of therapeutic vaccines such as DNA vaccines, peptide or protein vaccines and whole cell-based vaccines. The DNA vaccines have gained great attention since they are able to induce both humoral and CD4+ responses. Moreover, one of the most important advantages of DNA vaccines compared to protein or peptide ones is their ability to stimulate strong CTL responses (8,9). CEA, an oncofetal Ag, is a 200 kDa glycoprotein in which mannose, galactose, N-acetylglucosamine, fructose and sialic acid are most of the composition of the carbohydrate part (10). CEA is expressed in both cancerous and non-cancerous tissues and most people, especially cancer patients are not reactive against it (11,12). However due to its role in tumorigenesis, CEA has been investigated as a target for vaccine therapy for CEA-expressing cancers (13-15). Therapeutic vaccines have been tested for targeting CEA as a tumor-associated antigen (TAA) to induce CEA-specific humoral and cellular immunity in CEA+ tumors such as colon cancer (14,16). In this study, we developed CEA as a therapeutic DNA vaccine against colorectal cancer. We demonstrated the efficacy of CEA vaccine in the stimulation of humoral and cellular immunity in a mouse model.

MATERIALS AND METHODS

Animals and Cell Lines. Six- to 8-week-old female C57BL/6j mice were purchased from the Pasteur Institute Animal Laboratory, Tehran, Iran. All mice were kept in clean conditions and all experiments were conducted according to the approved protocols of Institution. pET28a plasmid was digested by *Bam HI* and *XhoI* restriction enzymes for excising CEA and ligating into pCDNA vector. Colon adenocarcinoma cell-line MC-38 cells (Iranian cell bank, Pasteur Institute of Iran) were grown in Dulbecco's Modified Eaegle's Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Sigma). HEK293 cells (Iranian cell bank, Pasteur Institute of Iran) were grown in DMEM medium (Gibco, USA) supplemented with 10% FBS, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 2 mM L-glutamine (Sigma).

Protein Expression in Cells. Different constructs of pCDNA3.1 were transfected to the HEK293 cells in the presence of Lipofectamine 2000 (Invitrogen AB, Stockholm, Sweden) according to the manufacturer's instructions. 48 h post transfection, the cells

were detached by trypsin and were washed in Phosphate buffer saline. Afterwards, the cells were lysed by laemmli buffer (Bio-Rad, Hercules, CA) and total protein samples were collected for western blot analysis for the following antibodies: a polyclonal anti-CEA antibody as the primary antibody and a Horseradish peroxidase (HRP) -conjugated goat anti-mouse IgG antibody as the secondary antibody (Santa Cruz, USA).

Preventive Immunization. Female five-to-six-week-old C57BL/6j mice (n=30) were randomly divided into five groups including pCEA (pCDNA3.1 plasmid expressing CEA), CEA protein (pET28a plasmid expressing CEA), pCEA/protein, pCDNA3.1 and PBS. pCEA (100 µg) in 50 µl of PBS, CEA protein (20 µg protein) and pCEA/protein (100 µg DNA conjugated in 20 µg protein) were subcutaneously (S.C) injected into mice. Group pCDNA3.1 and Group PBS as controls were respectively immunized with pCDNA3.1 and PBS at 7 day intervals 3 times. Following the last immunization, mice were challenged (subcutaneously) with 5×10^6 MC-38 tumor cells in 100 µl of PBS into the right flank. Palpable tumors usually developed on day 7. Tumor size was monitored and measured by caliper every other day. The following formula was used for the tumor volume calculation: $V = 0.5 \times D \times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter).

Enzyme Linked Immunosorbent Assay (ELISA). ELISA plates were coated (Griener, Germany) overnight at 4ºC with 2 µg/well CEA protein from Previous study (17) in 100 µl of carbonate/bicarbonate buffer (pH 9.6) and were then blocked with skim milk 5% for 2 h at room temperature. mice sera (1:100) in PBS were added to each well for 16 h at 4ºC and were then incubated with 100 µl of 1:5000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1 and IgG2a (Santa Cruz, USA) for 90 min at room temperature. The wells were washed and incubated for 10 min with 100 µl of TMB substrate (eBioscience, CA, USA) and the reaction was stopped by the addition of 50 µl of 2N H_2SO_4. Color density was read at OD450 using ELISA reader (ELx 800, BioTek, USA).

Splenocyte Proliferation Test. For the characterizing of splenocytes obtained from the immunized mice, we performed lymphocyte proliferation using a 5-bromo-2-deoxyuridine (BrdU) cell proliferation assay kit. For this purpose, after final immunization, mice were sacrificed and their spleens were removed and transferred to RPMI 1640 medium. The splenocytes were collected and subsequently suspended in lysis buffer (Roche, Germany) to remove erythrocytes. Then, the splenocytes (6×10^6 cells/ml) were resuspended in RPMI 1640 medium. Splenocytes from the all groups of vaccinated mice were seeded (6×10^6 cells/well, 200 µl) into 96-well plates in the presence of CEA plasmid or CEA protein (final concentration of 5 µg/ml) for 72 h at 37ºC in 5% CO_2. Proliferation of splenocytes was assessed by a 5-bromo-2-deoxyuridine (BrdU) cell proliferation assay kit (Cell Signaling, USA) according to the manufacturer's instruction. Proliferation responses of splenocytes were expressed as a stimulation index (SI) which was calculated by dividing the mean optical density at 450 nm of stimulated splenocytes by the unstimulated splenocytes.

Enzyme-Linked Immunosorbent Spot (ELISpot) Assay. IFN-γ ELISpot assay (Mabtech, Sweden) was performed to detect Ag-specific IFN-γ secreting T cells. After final immunization, the immunoplates were washed four times with PBS according to the manufacturer's instruction and were then blocked with RPMI 1640 supplemented with 10% FBS at 37ºC for 2 h. 3×10^5 splenocytes were seeded in a 96-well plate and were incubated at 37ºC in 5% CO_2 for 48 h in the presence of CEA plasmid or CEA protein (final concentration of 5 µg/ml). The plates were washed 5 times with PBS-T

(0.05% tween 20 in PBS) and were incubated at room temperature for 2 h with detection antibody (R4- 6A2-Biotin) (1 µg/ml) in PBS containing 0.5% FBS. After incubation, a 1:1000 dilution of Streptavidin- HRP in PBS-0.5% FBS was added to each well and then incubated at 37ºC for 1 h. The plates were then washed and 100 µl of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate was added to each well and was incubated at 37ºC for 30 min. The color development reaction was stopped by washing extensively with distilled water. The plates were air-dried overnight and the spots were counted using a dissecting microscope. The mean number of spot-forming units (SFU) per 10^6 splenocytes was used for data display.

Cytokine Assay. After final immunization, Mice were sacrificed and the spleens were removed. The splenocytes were then isolated and used for the IFN-γ, IL-2 and IL-4 production. Briefly, 3×10^6 cells were seeded in a 24-well plates in triplicate in the presence of CEA or CEA/protein (all at final concentration of 5 µg/ml) at 37ºC in 5% CO_2 for 72 h. The supernatants were harvested and secretion of IFN-γ, IL-2 and IL-4 were measured by mouse Th1/Th2 ELISA ready-set-go kit (eBioscience, USA) in accordance with the manufacturer's instruction.

Tumor Growth. In the immunotherapeutic experiment, C57BL/6j mice were divided into four groups (n=24): pCEA, CEA protein, pCEA/protein and PBS. All mice were challenged by subcutaneous (S.C) injection in the left flank with 5×10^6 MC-38 tumor cells. After one week, pCEA (100 µg) in 50 µl of PBS, CEA protein (20 µg protein) and pCEA/protein (100 µg DNA conjugated in 20 µg protein) were subcutaneously (S.C) injected into mice. For up to 4 weeks, the sizes of tumors in these mice were measured twice a week according to Carlsson's formula: (longest diameter) × (shortest diameter) 2) × 0.5.

Statistical Analysis. Microsoft Excel 2010 and GraphPad Prism software (version 6.0; GraphPad Software Inc., San Diego, CA, USA) were used for statistical data analysis and graphing. Tukey's post-hoc test was used to determine any significant differences between the groups. In all the cases, p-values<0.05 were considered as significant.

RESULTS

Expression of the CEA plasmid constructs.

To construct CEA expression plasmid, CEA genetic sequence was subcloned into the unique *BamH1* and *Xho1* restriction sites of the pCDNA expression vector (Figure 1A). To make sure that the constructed vector produces the Ags of interest, HEK 293 cells were transfected with the CEA constructs. Western blot analysis was used to detect the CEA proteins using polyclonal anti-CEA antibody (Santa Cruz, USA). Western blot analysis revealed a band of approximately 37 kDa which confirmed the production of CEA proteins (Figure 1B).

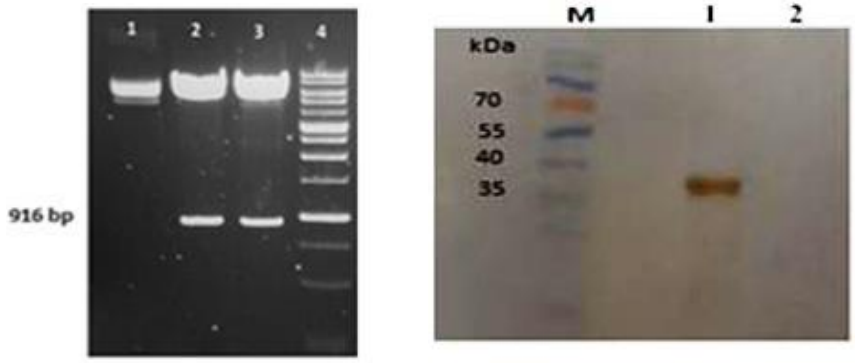

Figure 1. Subcloning and expression of the CEA. (A) Subcloning the gene CEA into the pCDNA vector. The lane number 1 is undigested pCDNA-CEA. The lanes numbers 2 and 3 are double digests of pCDNA-CEA with *BamH1* and *Xho1*. The lane 4 is molecular weight marker. **(B)** Western blot analysis for detection of CEA in transfected cells**.** The lane M is molecular weight marker. The lane number 1 is induced pCDNA-CEA cell lysate. The lane number 2 is induced pCDNA cell lysate.

Humoral responses.

ELISA was performed to measure the quantity of specific IgG antibody against CEA plasmid and pCEA/protein. Mean IgG antibody titers were significantly increased in mice immunized with the construct when it was compared to the control group ($p<0.0001$) (Figure 2A). Moreover, the results showed that the IgG2a/IgG1 levels were significantly increased in mice immunized with CEA constructs than the PBS and pCDNA control group ($p<0.05$). According to this result, a shift in immune responses toward T-helper 1 (Th1) was observed in the vaccinated mice. As shown in (Figure 2B), CEA/protein immunized mice showed the highest humoral responses among all other groups.

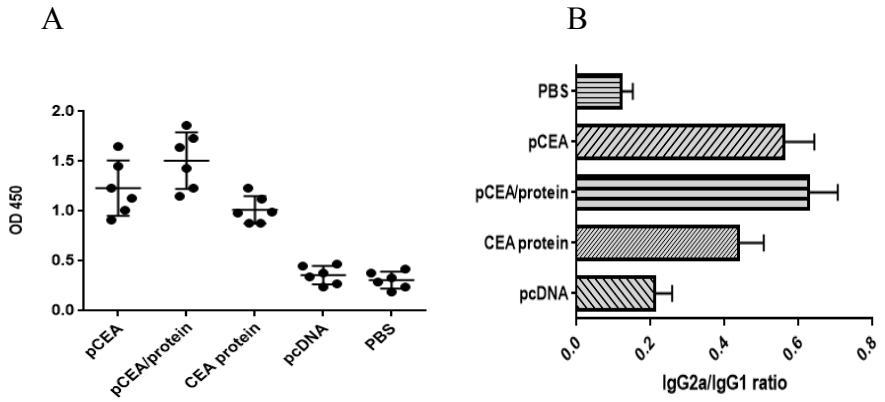

Figure 2. Analysis of the antibody responses. Anti-CEA plasmid, pCEA/protein antibody titers were measured by ELISA in 1:100 diluted sera of immunized mice after two weeks immunization. (A) Absorbance values of IgG. (B) The ratio of IgG2a/IgG1.

Lympho-proliferative response defective.

The lymphocyte proliferative response was measured after final immunization. As shown in Figure 3, pCEA/protein enhanced the proliferative response compared with other groups. The proliferative responses of pCEA, pCEA/protein, and CEA protein were higher than in the mice as control group ($p<0.05$). In this experiment, no statistically significant differences were found between the pCEA/protein and pCEA groups. The results showed that protein-booster has the potential to increase the lymphocyte proliferative response against pCEA/protein.

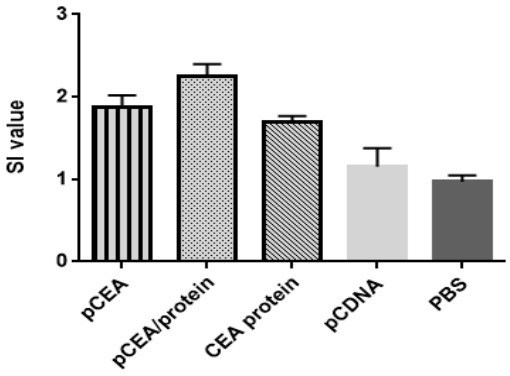

Figure 3. Proliferation of splenocytes. Mice were immunized with pCEA, and pCEA/protein. Mice that received PBS were regarded as controls. After final immunization, the spleen lymphocytes were cultured and Stimulation index (SI) values were calculated to determine the ratio of absorbance value at 450 nm of Ag-stimulated wells to that of non-stimulated wells. Statistical analysis was performed by the two-tailed Mann Whitney nonparametric test, and a p-value of ($p<0.05$) was considered as a statistically significant difference.

Elispot assay.

Two weeks after final immunization, the results demonstrated that the group of mice immunized with pCEA/protein could stimulate the highest number (~263 SFU/10^6 cells) of CEA specific IFN-γ secreting splenocytes compared to all other groups (Figure 4).

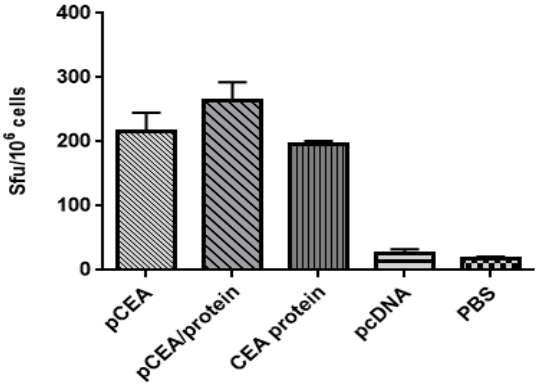

Figure 4. Concentration of IFN-γ in culture supernatants of splenocytes from immunized mice by ELISpot assay. These expriments were run in triplicate.

Small numbers of IFN-γ producing spleen lymphocytes were observed in the groups PBS (~14 FU/10^6 cells). In general, mice vaccinated with pCEA/protein had generated significantly stronger responses in terms of the number and magnitude of spots.

Cytokine profile.

After final immunization, the levels of IFN-γ, IL-2 and IL-4 in the lymphocytes culture supernatant were measured by ELISA method. As shown in Figure 5, mice immunized with the pCEA/protein showed higher levels of IFN-γ, IL-2 (Th1 cytokines) and IL-4 (Th2 cytokine). Stimulation of IFN-γ production was shown to be higher when the mice were immunized with the pCEA/protein and pCEA compared to pCDNA and PBS ($p<0.05$) and there were no significant differences between the pCDNA and PBS groups (Figure 5).

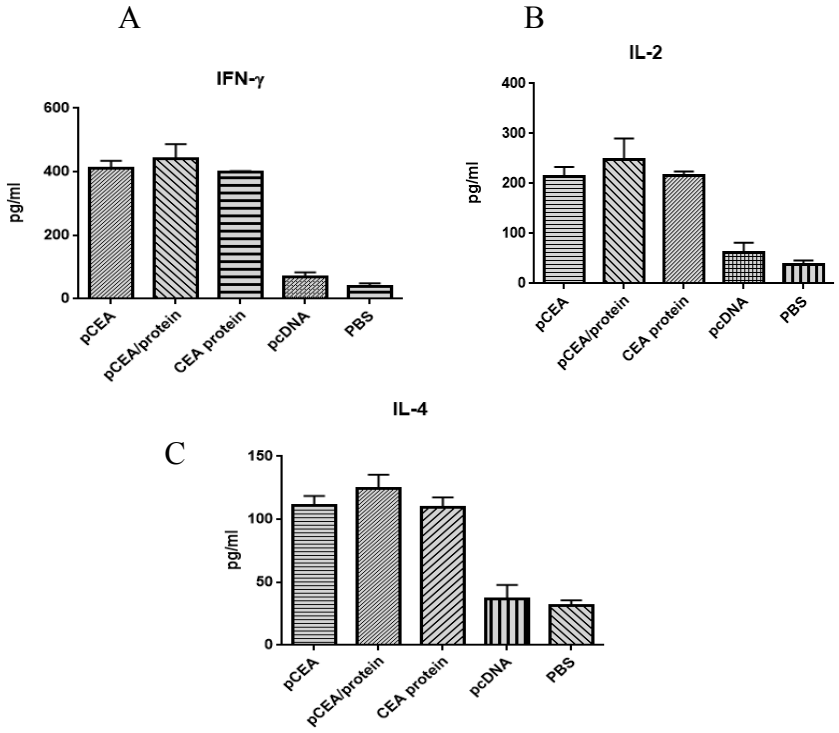

Figure 5. Analysis of cytokine assay. Antigen-specific cytokine production by spleen lymphocytes of vaccinated mice. Splenocytes were cultured in vitro with CEA. The supernatant of each group was harvested after 72 h of culture and released IFN-γ, IL-2, and ELISA measured IL-4. The PBS was used as a control. These expriments were run in triplicate.

Anti-tumor effect of vaccination.

We measured the reduction of tumor volume via therapeutic immunization in mice (n=24). In this study, mice were challenged with 5×10^6 MC-38 tumor cells. The vaccination groups (pCEA/protein) showed significantly reduced tumor size with control groups (Figure 6). Thus, the average tumor volume in the pCEA/protein groups was significantly lower than other groups.

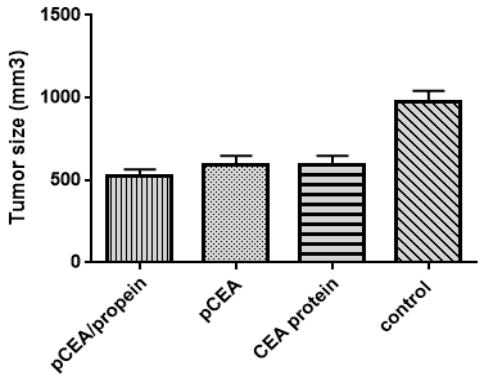

Figure 6. The tumor growth of the immunized mice. The reduction of tumor growth observed in C57BL/6j mice immunized with pCEA/protein, pCEA, CEA protein as compared to mice control group (p<0.05).

DISCUSSION

During the last decades, immunotherapy has emerged as a novel and efficient therapeutic strategy for treatment of different types of cancer. In this modality the individual's own immune system is utilized to fight against cancer cells through the production of neutralizing antibodies and/or induction of CTLs (18). It has been demonstrated that the injection of DNA plasmid elicits both humoral and cellular antigen-specific immune responses. Furthermore, DNA vaccines have some advantages over other types of conventional vaccines, including that they are more cost effective, they are easy to design and produce, they can be designed to carry different types of antigens and the safety of DNA vaccines has been demonstrated in various studies (19,20). However, DNA vaccines suffer from disadvantages such as poor immunogenicity and immune tolerance. This study aimed to examine whether a DNA vaccination strategy would be able to prevent colorectal tumor formation and be used as a therapeutic vaccine in colorectal cancer. In this study a therapeutic DNA vaccine expressing CEA antigen was designed and its efficacy in stimulation of both arms of immune responses was evaluated. In the first step of experiments, CEA-specific humoral and cellular immune responses were analyzed in immunized mice following the expression of CEA constructs. The construct was shown to be able to induce IgG production. Moreover, the IgG2a/IgG1 levels were significantly higher in mice immunized with CEA construct than in control groups. It is believed that the production of IgG2a is associated with the Th1 responses, whereas IgG1 production is associated with Th2 responses (21). Since there was a significant raised ratio of IgG2a/IgG1 in mice immunized with CEA, it can be postulated that there is a shift toward Th1 cell-mediated immune responses. This protective shift can be deduced from four leading observations: first, the in vitro antiproliferative activity was greater; second, the ELISPOT assays showed the larger number of cells which produce INF-γ; third, the proliferation of CEA-specific T-cells was higher, and fourth, the type 1 cytokines were secreted in higher levels. The vaccination showed a higher production of antigen-specific IFN-γ and IL-2 compared to the level of IL-4. In fact, pro-inflammatory Th1 responses are usually induced by DNA vaccines (22). The splenic T cells of mice

immunized with pcDNA-CEA produced high levels of IFN-γ *ex vivo*. This demonstrates that CEA-specific Th1 responses were induced as a result of vaccinating the mice with these plasmids. Humoral responses can be stimulated with the aid of activated T cells through direct T and B cell interaction and cytokines secreted by T cells. This, as a consequence, promotes B cell functional differentiation. In this experiment it was revealed that the mice immunized with the CEA construct develop high levels of corresponding antibodies nevertheless no specific antibody was detected in mice vaccinated with control plasmid. These high levels of antibodies may be utilized to evaluate the efficacy of the vaccination. Splenic T cells showed a stronger proliferative response in mice vaccinated with pcDNA-CEA. Additionally, T cells of mice immunized with CEA expressing plasmid produced higher concentrations of antigen-stimulated IFN-γ. Altogether, the DNA vaccine showed an effective capacity to stimulate both the humoral response through developing high titers of antigen-specific antibodies (Figure 2) and the CD4+ and CD8+ T-cell responses through production of antigen-specific IL-2 and IFN-γ (Figure 5).

Moreover, prime-boost strategies in which the DNA priming is followed by boosting with both infectious agents (23,24) and tumor protein have been shown to have remarkable impact on the production of specific antibodies (25,26). In our experiment, however, antibody titer was not significantly raised when the protein or the DNA were applied as a booster. In the present study it was demonstrated that CEA DNA vaccines are able to induce CD8+ T cell responses, and indeed have antitumor effects against an MC38 cell challenge. Furthermore, our data regarding the therapeutic effects of the DNA vaccines are in contrast to those of other studies. For instance, Schlom *et al.* showed that vaccinia virus-based CEA vaccines are capable of inducing therapeutic activity against MC38 cells which express CEA antigen on their surface (MC38/CEA) (13). One strategy for enhancing the efficacy of DNA vaccines to overcome immune-evading tumor cells is to design vectors which are capable of simultaneously targeting more than one tumor antigen. This is especially useful when one antigen is lost during the course of immunotherapy. It has been demonstrated that DNA vaccines targeting both CEA and HER2 antigens are able to confer a broader and more efficacious protective effect in animal models (27). In the present study, however, splenocytes derived from C57BL/6 mice immunized with pCEA showed different anti-tumor effects. This is because tumor growth was reduced in this group. In this study no significant difference was observed in the level of IL-4 among the study groups. The concentration of IL-4 was even very low and undetectable in the culture supernatant. This may be due to the increased levels of IFN-γ and IL-2 which down-regulate the production of T2 cytokines (28). IL-2 has a central role in regulating immune system responses. In CD4+ T cells it promotes proliferation and their differentiation into T-helper 1 (Th1) and T-helper 2 (Th2) cells. It also activates cytolytic activity of NK and CD8+ T cells (29). In the present study, the cultured splenocytes were stimulated with DNA vaccines and their supernatant was evaluated for IL-2 production. Like IFN-γ, mice immunized with DNA vaccine (CEA) produced the highest levels of IL-2. In conclusion, the present study demonstrated that vaccination with plasmid DNA alone or in combination with purified protein can trigger both humoral and cellular responses. More importantly, boosting with DNA plus protein appeared to be superior to the administration of CEA protein to elicit CEA-specific IgG. This strategy could be efficiently applied for cancer cases in which vaccinations need to be administered regularly over time to prevent tumor recurrence.

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