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SA-4-1BBL as the immunomodulatory component of a HPV-16 E7 protein based vaccine shows robust therapeutic efficacy in a mouse cervical cancer model

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Abstract

Cervical cancer is the leading cause of cancer-related deaths among women worldwide. Current prophylactic vaccines based on HPV (Human papillomavirus) late gene protein, L1 are ineffective in therapeutic settings. Therefore, there is an acute need for the development of therapeutic vaccines for HPV associated cancers. The HPV E7 oncoprotein is expressed in cervical cancer and has been associated with the cellular transformation and maintenance of the transformed phenotype. As such, E7 protein represents an ideal target for the development of therapeutic subunit vaccines against cervical cancer. However, the low antigenicity of this protein may require potent adjuvants for therapeutic efficacy. We recently generated a novel chimeric form of the 4-1BBL costimulatory molecule engineered with core streptavidin (SA-4-1BBL) and demonstrated its safe and pleiotropic effects on various cells of the immune system. We herein tested the utility of SA-4-1BBL as the immunomodulatory component of HPV-16 E7 recombinant protein based therapeutic vaccine in the E7 expressing TC-1 tumor as a model of cervical cancer in mice. A single subcutaneous vaccination was effective in eradicating established tumors in approximately 70% of mice. The therapeutic efficacy of the vaccine was associated with robust primary and memory CD4⁺ and CD8⁺ T cell responses, Th1 cytokine response, infiltration of CD4⁺ and CD8⁺ T cells into the tumor, and enhanced NK cell killing. Importantly, NK cells played an important role in vaccine mediated therapy since their physical depletion compromised vaccine efficacy. Collectively, these data demonstrate the utility of SA-4-1BBL as a new class of multifunctional immunomodulator for the development of therapeutic vaccines against cancer and chronic infections.

Keywords

costimulation; vaccine; SA-4-1BBL; adjuvant; human papillomavirus; cervical cancer

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1. Introduction

Cervical cancer continues to be a major healthcare problem and is the leading cause of cancer-related deaths among women worldwide. HPV is the leading cause of cervical cancer [1] and may also be the causative agent of some other tumors, such as head and neck [2]. Although two vaccines based on the structural L1 protein of HPV-16 and 18 subtypes forming virus like particles have demonstrated prophylactic efficacy in the clinic [3], they lack therapeutic efficacy against cervical cancer [4]. Therefore, the development of a therapeutic vaccine is of important significance to both of those individuals who are already exposed to HPV-infection and may develop cervical cancer and to those are with the active disease. The oncoproteins, E6 and E7, are consistently co-expressed in the majority of HPV-containing carcinomas and their metastatic lesions throughout the replicative cycle of virus. These proteins play a critical role in the induction and maintenance of malignant phenotype and tumor metastasis [5]. Most importantly, with the abundant expression in cervical cancer lesions, well characterized immunology, lack of antigenic drift and requirement for tumor growth makes E6 and E7 oncoproteins ideal tumor associated antigens (TAAs) for the development of therapeutic vaccines against HPV-associated cancer. Furthermore, these proteins are well conserved, and as such, vaccines based on E6 and E7 may have efficacy against tumors induced by a broad range of HPV subtypes [6,7].

Various vaccine approaches based on the E7 protein or peptides representing T cell epitopes have been developed and tested in preventive and therapeutic preclinical tumor models. Although effective in preventing the growth of E7 expressing transplantable tumors in mice [6,7], these vaccines have shown only moderate efficacy in therapeutic settings [8,9]. Although the exact mechanistic basis of vaccine failure is yet to be defined and is probably complex, active immune evasion mechanisms employed by the tumor may play a critical role. The success of E7 TAA-based therapeutic vaccines against cervical cancer, therefore, may require vaccine formulations containing adjuvants that not only generate E7-specific potent immune responses, but also overcome the tumor-mediate immune evasion mechanisms [9–12]. Costimulation plays a critical role for the generation of adaptive immune responses, and as such we recently proposed that vaccine formulations containing costimulatory ligands may have efficacy in therapeutic cancer settings [13]. We particularly focused on the 4-1BBL, a costimulatory member of the TNF family because of the critical role 4-1BB signaling plays in the generation and maintenance of CD8⁺ T cell memory [14,15], which is critical for the eradication of tumors [13,14,16]. Inasmuch as 4-1BBL has no function as a soluble trimeric molecule [17], we generated a chimeric recombinant SA-4-1BBL where the extracellular portion of this molecule was cloned C-terminus to core streptavidin (SA) [18]. This chimeric molecule exists as tetramers and oligomers with potent costimulatory activity on CD4⁺ and CD8⁺ T cells in soluble form [18,19]. Vaccination with SA-4-1BBL and E7 peptide representing the dominant CD8⁺ T cell epitope resulted in effective eradication of E7 expressing TC-1 tumors [13]. The therapeutic efficacy of the vaccine was superior over other vaccine formulations containing an agonistic antibody (Ab) to 4-1BB receptor or TLR agonists, such as LPS, MPL, and CpG [13,19].

In this study, we tested the efficacy of SA-4-1BBL as the immunomodulatory component of E7 protein based vaccine in the TC-1 tumor model as a prelude to Phase I clinical trials for cervical cancer. The whole E7 protein as the antigenic component of vaccine alleviates the concerns related to the use of a single peptide representing CD8⁺ T cell dominant epitope that include; i) saturation of immune response due to antigen exhaustion, ii) lack of CD4⁺ T cell help which can limit antitumor efficacy of vaccine, iii) lesser magnitude and duration of immune response towards a single epitope as compared to collective responses to multiple epitopes, iv) higher possibility of immune-edited escape variants, and v) requirement for HLA compatibility that will limit target patient population. We herein demonstrated that a

single vaccination with SA-4-1BBL and a recombinant whole E7 protein resulted in the eradication of established tumors in 70% of mice. The therapeutic efficacy of the vaccine was associated with robust primary and memory T cell responses, Th1 cytokines, enhanced intratumoral CD4⁺ and CD8⁺ T cell infiltration, and NK cell function. Taken together, these data corroborate the utility of SA-4-1BBL as a novel multifunctional immunomodulatory component of therapeutic vaccines and justify testing the E7 protein based vaccine formulation in human clinical trials.

2. Materials and methods

2.1. Mice and cell lines

C57BL/6.SJL and C57BL/6 mice (6–8 wk old) were purchased from The Jackson Laboratory or bred in our animal facility at the University of Louisville. C57BL/6 *4-1BB*^{−/−} mice were kindly provided by Dr. A.T. Vella of University of Connecticut, Farmington, CT, with permission from Dr. B.S. Kwon of University of Ulsan, Korea. All animals were cared for in accordance with institutional and National Institute of Health guidelines. TC-1 cell line was purchased from American Type Culture Collection (Manassas, VA).

2.2. Reagents

Fluorochrome-conjugated Abs (anti-CD4-APC, anti-CD8-PerCP, anti-CD25-PE, anti-CD19-APC, anti-I-A/I-E-PE, anti-4-1BB-bio, anti-4-1BBL-PE, anti-IFN- γ -PE, anti-IL-2 PerCpCy5.5, anti-IL-2 PE, anti-CD45.1-APC, anti-CD45.2-PE, anti-CD44-APC and SA-PerCP) and isotype controls were purchased from BD PharMingen and eBioscience. HPV-16 E7 peptide (E7₄₉₋₅₇ RAHYNIVTF) was purchased from CPC Scientific, Inc.

2.3. Cloning, expression, and purification of HPV16-E7 and SA-4-1BBL

A modified form of the E7 antigen of HPV-16 was cloned using a previously reported cDNA [20] as template for PCR. The PCR product was subcloned into the pTWIN-1 bacterial expression vector (New England Biolabs) using Nde I and BamH I restriction sites. To alleviate concerns about using the oncogenic form of native E7 protein [21], site-directed mutagenesis was performed to replace Rb binding Cys24 and Glu26 with glycines (Cys24Gly-Glu26Gly; underlined). The construct also contains a C-terminal 6X-histidine tag for purification. After transformation into C2566 competent *E. coli* cells (New England Biolabs), cultures were grown at 37°C and induced using IPTG. Cells were harvested 3 hours after induction, pelleted, and resuspended in 100 ml of lysis buffer (20 mM Tris, pH 7.0, 500 mM NaCl, 5 mM imidazole, 5 mM β -ME, 10 μ M ZnCl₂). Cells were lysed by ultrasonication and inclusion bodies and insoluble material were pelleted at 10,000 $\times$ g for 10 min. Pellet was washed three times by resuspending in lysis buffer containing 1% Triton X-100, rotating 30 min at room temperature, and repelleting at 35,000 $\times$ g for 30 min. The final pellet was resuspended in 100 ml lysis buffer containing 6M Guanidine-HCl and rotated at RT overnight to solubilize inclusion bodies. After, centrifugation at 35,000 $\times$ g for 30 min at 4°C to remove the insoluble material, supernatant collected for IMAC purification using Talon® cobalt resin according to the manufacturer's protocol (Clontech) with the exception of including 0.1% Triton X-114 to remove endotoxin. All purification steps were performed in the presence of 10 μ M ZnCl₂ and 5 mM β -ME to assist with proper folding and reduce oligomerization. Protein was dialyzed against PBS, concentrated using an Amicon Ultra MWCO 10,000 and sterile filtered using a 0.22 μ m filtration device. Protein concentration was measured using BCA and Bradford methods (Pierce). E7- C24G/E26G Sequence: R S M H G D T P T L H E Y M L D L Q P E T T D L Y G Y G Q L N D S S E E E D E I D G P A G Q A E P D R A H Y N I V T F C C K C D S T L R L C V Q S T H V D I R T L E D L L M G T L G I V C P I C S Q K P G H H H H H H. Endotoxin levels for the

E7 protein was 0.021 EU/ μ g E7. Construction, expression, purification, and characterization of SA-4-1BBL (endotoxin level 0.004 EU/ μ g protein) were recently described [18].

2.4. Flow cytometry

For phenotyping and sorting, spleens, lymph nodes, and tumors were processed into single-cell suspensions, and cells were labeled with saturating concentrations of fluorochrome-conjugated Abs. Isotype matched Abs with the same fluorochrome were used as controls. For intracellular cytokine staining, lymph node cells were resuspended in MLR medium at 2×10^6 /ml and stimulated with PMA (5 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma) for 2 hrs in a 37°C, 5% CO₂ incubator. GolgiPlug (1 μ l/ml, BD Pharmingen) was added to the activation mixture and cells were incubated for an additional 4 hrs. Cells were then stained with anti-CD4-APC and anti-CD8-PerCP, fixed with 4% paraformaldehyde, and stained with PE-conjugated anti-mouse IFN- γ , PE or PerCPy5.5-conjugated anti-mouse IL-2 or isotype control in permeabilization buffer containing saponin. Data was analyzed using CellQuest (BD Biosciences) and FlowJo (Tree Star) software.

2.5 In vitro lymphocyte proliferation

Splenocytes from naive, immunized, and long term boosted mice were cultured (2×10^5 /well) with 5 μ g/ml of soluble E7 for 5 days in complete MLR medium. Cultures were pulsed with [³H]-thymidine for the last 16 hrs of the culture period, and harvested on a Tomtec Harvester 96 (Tomtec Inc., Hamden, CT) for quantification of incorporated radioactivity. Results were expressed as mean $\pm$ SD cpm of triplicate wells.

2.6. In vivo cytotoxicity assay

A standard *in vivo* killing assay was performed by injecting peptide-pulsed target cells into congenic immunized mice as previously described [13]. In brief, a population of C57BL/6.SJL (CD45.1) spleen cells were labeled with 2.5 μ M fluorescent dye CFSE (CFSE^{high}) while a second population was labeled with 0.25 μ M CFSE (CFSE^{low}). CFSE^{high} cells were then pulsed with 2 μ g/ml of E7₄₉₋₅₇ peptide representing the dominant CD8⁺ T cell epitope for E7 for 90 min at 37°C in a 5% CO₂ incubator. CFSE^{high} and CFSE^{low} cells were then extensively washed to remove free peptide, mixed at 1:1 ratio, and injected i.v. into C57BL/6 (CD45.2) mice 7 days after vaccination. Spleens were harvested 48 hrs later, processed into single cell suspension, stained with APC-labeled anti-mouse CD45.1 Ab and analyzed by multiparameter flow cytometry to determine the ratio of CFSE^{high}/CFSE^{low} target cells. The percentage of *in vivo* killing was calculated by the following formula: $[1 - ((\text{CFSE}^{\text{high}}/\text{CFSE}^{\text{low}} \text{ for experimental})/(\text{CFSE}^{\text{high}}/\text{CFSE}^{\text{low}} \text{ for naive}))] \times 100$.

2.7. Tumor model and vaccination

To establish tumors, 1×10^5 live TC-1 cells were resuspended in 200 μ l of PBS and injected s.c. into the right back flank of naive syngenic C57BL/6 mice. Tumor growth was monitored 2–3 times per week and tumor size was measured in mm using a caliper. Average tumor size was calculated by measuring two perpendicular diameters. Animals bearing tumors were euthanized when tumors reached a size of 15 mm in one of the two perpendicular diameters or earlier if tumors ulcerated or animal showed signs of discomfort. A depleting Ab (clone PK136, Bioexpress) against mouse NK1.1 molecule was used at 500 μ g/mice via intra peritoneal injection to deplete NK cell one day before vaccination. The depletion of these cells was complete as monitored at day 5 post treatment. For therapeutic studies, mice were immunized s.c. on day 6 post-tumor challenge with various vaccine formulations containing 50 μ g of E7 protein alone or in combination with 25 μ g of SA-4-1BBL or 10 μ g of SA (equimolar) as control. The quantities of SA-4-1BBL and E7 used represent optimum doses established by published literature [13] and studies in this report.

2.8 Analysis of tumor infiltrating T cells

C57BL/6 mice bearing TC-1 tumors of ~3 mm in diameter were injected s.c. with 50 µg of E7 protein mixed with 25 µg SA-4-1BBL or 10 µg SA. Tumors were harvested 7 days later and cut into 2-mm pieces after removal of connective tissue by dissection. To isolate T cells, tumors were incubated in an enzyme mixture consisting of 2 mg of collagenase-P/ml, 1 mg of DNase I/ml, 10 U of penicillin/ml, and 10 µg of streptomycin/ml in PBS for 2 hrs at 37°C with occasional vortexing. The digested tissue was passed through a nylon mesh and the resultant cells were washed twice in PBS before being stained for flow cytometric analysis. Cells were stained with appropriate fluorochrome labeled anti-mouse CD3 and CD8 Abs and PE-conjugated anti-mouse CD45.2 Ab to selectively exclude CD45 negative tumor cells from the analysis. Three million cells were analyzed using multi parameter flow cytometry.

For immunofluorescence analysis, tumors from vaccinated mice described above were dissected, washed, embedded in OCT, snap-frozen, and cut into 5.0 µm sections with a cryostat. Sections were fixed with 4% PFA followed by blocking with PBS supplemented with 1% BSA and 5% goat serum for 30 min. at room temperature to avoid any nonspecific bindings. To assess the presence of tumor infiltrating T cells, sections were next incubated with rat anti-mouse CD4 and CD8 Ab (PharMingen) for 1 hr at room temperature. After 3 washes with PBS, sections were incubated with 1/400 dilution of goat anti-rat Alexa 647 (2 mg/ml, Invitrogen), followed by counterstained with Hoechst 33342, and analyzed using confocal microscopy (Leica TCS SP5). A minimum of 4 fields for each tumor section were analyzed to assess the infiltration of T cells.

2.9. NK cell cytotoxicity assay

E7+SA-4-1BBL long term survived mice were boosted using 50 µg E7 protein in combination with 25 µg SA-4-1BBL. Seven days later, splenocytes were harvested from these long term boosted and naïve mice and cultured (5×10^6 cells/ml) in complete MLR medium supplemented with 10 µg/ml E7 and 50 IU/ml rIL-2 for 5 days as described previously [13]. Live lymphocytes were recovered by centrifugation over a ficoll gradient and used as effectors against [³H]-thymidine labeled YAC-1 target cells at various E:T ratios for 4 hrs as previously described [22].

2.10 Statistical analysis

Statistical analysis was done using the Student's *t*-test or ANOVA. The survival assays were analyzed using long-rank test in SPSS software. For each test, *p* value less than 0.05 and 0.001 were considered significant (*) and very significant (**), respectively. Error bars represent ± SD.

3. Results

3.1. Cloning, expression, purification, and characterization of recombinant E7 protein

We generated a mutated form of HPV-16 E7 protein that has Cys24 and Glu26 replaced with Gly to abolish its interaction with the retinoblastoma tumor suppressor protein [20], thereby warding off concerns for its tumorigenic potential as a component of vaccine. The modified E7 protein was expressed in bacteria as inclusion bodies, denatured with Guanidine-HCl, and purified using IMAC, taking advantage of a C-terminus 6X-His tag. The purified protein runs approximately as a 16 kDa band on SDS-PAGE and reacts with antibodies to E7 protein (Fig. 1). The identity and purity of the E7 protein was further confirmed using trypsin digest and nano LC/MS/MS (data not shown).

3.2. Vaccination with E7 protein and SA-4-1BBL generates primary T cell responses

Generation of T cell-mediated proliferative and killing responses against tumors is critical for therapeutic efficacy of vaccines. Therefore, we immunized naïve mice s.c. with E7 protein mixed with SA-4-1BBL and a week later, assessed E7₄₉₋₅₇ peptide-specific *in vivo* killing response [13]. Mice vaccinated with E7 protein mixed with SA-4-1BBL generated significantly higher *in vivo* killing response as compared with those immunized with the E7 protein in combination with control SA protein or PBS (Fig. 2A). Consistent with the killing response, E7+SA-4-1BBL vaccine generated more vigorous *in vitro* proliferative response to the E7 protein as compared with E7 mixed with SA or PBS (Fig. 2B). Taken together, these data demonstrate that E7 protein is antigenically active and SA-4-1BBL serves as a potent immunostimulatory component of the vaccine formulation for the generation of effective T cell proliferative and killing responses.

3.3. Vaccination with E7 protein and SA-4-1BBL eradicates established TC-1 tumors and generates long term tumor specific memory response that requires 4-1BB signaling for efficacy

We next tested whether E7+SA-4-1BBL vaccine would offer a therapeutic efficacy against established TC-1 tumors expressing E7 protein as a TAA [7]. C57BL/6 mice were challenged with 1×10^5 live TC-1 tumor cells s.c. on the right flank and then vaccinated s.c. once on day 6 post tumor challenge with E7+SA-4-1BBL or E7+SA or PBS as controls. Vaccination with E7+SA-4-1BBL resulted in 70% tumor-free survival over the 90 day observation period (Fig. 3A and Supplement, Fig. S1). In marked contrast, all mice vaccinated with E7+SA expired from tumor-burden within 55 days (Fig. 3A). This survival rate, although slightly improved over the control mice injected with PBS, was not statistically significant. Importantly, long-term survivors did not develop tumor when re-challenged with live TC-1 cells 60 days after initial tumor inoculation, demonstrating the existence of long-term immunologic memory.

The vaccine efficacy was strictly dependent on 4-1BB signaling as all mice deficient for 4-1BB receptor (4-1BB^{-/-}) did not generate an enhanced therapeutic response as compared with those vaccinated with E7+SA and expired from tumor burden within 50 days (Fig. 3B and Supplement, Fig. S2). However, vaccination with E7, irrespective of SA-4-1BBL, significantly ($p < 0.05$) improved the survival of mice as compared with PBS controls. Interestingly, control 4-1BB^{-/-} mice injected with PBS had shorter survival time as compared to wild type mice (Fig. 3A), further suggesting the importance of 4-1BB signaling in eliciting immune responses against tumors.

3.4. The therapeutic efficacy of E7 protein and SA-4-1BBL vaccine is associated with the generation of robust T cell proliferative and effector responses

To elucidate the mechanistic basis of immune responses involved in TC-1 tumor therapy, long term surviving (>90 days) mice that had eradicated tumors were boosted with the E7+SA-4-1BBL and then analyzed 7 days later for various immune responses. Mice that were boosted with E7+SA-4-1BBL generated robust E7₄₉₋₅₇ peptide-specific *in vivo* killing response (Fig. 4A, specify it in the figure legend as well). Similarly, long term boosted mice generated significantly higher E7 protein-specific *in vitro* lymphoproliferative response as compared with naïve animals (Fig. 4B). Importantly, the long-term boosted mice also generated significant intracellular IL-2 and IFN- γ responses in both CD4⁺ and CD8⁺ T cells as compared with naïve mice (Fig. 4C, D and Supplement, Fig. S3).

3.5. Vaccination with E7 protein and SA-41BBL generates long term T cell memory pool and enhanced intratumoral CD4⁺ and CD8⁺ T cells

Signaling via 4-1BB has been shown to play a critical role not only for the T cell survival but also in the establishment of long-term memory [15,23,24]. Therefore, we analyzed whether the immunotherapeutic efficacy of the vaccine was associated with enhanced total CD44^{hi} T cell memory pool. Mice with effective E7+SA-4-1BBL therapy had a significant increase in the total peripheral CD44^{hi} T cell memory pool for both CD4⁺ and CD8⁺ T cell subsets as compared with naive animals (Fig. 5A).

Although the peripheral activation of TAA-specific T cells demonstrates the immune efficacy of the vaccine, such a response may not generate a clinical response in the absence of infiltration of these cells into the tumor [25,26]. To assess whether vaccination with E7+SA-4-1BBL enhances intratumoral T cells, mice with established tumors of 3–5 mm in diameter were vaccinated and tumors were surgically removed 7 days later for analysis. Vaccination with E7+SA-4-1BBL indeed resulted in significantly higher number of CD4⁺ and CD8⁺ T cells into the tumor as compared with immunization with E7+SA or PBS as determined by flow cytometry (Fig. 5B) and immunofluorescence microscopy (Fig. 5C).

3.6. NK cells play a critical role in vaccine efficacy

NK cells up regulate 4-1BB expression following activation, and signaling via this receptor using agonistic 4-1BB Abs were previously shown to enhance NK cell-mediated helper activity for CD8⁺ T cells and IL-2 responsiveness, but not the direct cytotoxicity [27,28]. To test if vaccination with SA-4-1BBL enhances NK cell-mediated cytotoxicity, long-term mice that had undergone successful immunotherapy were boosted with E7+SA-4-1BBL. Splenocytes from these mice were harvested 7 days later and tested for NK cell killing response using YAC-1 tumor cells as target. As shown in Fig. 6, E7+SA-4-1BBL vaccinated mice had potent NK cell specific killing activity against target cells as compared with naive controls. Importantly, the enhanced killing activity was relevant to therapy as depletion of NK cells one day before vaccination using an Ab to NK1.1 significantly compromised the E7+SA-4-1BBL vaccine efficacy against established TC-1 tumor (Fig. 3A and Supplement, Fig. S1).

4. Discussion

Despite intense efforts and impressive preclinical data, the therapeutic efficacy of TAA-based subunit cancer vaccines in the clinic remains to be realized. Although there are several reasons for the lack of clinical translation such as weak immunogenic nature of TAAs, immune tolerance to such antigens and the disease and treatment status of cancer patients, an elaborate set of immune evasion mechanisms employed by the progressing tumor may play the most critical role for the failure of therapeutic subunit cancer vaccines. Therefore, vaccine formulations which not only generate new immune responses and/or boost ongoing ones in cancer patients, but also reverse the immune suppressive or evasive mechanisms employed by progressing tumor may stand the best chance for eliciting an effective anti-tumor immune response to the magnitude and duration required for the clearance of established tumors.

Towards this end, we recently proposed that costimulatory ligands of the TNF family can be used as adjuvant of choice for the development of effective therapeutic vaccines due to their critical roles in modulating innate, adaptive, and regulatory immunity. As such, we generated a chimeric form of 4-1BBL (SA-4-1BBL) with potent costimulatory activity in soluble form [18]. The choice of 4-1BBL primarily stems from the critical role of 4-1BB signaling in the generation of primary and long-term memory CD8⁺ T cell responses that are

critical for the eradication of tumors [13–15]. Vaccination with SA-4-1BBL and a mutated form of HPV-16 E7 protein resulted in E7 protein-specific CD4⁺ and CD8⁺ T cell primary and long-term memory responses. Importantly, a single s.c. vaccination was effective in eradicating established E7 expressing TC-1 tumors in 70% of mice with tumor-free survival over a 90-day observation period. Mice with effective immunotherapy did not develop tumors when re-challenged 60 days later with live TC-1 cells, demonstrating the existence of long-term immunological memory. The long-term memory response was further substantiated by increased memory pool for both CD4⁺ and CD8⁺ T cells and enhanced T cell proliferative, killing, and Th1 cytokine responses in long-term surviving mice with effective therapy. The better therapeutic efficacy of the E7+SA-4-1BBL vaccine also correlated with significantly higher number of tumor-infiltrating CD4⁺ and CD8⁺ T cells as compared to controls. This is clinically important as increase in T cell infiltration into human colorectal cancer has been associated with better prognosis [26,29].

Importantly, we demonstrated that immunization with E7 protein and SA-4-1BBL was also effective in generating potent NK cell killing response and this response was relevant to the therapeutic efficacy of the vaccine as shown by the NK cell depletion experiment. This observation is consistent with the previous observation demonstrating the critical role NK cells play in killing class I negative tumors [30]. HPV E7 protein has been implicated in the down-regulation of MHC class I molecules on the cell surface by affecting the transcription regulation of MHC class I genes and those associated with antigen processing and presentation as a mechanism of immune escape [31]. We (Sharma *et al.*, unpublished observations) and others [30,32] have demonstrated a decrease in the levels of MHC class I molecules on the surface of TC-1 cells as a function of tumor progression. It has previously been shown that NK cells up regulate 4-1BB expression following activation, and signaling via this receptor using an agonistic Ab enhances NK cell-mediated helper activity for CD8⁺ T cells and IL-2 responsiveness, but not the direct cytotoxicity [27,28]. The helper function of NK cells for the generation of CTL responses was further substantiated in the poorly immunogenic B16-F10 melanoma model using a combined IL-12 gene transfer and an agonistic Ab to 4-1BB as a means of immunotherapy [33].

However, our data demonstrates for the first time, to our knowledge, that 4-1BB signaling via SA-4-1BBL improves the killing activity of NK cells. This obvious discrepancy between our data and the previous publications may be due to the quantitative as well as qualitative differences and toxicities associated with the use of agonistic anti-4-1BB Abs *vs.* SA-4-1BBL. For example, we have recently shown that SA-4-1BBL has potent costimulatory activity on both CD4⁺ and CD8⁺ T cells *in vitro* and *in vivo*, whereas agonistic 4-1BB Ab had costimulatory activity only on CD8⁺ T cells with lack/minimal effect on CD4⁺ T cells [19]. This strong and broad costimulatory activity of SA-4-1BBL translated into better therapy in the TC-1 tumor model as compared with the agonistic Ab [19]. Most importantly, the use of agonistic Ab at therapeutic doses is associated with the depletion of NK cells [19,34,35] whereas SA-4-1BBL not only lacks such a negative effect on NK cells [19], but also enhances NK cell killing response as shown in this study.

The therapeutic efficacy of whole E7 protein and SA-4-1BBL vaccine in the TC-1 tumor model was comparable to the efficacy generated by using a synthetic peptide representing the dominant CD8⁺ T cell epitope for E7 protein as the antigenic component of the vaccine [13]. The comparable therapeutic efficacies of the peptide and whole E7 protein-based vaccines may be due to the vaccine formulations and the tumor model used in these studies. Both vaccine formulations included equal amounts of antigen (50 µg each), and therefore, more molar amounts of the peptide. The excess amount of the peptide and its faster kinetics of presentation by MHC class I molecule may allow robust generation of a CD8⁺ T cell response that curbs the tumor growth. Inasmuch as the TC-1 tumor is a transplantable tumor

model and has fast growth kinetics, antigen escape variants due to immunological pressure may not occur within the time frame of the experiment. However, this may be totally different in a spontaneous tumor setting [36], where immunological pressure may likely to give rise to antigen-loss variants. Therefore, either whole E7 protein-based vaccine or those with both E6 and E7 proteins [37] may have better efficacy in a spontaneous setting due to the availability of not only multiple CD8⁺, but also CD4⁺ T cell epitopes [38]. Consistent with this notion, we demonstrated the potent primary as well as memory CD4⁺ and CD8⁺ T cell responses in mice vaccinated with E7 protein and SA-4-1BBL. Although CD4⁺ T cell help appears not to be required for mounting primary immune responses, it is critical for the generation and maintenance of long term memory and recall responses [39,40].

The development of nontoxic adjuvants that not only activate effector arm of the immune system against tumor, but also overcome various immune evasion mechanisms by the progressing tumor is key to the success of therapeutic subunit vaccines against cancer. Importantly, we previously reported that the pleiotropic effects of SA-4-1BBL on cells of innate, adaptive, and regulatory immunity [13] and treatment with SA-4-1BBL at therapeutic doses did not result in detectable signs of acute toxicity recently reported for agonistic Abs to 4-1BB [19] as assessed by lymphadenopathy, lymphocyte proliferation, systemic cytokine response, and gross pathology [19]. Taken together, our findings support the notion that SA-4-1BBL has the potential to serve as an effective and safe adjuvant as component of therapeutic vaccines. Testing its therapeutic efficacy in clinical trials, and if effective, this molecule may serve as safe and effective platform for the development of therapeutic vaccines against cancer and chronic infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Characterization of the purified recombinant E7 protein. IMAC purified recombinant E7 protein was run on an SDS-PAGE and stained with Coomassie (A) or analyzed using antibodies against E7 in Western blots (B). Proteins of the indicated kDa used as standard size markers (M).

Fig. 2. Vaccination with E7 protein and SA-41BBL generates potent primary T cell responses. (A) *In vivo* endogenous CTL killing response. Naïve C57BL/6 (CD45.2⁺) mice were immunized s.c. with E7 (50 µg) + SA-4-1BBL (25 µg). Seven days post vaccination, mice received E7₄₉₋₅₇-pulsed syngeneic splenocytes from C57BL/6.SJL (CD45.1⁺) as targets. *In vivo* peptide-specific killing was assessed 2 days later, and expressed as percent lysis for each histogram (n = 3–4; ** *p* < 0.001 compared with E7+SA or PBS controls). (B) E7+SA-4-1BBL treated mice generate vigorous *in vitro* proliferative responses to the E7 protein. Splenocytes from (A) were pulsed with 5 µg of E7 protein/ml/10⁶ cells and incubated for 5 days, and proliferation was assessed by [³H] thymidine incorporation. Results are representative of three independent experiments (** *p* < 0.001).

Fig. 3.

Vaccination with E7 protein and SA-4-1BBL eradicates established TC-1 tumors and NK cells play an important role in vaccine efficacy. (A) C57BL/6 mice were challenged with 1×10^5 live TC-1 cells and vaccinated once s.c. on day 6 post-tumor challenge with E7 protein (50 μ g) mixed with SA-4-1BBL (25 μ g) or an equimolar quantity of SA (10 μ g). The role of NK cells in the observed vaccine efficacy was tested by depleting these cells with i.p. injection of 500 μ g anti-NK1.1 Ab/mouse one day before vaccination. * $p < 0.05$ for E7+SA-4-1BBL vs. NK depleted/E7+SA-4-1BBL group. ** $p < 0.001$ for E7+SA-4-1BBL vs. PBS and E7+SA groups. (B) The vaccine efficacy of E7+SA-4-1BBL is strictly dependent on 4-1BB signaling. C57BL/6 4-1BB^{-/-} mice were vaccinated as in (A). $p < 0.05$ for E7+SA-4-1BBL or E7+SA vs. PBS group.

Fig. 4. Therapeutic efficacy of E7 and SA-4-1BBL vaccine is associated with the generation of potent T cell effector and Th1 cytokine responses. (A) Long-term mice with effective therapy were boosted with E7+SA-4-1BBL and 7 days later tested for (A) E7₄₉₋₅₇-specific *in vivo* killing response (n = 3, ** $p < 0.001$ vs. naive control), (B) E7-specific proliferation, (C) intracellular IL-2 response (n = 3, ** $p < 0.001$ vs. naive control), and (D) IFN- γ response (n = 3, * $p < 0.05$ vs. naive control). Naive mice were used as controls.

Fig. 5.

Vaccination with E7 protein and SA-4-1BBL results in enhanced long-term peripheral T cell memory pool and infiltration into the tumor. (A) Splenocytes from long-term tumor-challenged mice with effective immunotherapy were harvested 7 days after boosting with E7+SA-4-1BBL and subjected to phenotypic analysis using various T cell markers in multiparameter flow cytometry. Data is representative of two independent experiments. (B, C) Mice with TC-1 tumors of 3–5 mm in diameter were injected with E7+SA-4-1BBL, E7+SA, or PBS. Tumors were harvested 7 days later and subjected to analysis using flow cytometry (** $p < 0.001$) or confocal microscopy (C). Data are representative of 3–4 mice per treatment group per panel.

Fig. 6.

Mice with effective immunotherapy generates robust NK cell killing response. Long-term mice with effective immunotherapy were boosted by s.c. vaccination with E7+SA-4-1BBL. Splenocytes were harvested 7 days later and pulsed with 5 μ g of E7/ml/ 10^6 cells in the presence of 50 IU IL-2 and incubated for 5 days. The viable cells were isolated using Ficoll gradient centrifugation and used as effectors against [3 H] labeled YAC-1 cells as targets at various effector:target (E:T) ratios. Percent specific cytotoxicity is graphed separately for 3 experimental mice. Effector cells prepared from splenocytes harvested from unimmunized naive mice cultured under similar conditions were used as controls.