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DNA vaccine with α-galactosylceramide at prime phase enhances anti-tumor immunity after boosting with antigen-expressing dendritic cells

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Abstract

DNA vaccines contribute to a promising new approach for the generation of cytotoxic T lymphocytes (CTL). DNA vaccines do have several disadvantages, including poor immunogenicity and oncogene expression. We used the natural killer T-cell (NKT) ligand α-galactosylceramide (α-GalCer) as an adjuvant to prime initial DNA vaccination; and used the potent immune-stimulatory tumor antigen-expressing dendritic cells (DCs) as a booster vaccination. A DNA vaccine expressing human papillomavirus (HPV) type 16 E7 (pcDNA3-CRT/E7) was combined with α-GalCer at the prime phase, and generated a higher number of E7-specific $CD8^+$ T-cells in vaccinated mice than vaccine used at boost phase. Therefore, priming with a DNA vaccine in the presence of α-GalCer and boosting with E7-pulsed DC-1 led to a significant enhancement of E7-specific $CD8^+$ effector and memory T-cells as well as significantly improved therapeutic and preventive effects against an E7-expressing tumor model (TC-1) in vaccinated mice. Our findings suggested that the potency of a DNA vaccine combined with α-GalCer could be further enhanced by boosting with an antigen-expressing DC-based vaccine to generate anti-tumor immunity.

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Keywords

DNA vaccine; α-Galactosylceramide; Anti-tumor immunity

1. Introduction

Cancer is a worldwide leading cause of death, and several malignancies are incurable by conventional therapies. Therefore, new anti-tumor immunotherapies are necessary to improve the outcome of patients with advanced cancer. DNA vaccines are a potentially valuable form of antigen-specific immunotherapy because of their safety, ease of production, stability, and expression of the tumor-specific antigens for longer time periods than RNA or protein-based vaccines [1]. Further, DNA vaccines do not elicit neutralizing antibody production in the patients, unlike live vector vaccines, and can therefore be repeatedly boostered [2]. However, DNA vaccines also have several significant disadvantages. Some DNA vaccines may express oncogenes which could potentially integrate into host genomes [3]. DNA vaccines also result in relatively poor immunogenicity [4].

Dendritic cells (DCs) can pick up antigens and activate naïve and memory $CD4^+$ and $CD8^+$ T-cells, and this characteristic may allow them to trigger specific anti-tumor immunity [5]. DCs present antigen and prime T-cells, and therefore vaccines utilizing DCs should stimulate superior protective and therapeutic immune responses in cancer patients when compared to other vaccination strategies [6]. Further, DC-based vaccines may circumvent tumor-mediated immune suppression [7,8]. However, individualized DC-based vaccine are difficult to prepare and costly; vaccine preparation requires harvesting of DCs from patients and *in vitro* exposure to tumor antigens in large-scale culture. Furthermore, the route of administration is likely to be important for DC-based vaccination because the DCs must home to the lymphoid organs to interact with the majority of naïve T-cells [9].

Several alternate immunostimulants and adjuvants have been developed and applied, including CpG-oligodeoxynucleotide (ODN) [10], polyactide-co-glycolide (PLG) [11], and the NKT-cell ligand α-galactosylceramide (α-GalCer) [12]. α-GalCer is a glycolipid originally extracted from marine sponges, and is presented by the CD1d molecule on DCs [13]. Several studies have reported that α-GalCer may be used as a systemically delivered vaccine adjuvant for the induction of potent natural killer cell-dependent anti-tumor cytotoxic responses [14,15]. α-GalCer enhanced anti-tumor immunity in mice when administered in combination with various types of vaccines [16–18]. Previous studies have demonstrated that α-GalCer and tumor cells are cross-presented by DCs *in vivo* to induce T-cell-mediated immunity [16] and can stimulate splenic DCs maturation, not DCs from bone marrow progenitors in mice [19]. These data suggest that α-GalCer may function as a potent adjuvant for DNA and DC-based vaccines.

We co-administered DNA vaccines or tumor antigen-loaded DC vaccines with α-GalCer in the present study to start early immunotherapy and improve anti-tumor efficacy. We examined several vaccine protocols to determine which combination of DNA-or DC-based vaccines and α-GalCer would most effectively prime naïve $CD8^+$ T-cells to generate and maintain E7-specific $CD8^+$ T-cell immune responses after boosting. Our data suggested that priming with a DNA vaccine and α-GalCer followed by boosting with peptide-pulsed DC most effectively induced E7-specific $CD8^+$ T-cell immune responses. These data suggested that initial co-administration of a DNA vaccine and α-GalCer and a subsequent booster with a DC-based vaccine might generate robust anti-tumor immunity.

2. Materials and methods

2.1. Antibodies(Abs), peptide, α-GalCer, cell line and mice

The HPV-16E7 (RAHYNIVTF) peptide was synthesized at ≥ 90% purity by Macromolecular Resources (Denver, CO, USA). Anti-CD8 (PE-conjugated, clone Ly-1) and anti-IFN-γ (FITC-conjugated, clone XMG1.2) antibodies were purchased from BD Pharmingen. α-GalCer (2S, 3S, 4R-1-O [a-galactopyranosyl]-2[N-hexacosanoylamino]-1,3,4-octadecanetriol) was purchased from Toronto Research Chemicals (Ontario, Canada) and diluted in phosphate-buffered saline. HPV-16 E7-expressing murine tumor cells (the TC-1 cell line) were used for the tumor model [20], and the DC-1 cell line was used as a dendritic cell model. All cells were maintained in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, 50 uM β-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Female C57BL/6 mice (6–8 weeks of age) were purchased from the Chung-Ang Laboratory Animal Service (Seoul, Korea), and housed the Animal Facility of the Pre-Clinical Research Center in Chung-Ang University. All animals were maintained under specific pathogen-free conditions. All procedures were performed according to previously approved protocols and in accordance with the recommendations for the proper use and care of laboratory animals of the Ethics Committee of the College of Medicine, Chung-Ang University.

2.2. Plasmid DNA construct and DNA preparation

The generation of pcDNA3-CRT/E7 has been described previously [21]. Plasmid constructs were confirmed by DNA sequencing. Amplification and purification of DNA were previously described [22].

2.3. DNA vaccination and DC immunization

Intramuscular (i.m.) DNA vaccination was performed with 100 μg of pcDNA3-CRT/E7 DNA/mouse; mice received booster vaccines 1 week later. DC-1 cells were pulsed with HPV-16 E7 (aa 49–57) peptide (RAHYNIVTF, 10 μg/ml) at 37 °C for 3 h. DC-1 cells were washed with RPMI-1640, supplemented with 10% FBS and Hank's balanced salt solution, and re-suspended in Hank's balanced salt solution at final concentration of 1×10^7 cells/ml. DC-1 cells (100 μl/mouse) were injected into the footpads of mice. The mice were boosted once with the same immunization regimen 1 week later. α-GalCer (2 μg) was mixed with either a DNA construct or E7-pulsed DC-1 cells and injected as previously described.

2.4. Maturation of splenic DCs with α-GalCer

DCs were isolated from spleen of mouse administered several adjuvant using previous study methods [19]. Splenocytes from mice administrated with α-GalCer (2 μg) or LPS (25 μg) were harvested by homogenization followed by treatment with collagenase. Collagenase-treated splenocytes were suspended with sterile PBS in the presence of 5% BSA. We separated CD11c+ fractions using anti-CD11c coated magnetic beads (Miltenyi Biotech, Auburn, CA, USA). Then we stained cells with PE-conjugated anti-CD40, CD80, CD86, Db, I-Ab. All antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Flow cytometry was performed on a Becton-Dickinson FACSCalibur with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA, USA).

2.5. Intracellular cytokine staining and flow cytometric analysis

Splenocytes were harvested from mice ($n = 5$ per group) either 1 week or 60 days after the last vaccination. A total of 5×10^6/mouse of pooled splenocytes from each vaccination group were incubated for 16 h with E7 peptide (1 μg/ml, aa 49–57, RAHYNIVTF) [23]

containing an MHC class I epitope for the detection of E7-specific $CD8^+$ T-cell precursors in the presence of GolgiPlug (BD Pharmingen). Stimulated splenocytes were washed twice with FACS buffer (PBS containing 5% BSA); cell surface marker staining for CD8 and intracellular cytokine staining for IFN-γ were performed using previously described conditions [22,24]. Flow cytometry was performed on a Becton-Dickinson FACSCalibur with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA, USA).

2.6. In vivo tumor treatment experiment using TC-1 tumor cells

C57BL/6 mice (n = 5 per group) were challenged with 2×10^5 TC-1 tumor cells/mouse by subcutaneous (s.c.) injection in the right hind leg for the *in vivo* tumor treatment experiment. Mice were vaccinated in various protocols twice with a one-week interval between the injections starting at the third day after challenge with TC-1 tumor cells. Mice were monitored for evidence of tumor growth twice per week by inspection and palpitation, as previously described [20]. Tumor volumes were measured starting at day 7 after tumor challenge.

2.7. In vivo tumor protection and antibody depletion experiment

To identify the subset of lymphocytes that are important for the anti-tumor effects, an *in vivo* antibody depletion experiment was performed. The *in vivo* antibody depletions were started immediately after last vaccination. MAb GK1.5 was used for CD4 depletion, MAb 2.43 was used for CD8 depletion, and MAb PK136 was used for NK1.1 depletion. Flow cytometry analysis revealed that the >95% of the appropriate lymphocyte subset were depleted with a normal level of other subsets. All Antibodies were purchased from Harlan (Indianapolis, IN, USA). C57BL/6 mice (n = 5 per group) were subcutaneously challenged with 2×10^5 TC-1 tumor cells/mouse in the right hind leg 1 week after last vaccination. Mice were monitored for evidence of tumor growth twice per week by inspection and palpitation, as previously described [20].

2.8. Long-term in vivo tumor protection experiment

Mice (n = 5 per group) were vaccinated with various vaccination protocols for long-term tumor protection experiments; mice were boosted with the same protocol as the initial vaccination after 1 week. Mice were challenged with TC-1 tumor cells (5×10^5/mouse) s.c. in the right hind leg at day 60 after booster vaccination. Tumor growth was monitored by twice weekly visual inspection and palpation, as previously described [20].

2.9. Tumor measurement and conditional survival

Three-dimensional tumor sizes were measured three times per week with Vernier calipers. Tumor sizes were approximated by multiplication of measured lengths. Tumors were measured every other day from day 25 after tumor cell challenge, and mice with tumor sizes >17 mm in diameter or with projected tumor volumes >10% body weight or >2500 mm^3 were sacrificed. Tumor volumes were calculated using the following formula: $V = (L \times W \times D)$; V is tumor volume, L is length, W is width, and D is depth. All procedures were performed according to approved protocols and in accordance with recommendations of the ethics committee of College of Medicine, Chung-Ang University, for the proper use and care of laboratory animals.

2.10. Statistical analysis

All data were expressed as mean ± standard deviation (SD) values and were representative of at least two different experiments. Data for intracellular cytokine staining, flow cytometric analysis, and tumor treatment experiments were evaluated by analysis of

variance. Comparisons between individual data points were made using the Student's *t*-test. Statistical significance was defined at *P*-values <0.05.

3. Results

3.1. Co-administration of pcDNA3-CRT/E7 with a-GalCer at prime phase enhances E7-Specific CD8$^+$ T-cell responses

DNA vaccination is capable of inducing antigen-specific CD8$^+$ T-cell responses. However, the overall immunogenicity of DNA vaccination is relatively low. DNA vaccines frequently require adjuvants and multiple applications of DNA or heterogonous boosting protocols [25,26]. We measured E7-specific CD8$^+$ T-cell immune responses in vaccinated mice using intracellular IFN-γ staining and flow cytometric analysis after priming to determine the effects of α-GalCer on the quantity of E7-Specific CD8$^+$ T-cell precursors generated by co-administration of the CRT/E7 vaccine construct with α-GalCer. Mice vaccinated with CRT/E7 and α-GalCer generated more E7-specific CD8$^+$ T-cell precursors compared to mice vaccinated with CRT/E7 alone (Fig. 1A and B). We also tested the effects of α-GalCer as an adjuvant at either the prime or boost phase. Mice were vaccinated with CRT/E7 in the presence of α-GalCer as prime or boost regimen; intracellular cytokine staining and flow cytometry were performed 1 week after the last vaccination. Mice primed with CRT/E7 and α-GalCer and boosted with CRT/E7 generated the strongest E7-Specific CD8$^+$ T-cell responses compared to mice primed and boosted with CRT/E7 or mice primed with CRT/E7 and boosted with α-GalCer + CRT/E7 (Fig. 1C and D). We investigated the effects of α-GalCer as an adjuvant on dendritic cell (DC)-based vaccines. Mice were vaccinated with E7-pulsed DC-1(DC-1-E7) with or without α-GalCer and boosted 1 week post-priming. Mice who received DC-1-E7 with α-GalCer at the prime or boost phases generated greater E7-Specific CD8$^+$ T-cell responses compared to mice vaccinated without α-GalCer. However, mice were vaccinated DC-1-E7 with α-GalCer at both prime and boost phase did not enhance E7-Specific CD8$^+$ T-cell immune responses (Supplemental Fig. 1). Previous studies have shown that α-GalCer stimulate the splenic DC maturation [19]. We also examined the splenic DC responses to α-GalCer after intramuscular administration. Splenic DC from mice injected α-GalCer (2 μg) or LPS (25 μg) expressed significant high level of CD40, CD86 on the surface compared with mice non-injected control group. However, CD80, D^b (MHC class I) and I-A^b (MHC class II) expression were not significantly changed (Supplemental Fig. 2).

Our data therefore suggested that CRT/E7 co-administration with α-GalCer at the prime phase generated greater E7-specific CD8$^+$ T-cell precursors after CRT/E7 boosting through the stimulation of maturation DCs.

3.2. Mice primed with pcDNA3-CRT/E7 vaccine and α-GalCer and boosted pcDNA3-CRT/E7 vaccine alone generated potent anti-tumor effects

We performed *in vivo* tumor treatment experiments using the previously characterized E7-expressing tumor model, TC-1 [20] to determine whether the enhanced E7-specific CD8$^+$ T-cells generated by the combination of CRT/E7 and α-GalCer could be translated into therapeutic anti-tumor effects. C57BL/6 mice were initially challenged with TC-1 cells (2×10^5/mouse) by subcutaneous injection into the right hind leg. Mice were vaccinated i.m. twice at 1 week intervals with various CRT/E7 and α-GalCer combinations three days after tumor challenge. Treated mice were monitored for tumor growth. We observed that mice primed with α-GalCer + CRT/E7 and boosted with CRT/E7 generated the best therapeutic anti-tumor effects among all tested vaccination regimens tested (Fig. 2A). The mice treated with the α-GalCer + CRT/E7 prime and CRT/E7 booster regimen demonstrated prolonged survival in vaccinated mice (Fig. 2B). Therefore, our data suggested that the therapeutic

anti-tumor effects of the DNA vaccines in tumor-challenged mice could be significantly improved by the addition of α-GalCer at the prime phase.

3.3. Priming with α-GalCer + CRT/E7 and boosting with E7-pulsed DC-1 further enhances E7-specific CD8$^+$ T-cell immune responses in vaccinated mice

DNA- and DC-based vaccines have advantages and disadvantages, as previously discussed. Mice were vaccinated with various combinations of the DNA vaccines, α-GalCer and DC-1-E7 to identify the best vaccine protocol. As shown in Fig. 3A and B, Mice primed with α-GalCer + CRT/E7 and boosted with DC-1-E7 generated two times the numbers of E7-specific CD8$^+$ T-cells compared to the mice primed with α-GalCer + CRT/E7 and boosted with CRT/E7 (Fig. 3A and B). Furthermore, we investigated the effects of α-GalCer on a DNA vaccine prime-dendritic cell vaccine boost regimen. Mice primed with α-GalCer + CRT/E7 and boosted with DC-1-E7 generated more E7-specific CD8+ T-cells than the mice primed with CRT/E7 and boosted with α-GalCer + DC-1-E7 (Fig. 3C and D). Our data suggested that the α-GalCer and DNA vaccine prime and DC-1-E7 boost regimen further enhanced E7-specific CD8$^+$ T-cell immune responses in vaccinated mice.

3.4. Therapeutic anti-tumor effects of α-GalCer + CRT/E7 can be further improved by boosting with E7-pulsed DC-1

We performed *in vivo* tumor treatment experiments using the previously characterized E7-expressing tumor model, TC-1, to determine whether the enhanced E7-specific CD8$^+$ T-cells generated by priming with α-GalCer + CRT/E7 and boosting with DC-1-E7 could be translated into improved anti-tumor effects [20]. C57BL/6 mice were initially challenged with TC-1 cells (2×10^5/mouse) subcutaneously. Mice were vaccinated twice i.m. at 1 week intervals with various combinations three days after tumor challenge. The α-GalCer + CRT/E7 prime-DC-1-E7 boost combination generated the best therapeutic anti-tumor effects among all tested combinations (Fig. 4A). Mice primed with CRTE7 and boosted with DC-1-E7 in the presence of α-GalCer also resulted in significantly prolonged survival; however, these mice began to die on the 43rd day after tumor challenge (Fig. 4B). These data suggested that the DNA vaccines in the presence of α-GalCer followed by antigen-expressing dendritic cells led to enhanced anti-tumor effects and prolonged survival in tumor-bearing mice through generation of E7-specific CD8$^+$ T-cells.

3.5. CD8$^+$ T-cells are important for the anti-tumor effect in mice primed with α-GalCer + CRT/E7 and boosted with E7-pulsed DC-1

Several studies have shown that α-GalCer presented by CD1d molecule expressing on dendritic cells efficiently stimulates NKT cells implicated in innate immunity [27,28]. We performed an *in vivo* antibody depletion experiment to determine the contribution of various lymphocyte subsets to tumor protection and treatment generated in mice vaccinated α-GalCer + CRT/E7 prime-DC-1-E7 boost combination. CD4, CD8, and NK depletion were initiated after vaccination. As shown in Fig. 5, mice immunized with α-GalCer + CRT/E7 prime-DC-1-E7 boost completely were protected tumor growth after TC-1 challenge, In contrast, 100% of nonvaccinated(naïve) mice and mice depleted of CD8$^+$ T-cells grew tumor within 2 weeks after TC-1 challenge. None of the mice depleted of CD4$^+$ T-cells and 80% of mice depleted of NK cells remained tumor-free conditions at day 60 after TC-1 challenge. These data suggested that CD8$^+$ T-cells are the major contributors to the observed anti-tumor effects generated by α-GalCer + CRT/E7 prime-DC-1-E7 boost combination.

3.6. Priming by CRT/E7 with α-GalCer and boosting with E7-pulsed dendritic cells leads to enhanced E7-specific CD8$^+$ memory T-cells in vaccinated mice

Therapeutic anti-tumor vaccines can generate long-term memory immune responses. Mice were vaccinated with various combinations of α-GalCer and DC-1-E7 to determine the effects of α-GalCer on the generation of long-term E7-specific CD8$^+$ memory T-cells. Control mice were also immunized with pcDNA3 and DC-1 alone. Splenocytes were collected and characterized for the presence of E7-specific CD8$^+$ memory T-cells by intracellular IFN-γ cytokine staining followed by flow cytometric analysis two months later. Mice primed with α-GalCer + CRT/E7 and boosted with DC-1-E7 generated the highest number of E7-specific CD8$^+$ memory T-cells. CRT/E7 priming followed by DC-1-E7 boosting in the presence of α-GalCer also produced significant levels of E7-specific CD8$^+$ memory T-cells in vaccinated mice (Fig. 6A and B). Our data therefore suggested that the addition of α-GalCer to the DNA vaccine led to increased E7-specific CD8$^+$ memory T-cell responses after boosting with antigen-expressing dendritic cells in vaccinated mice.

3.7. α-GalCer + CRT/E7 prime-E7-pulsed dendritic cells boost leads to long-term protection against TC-1 tumors in vaccinated mice

We performed long-term *in vivo* tumor protection experiments to determine whether the observed increases in E7-specific CD8$^+$ memory T-cells generated by priming with α-GalCer + CRT/E7 and boosting with DC-1-E7 could be translated into long-term protective anti-tumor effects. C57BL/6 mice were vaccinated with various combinations of the DNA vaccines, α-GalCer and DC-1-E7. Immunized mice were challenged s.c. with TC-1 tumor cells (5×10^5/mouse) two months after the last vaccination and were monitored for tumor growth. Mice primed with α-GalCer + CRT/E7 and boosted with DC-1-E7 demonstrated complete inhibition of tumor growth compared with mice vaccinated with other regimens (Fig. 7A). We also observed significantly prolonged survival in these mice. Mice primed with CRT/E7 and boosted with DC-1-E7 in the presence of α-GalCer also demonstrated time-limiting prevention of tumor generation in vaccinated mice for a period of time; tumors grew at day 25 after tumor challenge (Fig. 7B). These data suggested that mice primed with α-GalCer + CRT/E7 and boosted with E7-pulsed dendritic cells could further enhance the generation of E7-specific CD8$^+$ memory T-cells against the TC-1 tumor model in vaccinated mice.

4. Discussion

We demonstrated that the heterogenous α-GalCer + CRT/E7 prime and E7-pulsed DC-1 boost regimen generated the highest number of E7-specific CD8$^+$ effectors and memory T-cells and the best anti-tumor effects in vaccinated mice compared with other protocols in the present study. DNA vaccination represents an attractive strategy for cancer immunotherapy by combining vaccine stability, cost-effectiveness and safety. Additionally, enhancement of immune responses after plasmid DNA immunization has been noted in mouse models. Despite these advantages, DNA vaccines alone have not been sufficient to induce complete or sustained immunity and also displayed suboptimal potency in non-human primate models and in clinical trials [29].

The primary mechanism of α-GalCer to enhance and maintain immune response to DNA vaccination may associate to several steps in antigen uptake and presentation. While the mechanism of antigen recruitment and presentation after intramuscular injection of DNA is not entirely defined, previous studies agree that the likely pathway responsible for antigen presentation is either the direct transfection of muscle cells or transfection of dendritic cells (DCs) *in vivo* [26]. The NKT-cell ligand, α-GalCer, stimulates the full maturation of DCs *in situ* after a single injection and results in induction of CD8$^+$ T-cell immunity against co-

administered proteins [19]. We also observed that splenic DC from mice injected α-GalCer (2 μg) expressed significant high level of CD40, CD86 on the surface compared with mice non-injected control group. However, several clinical trials using or combined with α-GalCer only focused on enhancement of the NKT cell-specific immune responses. Motohashi et al. [30] showed that the administration of α-GalCer-pulsed IL-2/GM-CSF-cultured peripheral monocytes was well tolerated and was accompanied by the successful induction of NKT cell-dependent immune responses. Kunii et al. [31] also showed that intra-arterial infusion of activated Valpha24 NKT cells and the sub-mucosal injection of α-GalCer -pulsed APC have been shown to induce significant anti-tumor immunity. Several studies show that α-GalCer has a potent adjuvant effect on generation of antigen-specific cytotoxic T lymphocytes through stimulation of DCs but offer little therapeutic effects against tumor by co-administration of DNA vaccine inducing CTLs responses with α-GalCer as adjuvant. Our studies show the new measure to overcome low immunogenicity of DNA vaccine with α-GalCer for inducing strong tumor antigen-specific CTLs responses through stimulating maturation of DCs.

α-GalCer previously displayed adjuvant effects on HIV DNA vaccines after administration at priming, leading to the enhancement of both antigen-specific cellular and humoral responses. The adjuvant activity of α-GalCer is not restricted to certain antigens and is more profound when administered with low-dose DNA vaccines [12,32]. Our results are consistent with previous reports which demonstrated that mice primed with CRT/E7 in the presence of α-GalCer (which has adjuvant role for DC maturation) generated a significant number of E7-specific $CD8^+$ effector T-cells after CRT/E7 boosting as well as significant therapeutic anti-tumor effects against TC-1 tumors. Co-administration of E7-pulsed DC-1 (DC-1-E7) with α-GalCer at either the prime or boost phase generated greater E7-specific $CD8^+$ T-cell precursors compared to mice vaccinated without α-GalCer.

However, mice boosted with CRT/E7 and α-GalCer did not enhance or suppress immune responses and mice co-administered CRT/E7 with α-GalCer both at prime and boost phase produced only 20% E7-specific $CD8^+$ T-cells when compared with mice primed and boosted with CRT/E7 alone. Previous report also showed that the adjuvant activity of α-GalCer was most profound when co-administered at the priming, but not at the boosting phase and mice that received α-GalCer during both priming and boosting had reduced $CD8^+$ IFN-γ T-cell responses. [19]. It appears that mice exposed to α-GalCer frequently resulted in a minimal production of cytokines by NKT cells, leading to a failed adjuvant effect in the boosting phase [33]. However, we observed that 80% of the mice depleted NK cells remained tumor-free conditions at day 60 after tumor challenge. We need further investigation to find out the specific mechanism of NKT cells or α-GalCer for DC maturation and generation of antigen-specific $CD8^+$ T cell responses after DNA vaccination. The induction of an effective immune response against an established tumor via DNA vaccines may require repeated immunization. Heterologous prime-boost vaccination with a different agent (recombinant virus or protein vaccine) may eliminate the poor efficacy of boosting with the same viral vector or DNA vaccine [34–36]. After intramuscular injection of a naked plasmid DNA, myocytes are the predominant cells transfected; however, DCs and macrophages within the muscle tissue are also transfected [37,38]. DNA vaccines are more safe than live attenuated viral vector and relatively simple and inexpensive to design and create. Although the exact mechanism of cellular macromolecule entry is still unknown, larger molecules such as naked plasmid DNA are thought to enter through a multistep mechanism. Electroporation (EP) is a method whereby cellular membranes are transiently destabilized to facilitate the entry of foreign molecule into cells and tissues [39]. Recently, EP can boost cellular expression of DNA plasmids and immunogenicity across several species, different types of cells and tissues, even human clinical trials. For instance, it has been shown that EP can be used to enhance CRT/E7 DNA vaccine in the preclinical model

[40]. Furthermore, the intramuscular administration of clinical grade CRT/E7 DNA vaccine followed by electroporation will be evaluated in patients with advanced head and neck cancer (Dr. Sara Pai, personal communications). However, the clinical application of EP technology may require appropriate devices that can provide a precise control of voltage and current to prevent excessive tissue damage. For this reason, it is necessary to consider the development of a more available and potent adjuvant system to enhance DNA vaccine immunogenicity.

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) and mediate effective *in vivo* and *in vitro* immune responses [41]. Administration of DCs with tumor-specific antigens may elicit anti-tumor cell-mediated immune responses and has utility as an anti-cancer therapy. However, the preparation of individualized DC-based vaccines is difficult to generate because it requires large scale culture, high costs, and prolonged time periods. For these reasons, appropriate combinations of DNA- and DC-based vaccines in prime-boost regimens might be more effective than either alone. Interestingly, we observed that priming with α-GalCer + CRT/E7 and boosting with E7-pulsed DC-1 further enhanced E7-specific $CD8^+$ T-cell immune responses in vaccinated mice compared to mice primed with CRT/E7 and boosted with α-GalCer + DC-1-E7. We additionally observed that the α-GalCer + CRT/E7 prime and DC-1-E7 boost combination generated the best therapeutic anti-tumor effects and led to significant prolonged survival in vaccinated mice among all tested DNA-DCs vaccine combinations.

An important feature of a therapeutic anti-tumor vaccine is the ability to generate long-term memory immune responses. Mice primed with α-GalCer + CRT/E7 and boosted with DC-1-E7 demonstrated complete inhibition of tumor growth compared with mice vaccinated other vaccination regimens. We also demonstrated that priming with CRT/E7 followed by DC-1-E7 in the presence of α-GalCer also produced significant levels of E7-specific $CD8^+$ memory T-cells in vaccinated mice. However, mice primed with CRT/E7 and boosted with DC-1-E7 in the presence of α-GalCer started to die on the 43^{rd} day after tumor challenge. α-GalCer may influence the APC function or generation of E7-specific $CD8^+$ memory T-cell, and may result in the enhancement of protection against TC-1 tumor cells. The specific mechanisms for the observed effects of α-GalCer on antigen-specific T-cells at prime phase warrant further investigation.

In summary, our data suggests that the enhancement of DNA vaccine potency by the co-administration of α-GalCer and DCs-based vaccine led to enhanced antigen-specific $CD8^+$ T-cell activity and potent *in vivo* anti-tumor effect. These findings may have significant therapeutic utility. This innovative strategy may be a highly useful approach in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.vaccine.2010.08.079.

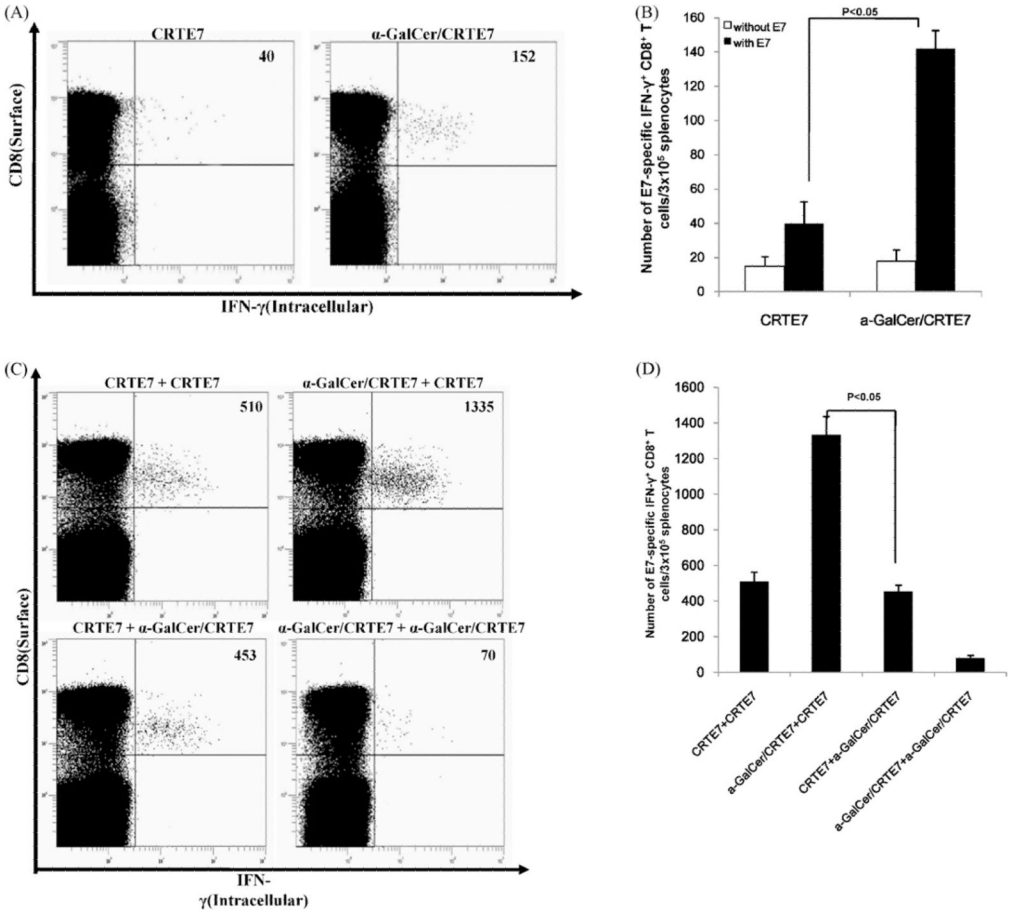

Fig. 1.

Characterization of E7-specific $CD8^+$ T-cell immune responses in mice primed with DNA vaccine and α-GalCer. (A) C57BL/6 mice (n = 5 per group) were immunized with combination DNA vaccines (100 μg of DNA/mouse), either with or without α-GalCer (2 μg per injection). Splenocytes were harvested 1 week post-vaccination and were tested for E7-specific $CD8^+$ T-cells by intracellular IFN-γ staining followed by flow cytometry. (B) Bar graphs depicting numbers of E7-specific IFN-γ-secreting $CD8^+$ T-cells per 3×10^5 splenocytes after administration of a single vaccination (mean ± SD values). (C) C57BL/6 mice (5 mice per group) were immunized with the DNA vaccine (100 μg/mouse) either with or without in α-GalCer (2 μg per injection). Mice received vaccination boosters of both the DNA vaccine and α-GalCer. Representative flow cytometry data for the E7-specific $CD8^+$ T-cells are also described. Numbers in the upper right-hand corner represent the number of E7-specific IFN-γ-secreting $CD8^+$ T-cells per 3×10^5 pooled splenocytes. (D) Bar graphs depicted the numbers of E7-specific IFN-γ-secreting $CD8^+$ T-cells per 3×10^5 splenocytes (mean ± SD values). The data presented in this figure are from one of two representative experiments.

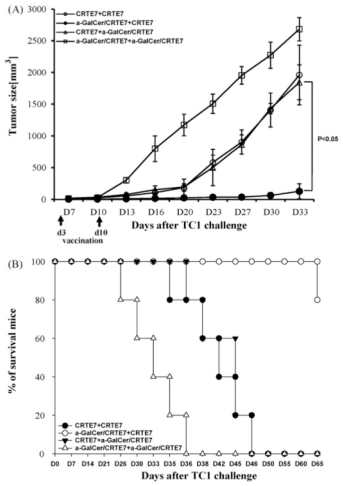

Fig. 2.

In vivo tumor treatment experiments. C57BL/6 mice (n = 5 per group) were initially challenged with TC-1 tumor cells (1×10^5/mouse) by s.c. injection. Mice were injected with DNA (100 μg/mouse) with or without added α-GalCer (2 μg per injection) three days after tumor challenge. The mice were vaccinated 1 week later with various vaccine combination regimens, and were monitored twice weekly for evidence of tumor growth by inspection and palpation. Tumor volumes were measured starting at day 7 after tumor challenge. (A) Line graph depicting the tumor volumes in mice of different tumor treatments (means ± SD). (B) Kaplan–Meier survival analysis of tumor treatment experiments in mice. Data shown are from one of two representative experiments.

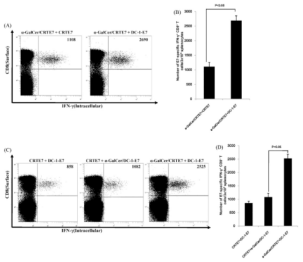

Fig. 3.

Characterization of E7-specific $CD8^+$ T-cell immune responses in mice primed with a α-GalCer + DNA vaccine and boosted with DC-1-E7. C57BL/6 mice (n = 5 per group) were immunized with various combination DNA vaccines (100 μg DNA/mouse) with α-GalCer (2 μg per injection). Mice received booster vaccinations 1 week later with various vaccine combination regimens. Splenocytes were harvested 1 week after the last vaccination and were tested for E7-specific $CD8^+$ T-cells by intracellular IFN-γ staining and flow cytometry. (A and C) Representative flow cytometry data for E7-specific $CD8^+$ T-cells. Numbers in the upper right-hand corner represent the number of E7-specific IFN-γ-secreting $CD8^+$ T-cells per 3×10^5 pooled splenocytes. (B and D) Bar graphs depicting numbers of E7-specific IFN-γ-secreting $CD8^+$ T-cells per 3×10^5 splenocytes (means ± SD). Data presented are from one of two representative experiments.

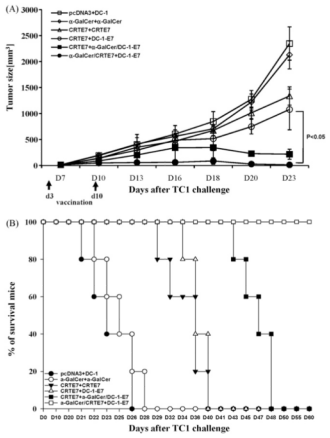

Fig. 4.

In vivo tumor treatment experiments. C57BL/6 mice (n = 5 per group) were initially challenged with a s.c. injection of TC-1 tumor cells (2×10^5/mouse). Mice received DNA injections (100 μg of DNA/mouse) three days after tumor challenge with or without added α-GalCer (2 μg/injection). The mice were boosted 1 week later with various vaccine combination regimens, including DNA vaccine or E7-pulsed DC-1 in the presence or absence of α-GalCer. The mice vaccinated with pcDNA3 or α-GalCer was used as controls. The mice were monitored for evidence of tumor growth by twice weekly inspection and palpation. Tumor volumes were measured starting at day 7 after tumor challenge. (A) Line graph depicting tumor volumes in mice of different tumor treatments (mean ± SD values). (B) Kaplan–Meier survival analysis in mice from the tumor treatment experiments. The data shown here are from one of two representative experiments.

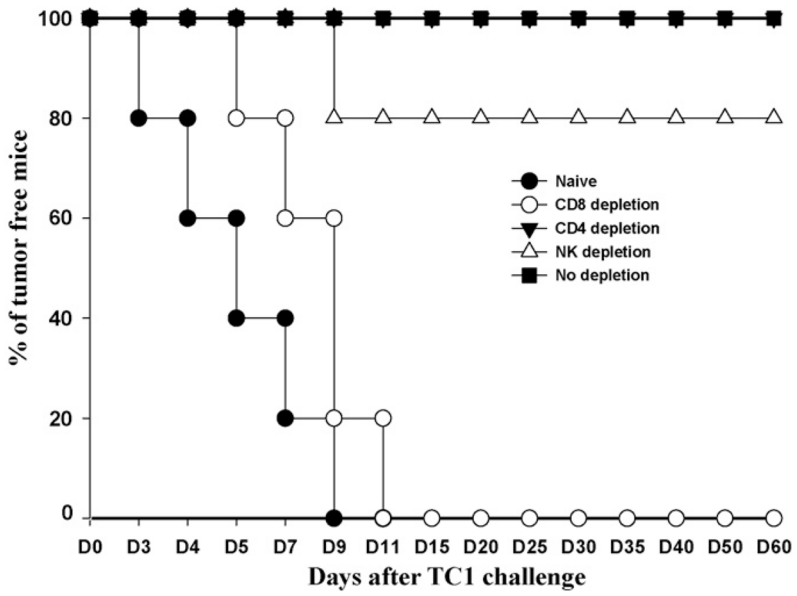

Fig. 5.

In vivo tumor protection experiment and *in vivo* antibody depletion experiment in α-GalCer + DNA vaccine primed and DC-1-E7 boosted mice. An *in vivo* tumor protection experiment was performed to determine if the observed enhancement in E7-specific $CD8^+$ T-cells mediated by α-GalCer + DNA vaccine prime-DC-1-E7 boost combination also led to a significant E7-specific anti-tumor protection response. Mice (n = 5 per group) were primed with 100 μg of DNA vaccine in the presence of α-GalCer and boosted with E7-pulsed DC-1. *In vivo* antibody depletion was performed to determine the effect of lymphocyte subsets on tumor protection. CD4, CD8 or NK depletion was initiated after last vaccination. At 1 week after the last vaccination, mice were challenged with 2×10^5 TC-1 tumor cells/mouse in the right hind leg. Tumor growth was monitored by visual inspection and palpation twice or thrice weekly. The data shown here are from one of two representative experiments.

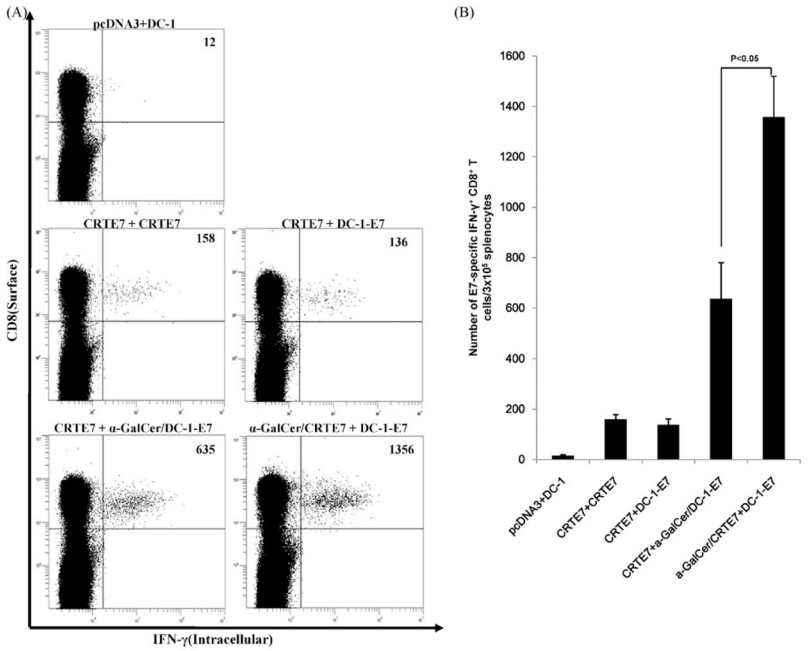

Fig. 6.

Characterization of long-term E7-specific $CD8^+$ T-cell immune responses in mice primed with α-GalCer + DNA vaccine and boosted DC-1-E7. C57BL/6 mice (n = 5 per group) were immunized with various DNA vaccine combinations (100 μg of DNA/mouse) and α-GalCer (2 μg/injection). The mice were boosted at 1 week later with various vaccine combination protocols. Splenocytes from vaccinated mice were harvested 2 months after the last vaccination and tested for E7-specific $CD8^+$ T-cells by staining for intracellular IFN-γ followed by flow cytometry. (A) Representative flow cytometric data for the E7-specific $CD8^+$ T-cells. Numbers in the upper right-hand corner represent the number of E7-specific IFN-γ-secreting $CD8^+$ T-cells per 3×10^5 pooled splenocytes. (B) Bar graphs depicting the numbers of E7-specific IFN-γ-secreting $CD8^+$ T-cells per 3×10^5 splenocytes (mean ± SD values). The data presented in this figure are from one of two representative experiments.

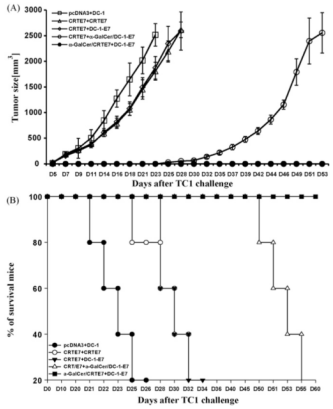

Fig. 7.

Long-term *in vivo* tumor protection experiments. C57BL/6 mice (*n* = 5 per group) were immunized with various DNA vaccine combinations (100 μg of DNA/mouse) in the presence or absence of α-GalCer (2 μg/injection). The mice were boosted at 1 week later with various vaccine combination regimens. Mice were challenged by s.c. injection of 5×10^5 of TC-1 cells/mouse two months after the last vaccination. The mice were monitored for evidence of tumor growth by twice weekly inspection and palpation. Tumor volumes were measured starting at the 7th day after tumor challenge. (A) Line graph depicting tumor volumes in mice challenged with TC-1 cells (mean ± SD values). (B) Kaplan–Meier survival analysis in mice challenged with TC-1 cells. The data shown here are from one of two representative experiments.