Costimulation as a Platform for the Development of Vaccines: A Peptide-Based Vaccine Containing a Novel Form of 4-1BB Ligand Eradicates Established Tumors

Rajesh K. Sharma, Kutlu G. Elpek, Esma S. Yolcu, Rich-Henry Schabowsky, Hong Zhao, Laura Bandura-Morgan, and Haval Shirwan

Institute for Cellular Therapeutics, Department of Microbiology and Immunology, and James Graham Brown Cancer Center, University of Louisville, Louisville, Kentucky

Abstract

Vaccines represent an attractive treatment modality for the management of cancer primarily because of their specificity and generation of immunologic memory important for controlling recurrences. However, the efficacy of therapeutic vaccines may require formulations that not only generate effective immune responses but also overcome immune evasion mechanisms employed by progressing tumor. Costimulatory molecules play critical roles in modulating innate, adaptive, and regulatory immunity and have potential to serve as effective immunomodulatory components of therapeutic vaccines. In this study, we tested the function of a novel soluble form of 4-1BB ligand (4-1BBL) costimulatory molecule in modulating innate, adaptive, and regulatory immunity and assessed its therapeutic efficacy in the HPV-16 E7-expressing TC-1 cervical cancer and survivin-expressing 3LL lung carcinoma mouse models. Vaccination with 4-1BBL activated dendritic cells and enhanced antigen uptake, generated CD8+ T-cell effector/memory responses, and endowed T effector cells refractory to suppression by CD4+CD25+FoxP3+ T regulatory cells. Immunization with 4-1BBL in combination with an E7 peptide or survivin protein resulted in eradication of TC-1 and 3LL tumors, respectively. 4-1BBL was more effective than TLR agonists LPS, MPL, and CpG, and an agonistic 4-1BB antibody as a component of E7 peptide-based therapeutic vaccine for the generation of immune responses and eradication of TC-1 established tumors in the absence of detectable toxicity. Therapeutic efficacy was associated with reversal of tumor-mediated nonresponsiveness/anergy as well as establishment of long-term CD8+ T-cell memory. Potent pleiotropic immunomodulatory activities combined with lack of toxicity highlight the potential of 4-1BBL molecule as an effective component of therapeutic cancer vaccines. [Cancer Res 2009;69(10):4319–26]

Introduction

Regardless of many advances in vaccinology, the therapeutic potential of cancer vaccines remains to be realized. This is partially due to an array of immunoevasive and suppressive mechanisms employed by progressing tumors (1, 2). Therefore, the success of therapeutic vaccines will require formulations that are effective not only in generating new immune responses and/or boost the existing ones but also in their ability to overcome immune evasion mechanisms. In this context, the discovery and development of novel adjuvants with potent immunomodulatory activities on cells of innate, adaptive, and regulatory immunity without adverse toxicity at therapeutic doses is of significant importance in the field of cancer immunotherapy.

Costimulatory signals transduced via CD28 and TNFR family members play paramount roles in modulating innate, adaptive, and regulatory immunity (3), and as such, agonistic ligands for this class of immunomodulatory receptors have potential to serve as effective components of therapeutic cancer vaccines. Consistent with this notion is the demonstrated efficacy of agonistic antibodies against costimulatory receptors in various therapeutic preclinical tumor settings (4–7). The use of agonistic antibodies, however, may be associated with severe toxicity as shown in selected settings in rodents (8, 9) and human (10). We hypothesized that signaling by natural ligands may have better efficacy and safety compared with agonistic antibodies and herein tested this notion using 4-1BB ligand (4-1BBL), a member of the TNFR family, as the immunomodulatory component of vaccines. The choice of 4-1BBL was because of the pleiotropic effects of 4-1BB signaling on various cells of innate (11), adaptive (5, 12), and regulatory (13, 14) immunity as well as the robust therapeutic efficacy of agonistic 4-1BB antibodies in various rodent cancer models (4, 5). Inasmuch as the natural 4-1BBL functions as a cell membrane-bound protein and has no activity in soluble form (15), we recently generated a novel form of this ligand by fusing the extracellular domain of murine 4-1BB to the COOH terminus of a modified core streptavidin (SA-4-1BBL; ref. 16). The choice of streptavidin is because it exists as stable tetramers and oligomers (17) and as such serves as a chaperone to enable the chimeric SA-4-1BBL to exist as multivalent tetramers and oligomers (18, 19) with the ability to cross-link 4-1BB receptor on immune cells for potent signal transduction (16).

In the present study, we showed that soluble SA-4-1BBL protein served as an effective immunomodulatory component of vaccines by activating dendritic cells and enhancing antigen uptake, stimulating primary T-cell responses, maintaining long-term memory, and licensing T effector (Teff) cells to overcome the suppressive effect of CD4+CD25+FoxP3+ T regulatory (Treg) cells. A single vaccination with SA-4-1BBL mixed with survivin protein or a dominant CD8+ T-cell epitope for E7 was effective in eradicating established 3LL and TC-1 tumors, respectively, without detectable toxicity. Importantly, SA-4-1BBL had better activity than TLR agonists, LPS, MPL, and CpG, and an agonistic antibody to 4-1BB in
modulating various immune responses and eradicating TC-1 tumors. Collectively, our findings provide a strong rationale for further developing this novel form of soluble 4-1BBL as an immunomodulatory component of therapeutic vaccines against cancer and infections.

**Materials and Methods**

**Mice.** C57BL/6.SJL and C57BL/6 mice were bred in our animal facility at the University of Louisville. 4-1BB knockout mice were generously provided by Dr. A.T. Vella (University of Connecticut), with permission from Dr. B.S. Kwon (University of Ulsan). All animals were cared for in accordance with institutional and NIH guidelines.

**Reagents.** Construction, expression, purification, and characterization of SA-4-1BBL (endotoxin level 0.004 EU/μg protein) were recently described (16). Anti-41BB agonistic antibody (clone 3H3) was kindly provided by Dr. R. Mittler (Emory University; ref. 9). Fluorochrome-conjugated antibodies (anti-CD8-PerCP, anti-I-A/I-E-PE, anti-CD86-APC, anti-IFN-γ-PE, anti-CD45.1/2-APC) and isotype controls were purchased from BD Pharmingen and eBioscience. HPV-16 E7 peptide (E749-57 RAHYNIVTF) and CpG oligonucleotide (G*GGGGACGATCGTCG*G*G*G*G; * represents phosphorothioate linkage) were purchased from CPC Scientific and Operon Biotechnologies, respectively. LPS and MPL were purchased from Sigma-Aldrich and InVivogen, respectively.

**Intracellular cytokine staining.** Lymph nodes were processed into single-cell suspensions and stimulated with phorbol 12-myristate 13-acetate/ionomycin for intracellular cytokine staining as described (20).

**In vivo antigen uptake by dendritic cells.** FITC-labeled ovalbumin (25 μg) mixed with SA-4-1BBL (25 μg) or equimolar quantity of SA in PBS was injected subcutaneously into the right flank of C57BL/6 mice. Animals injected with ovalbumin-FITC alone served as control. Draining lymph nodes were harvested 3 h later and processed into a single-cell suspension. After blocking Fc receptors, cells were stained with APC-conjugated anti-mouse CD11c antibody. FITC+CD11c+ cells were analyzed using flow cytometry.

**T-cell proliferation and suppression assays.** The effect of soluble SA-4-1BBL protein on CD8+ T-cell proliferation was determined as described previously (16). For suppression assay, CD4+CD25+ Treg and CD4+CD25−Teff cells were sorted from wild-type and 4-1BB knockout C57BL/6 mice by flow cytometry. Treg cells were cocultured at various ratios with a fixed number of Teff cells (2.5 × 10^4 per well) in U-bottomed 96-well plates in the presence of anti-CD3 antibody, irradiated (2,000 cGy) syngeneic splenocytes (1 × 10^5 per well), 1 μg/mL SA-4-1BBL, or equimolar (0.4 μg/mL) quantity of SA protein. Cells were cultured for 3 days, pulsed with [3H]thymidine during the last 16 h of culture, harvested, and analyzed for proliferation as described (16).

![Figure 1](image-url)
In vivo cytotoxicity assay. B6.SJL (CD45.1) spleen cells were labeled with 2.5 μmol/L CFSE (CFSE<sup>high</sup>) and 0.25 μmol/L CFSE (CFSE<sup>low</sup>). CFSE<sup>high</sup> cells were then pulsed with either 2 μg/mL E<sub>749-57</sub> or a survivin peptide as a control for 90 min at 37°C in a 5% CO<sub>2</sub> incubator. After extensive washing, CFSE<sup>high</sup> and CFSE<sup>low</sup> cells were mixed at a 1:1 ratio and injected intravenously into C57BL/6 (CD45.2) mice 5 days after vaccination. Spleens were removed 48 h later, processed into single-cell suspension, stained with APC-labeled CD45.1 antibody, and analyzed by flow cytometry to determine the ratio of CFSE<sup>low</sup>/CFSE<sup>high</sup> target cells. The percentage of in vivo killing was calculated by the formula: (1 - [(CFSE<sup>low</sup> / CFSE<sup>high</sup>) for test] / (CFSE<sup>low</sup> / CFSE<sup>high</sup> for naive)] × 100.

Tumor models and vaccination. One hundred thousand TC-1 or 3LL cells were injected subcutaneously into the right flank of C57BL/6 mice. Tumor growth was monitored two to three times per week using caliper. Animals bearing tumors were euthanized when tumors reached a size of 15 mm in diameter or earlier if tumors ulcerated or animal showed signs of discomfort. For TC-1 therapeutic studies, mice were immunized subcutaneously 10 days post-tumor challenge with 50 μg E<sub>749-57</sub> peptide alone or in combination with 25 μg SA-4-1BBL, 10 μg SA as control protein, 25 μg LPS, 25 μg MPL, 10 μg CpG, or 100 μg 3H3 antibody against 4-1BB. The quantities of LPS, MPL, and CpG used represent optimum doses established by published literature (21) and studies in this report. For tumor recurrence studies, tumors were removed surgically when they reached an average size of 4 mm in diameter. The mice were left to recover from surgery for 48 h and then vaccinated subcutaneously with 50 μg E<sub>749-57</sub> plus 25 μg SA-4-1BBL or an equimolar control SA (10 μg) protein. For 3LL tumors, subcutaneous vaccination was done using 25 μg SA-4-1BBL mixed with 50 μg recombinant mouse survivin on day 6 post-tumor challenge.

Analysis of tumor-infiltrating CD8<sup>+</sup> T cells. Mice bearing TC-1 tumors of ~3 mm in diameter were injected subcutaneously with 50 μg E<sub>749-57</sub> peptide mixed with 25 μg SA-4-1BBL, 25 μg MPL, 25 μg LPS, or 10 μg CpG. Tumors were harvested 7 days later, digested in 2 mg collagenase P/mL, and 1 mg DNase I/mL in PBS for 2 h at 37°C with occasional shaking. The resultant cells were washed and stained with anti-mouse CD3 and CD8. Anti-CD45.2 antibody was used to selectively exclude CD45<sup>+</sup> tumor cells from analysis.

Statistical analyses. Statistical analyses were done using Student's t test, ANOVA, or log-rank test using SPSS software. For each test, P values less than 0.05 and 0.001 were considered significant (*) and very significant (**), respectively.

Results

SA-4-1BBL has pleiotropic effects on cells of innate, adaptive, and regulatory immunity. The 4-1BB receptor is constitutively expressed on a subpopulation of immature dendritic cells and CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells (14, 16) and inducibly expressed on activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells (22, 23). Therefore, we first investigated the effect of SA-4-1BBL treatment on each of these cell populations. In vitro treatment of bone marrow-derived primary immature dendritic cells or the JAWS II dendritic cell line with...
SA-4-1BBL for 48 h resulted in the activation of both cell types as assessed by the up-regulated expression of CD86 and MHC class II molecules, and this effect was comparable with that obtained using the TLR4 agonist LPS (Fig. 1A). However, SA-4-1BBL was more effective than MPL, a modified version of LPS, and TLR9 agonist CpG in up-regulating various activation markers on bone marrow-derived dendritic cells (Supplementary Fig. S1; Supplementary Table S1).

It has been shown that activation of immature dendritic cells with an agonistic antibody to CD40 or TLR agonists result in enhanced antigen uptake followed by maturation (24). To test if 4-1BB stimulation also enhances antigen uptake by dendritic cells, we injected mice with SA-4-1BBL and ovalbumin-FITC as a cognate antigen. There was a significant increase (>11%) in antigen uptake by dendritic cells isolated from mice treated with SA-4-1BBL compared with dendritic cells from mice treated with ovalbumin-FITC alone (1.7%) or antigen with control SA (0.85%; Fig. 1B).

We have recently shown that SA-4-1BBL as a soluble protein has robust costimulatory activity on CD4+ T cells (16). To test if the costimulatory activity of SA-4-1BBL also applies to CD8+ T cells, flow-sorted cells were used in a CD3 antibody-based proliferation assay. At suboptimal doses of anti-CD3 antibody stimulation, SA-4-1BBL showed potent costimulatory activity on CD8+ T cells (Fig. 1C).

CD4+CD25+FoxP3+ Treg cells constitutively express the 4-1BB receptor (14, 25, 26). We have recently shown that 4-1BB signaling into Treg cells results in their proliferation without a major effect on their suppressive function in the absence of the ligand (16). However, in the presence of 4-1BBL, Treg cells failed to suppress Teff cells and the lack of suppressive effect was associated with the 4-1BB signaling into Teff cells rather than Treg cells (16). To further confirm this finding, we performed CD3 antibody-based suppression studies using Teff and Treg cells obtained from 4-1BBL wild-type and knockout mice. Costimulation with SA-4-1BBL was effective in blocking the suppressive function of Treg cells on wild-type T eff cells but not knockout T eff cells, showing the importance of 4-1BB signaling into T eff cells for overcoming the suppressive function of Treg cells (Fig. 1D). The Treg cells from knockout mice showed better suppressive activity (Fig. 1D). Taken together, these data show that SA-4-1BBL has pleiotropic effects on cells of innate (dendritic cells), adaptive (Teff cells), and regulatory (Treg cells) immunity and as such has potential to serve as an effective immunomodulatory component of therapeutic cancer vaccines.

SA-4-1BBL serves as an effective immunomodulatory component of a therapeutic cancer vaccine. To test if the significant immunomodulatory activities of SA-4-1BBL translate into therapeutic efficacy, the TC-1 cell line expressing c-Ras and HPV-16 E7 and E6 oncogenes was used as a transplanted tumor model for cervical cancer. Immunization of naive mice with a synthetic E749-57 peptide representing the dominant CD8+ T-cell epitope in C57BL/6 mice (27) in combination with varying doses of SA-4-1BBL generated in vivo killing responses, in which 50 μg of the peptide and 25 μg SA-4-1BBL produced the most pronounced effect (Fig. 2A). The killing activity of this vaccine regimen was further confirmed using E7-expressing TC-1 cells as targets in an in vitro cytotoxicity assay (Supplementary Fig. S2). Consequently, this vaccine formulation was tested in two different therapeutic tumor settings for efficacy. A single subcutaneous injection of the vaccine formulation was effective in eradicating 10-day established TC-1 tumors in 75% of mice and retarding the tumor growth for the rest of the animals.
SA-4-1BBL vaccination reverses the tumor-mediated immune suppression. It has previously been reported that the initial antigen-specific killing response mounted by the host gradually decreases due to induction of antigen-specific anergy as a function of tumor growth in an E7+ tumor model (29). To confirm this finding and test the efficacy of our vaccine to boost tumor-specific CD8+ T-cell killing studies in unvaccinated tumor animals with small and large tumors. There was a significant reduction in the peptide-specific in vivo killing response in mice bearing large tumors compared with those with smaller tumors (Fig. 4A). Importantly, vaccination of mice bearing large tumors with SA-4-1BBL and E7 peptide restored the killing response to levels comparable with those observed for mice without tumors (Fig. 4B).

SA-4-1BBL has better efficacy than TLR agonists as the immunomodulatory component of a therapeutic cancer vaccine. There has been significant interest in using TLR agonists as adjuvants for therapeutic vaccines due to their stimulatory effects on innate immune responses (30). Inasmuch as 4-1BB signaling directly modulates innate, adaptive, and regulatory immunity, we hypothesized that SA-4-1BBL may have better immunostimulatory efficacy than TLR agonists. Initially, we compared the efficacy of SA-4-1BBL with MPL, a detoxified form of LPS used in the clinic (31). In a dose response study where 50 μg E749-57 peptide was used for vaccination in combination with various doses of SA-4-1BBL and MPL, we determined 25 μg/injection to be the optimal dose for both MPL and SA-4-1BBL for the generation of in vivo peptide-specific killing response (Fig. 5A). These doses of SA-4-1BBL and MPL were used for the rest of studies. SA-4-1BBL showed significantly better in vivo killing activity than MPL over various peptide doses tested (Fig. 5B). The better effect of SA-4-1BBL in generating in vivo killing responses translated to a better therapeutic efficacy against TC-1 tumors. A single vaccination with SA-4-1BBL and E749-57 peptide resulted in the eradication of tumors in 75% of mice compared with 30% to 42% achieved using LPS or MPL as components of the vaccine (Fig. 5C). Furthermore, administration of E749-57 peptide with 10 μg CpG, a dose that showed efficacy in previously published studies (21, 32), resulted in a survival rate (40%) comparable with those achieved using LPS and MPL. Importantly, vaccination with 50 μg of the peptide and 100 μg of an agonistic 4-1BB antibody (3H3; ref. 9) was less effective than peptide and SA-4-1BBL in eliminating TC-1 tumors (Supplementary Fig. S4).

The better efficacy of the SA-4-1BBL vaccine correlated with significantly higher percentages of tumor-infiltrating CD8+ T cells shown in Fig. 2B retained long-term peptide-specific CD8+ T effector memory responses as shown by in vivo killing (Fig. 3A). This effector memory response could further be boosted by revaccination, resulting in higher killing (Fig. 3A) and IFN-γ production (Fig. 3B). Consistent with the role of 4-1BB signaling in development and maintenance of memory, we observed a significant increase in total memory CD8+CD44high T-cell pool in long-term animals compared with naive mice (39.1% versus 14.7%; Fig. 3C). Importantly, we did not detect any sign of acute toxicity recently reported for agonistic antibodies to 4-1BB (9) in vaccinated mice as assessed by sizes of lymphoid tissues, lymphocyte proliferation, systemic cytokines, and gross pathology.1

1 R-H. Schabowsky and colleagues, submitted for publication.

with >80% survival for 90-day observation period (Fig. 2B; Supplementary Fig. S3). Vaccination of 4-1BB knockout mice with E749-57 peptide + SA-4-1BBL did not show better efficacy than E749-57 peptide alone (data not shown), showing that vaccine effect is primarily mediated through 4-1BB signaling. Furthermore, the surviving mice did not develop tumors when rechallenged with live TC-1 cells 60 days after initial tumor inoculation, showing long-term immunologic memory. Importantly, the therapeutic efficacy of SA-4-1BBL–based vaccine was also shown in the 3LL mouse lung carcinoma model where a recombinant survivin protein was used as a bona fide self-tumor-associated antigen (TAA; Fig. 2C).

Therapeutic cancer vaccines are well suited for the control/prevention of recurrences in patients who undergo tumor resection and chemotherapy, as such patients may have minimal levels of immunosuppressive/regulatory mechanisms that curb the efficacy of vaccines (28). Hence, we tested the efficacy of our vaccine formulation for preventing/controlling recurrences in a surgical tumor removal model in mice. A single subcutaneous vaccination with SA-4-1BBL and E749-57 peptide 2 days after resection of established tumors was effective in preventing recurrences in 90% of animals compared with 42.8% for the peptide alone and 0% for control groups (Fig. 2D). Tumor-free animals
compared with MPL or CpG as determined by flow cytometry (Fig. 5D) and immunofluorescence microscopy (Supplementary Fig. S5).

**SA-4-1BBL is more effective than TLR agonists in generating memory recall responses.** Given the demonstrated role of 4-1BB signaling in the maintenance of CD8\(^+\) T-cell memory pool and generation of secondary responses (33), we tested the efficacy of SA-4-1BBL to generate CD8\(^+\) T-cell memory responses in two different settings. First, memory and recall responses were investigated in long-term surviving mice that had undergone successful immunotherapy using E749-57 peptide in combination with SA-4-1BBL, LPS, MPL, or CpG. The long-term tumor-free mice were tested 90 days after single vaccination for \textit{in vivo} killing of the syngeneic target cells pulsed with E749-57 peptide. Mice vaccinated with 4-1BBL retained a significantly higher killing response than all other groups, indicating the existence of long-term effector memory response (Fig. 6A). The better efficacy of SA-4-1BBL in generating/maintaining long-term CD8\(^+\) T-cell memory was also shown in a second setting where naive mice that had been vaccinated 75 days earlier were rechallenged with the same vaccine formulation and tested for E749-57 peptide-specific \textit{in vivo} killing responses 7 days later (Fig. 6B).

**Discussion**

The role of immunosurveillance against spontaneous tumors and the potential for immunologic control of cancer have been well established. However, the translation of existing extensive knowledge in cancer immunobiology into successful TAA-based therapeutic cancer vaccines in the clinic remains to be realized. Although the exact nature of cellular and molecular mechanisms responsible for this deficiency is not known and most likely complex, the weak immunogenic features of TAAs, self-tolerance to these antigens, and various direct and indirect immunoevasive mechanisms employed by the progressing tumor are some possibilities (1,25,29). Therefore, the success of therapeutic vaccines most likely will depend on their ability to induce strong immune responses against tumors as well as overcome various immune evasion mechanisms. This may require vaccine formulations that are designed to contain immunomodulators having pleiotropic effects on various cells of the innate, adaptive, and regulatory immunity. We herein report that a novel form of soluble 4-1BBL molecule chimeric with core streptavidin, SA-4-1BBL (16), has potential to serve such an immunomodulator. SA-4-1BBL (a) activated
dendritic cells and enhanced antigen uptake, (b) generated primary and memory CD8⁺ T-cell responses, and (c) endowed T<sub>eff</sub> cells refractory to the suppressive function of CD4⁺CD25⁺FoxP3⁺ Treg cells. Importantly, these pleiotropic immunomodulatory functions of SA-4-1BBL translated into its efficacy as a component of TAAAs in eradicating established tumors in two different tumor models: a xenogeneic TAA peptide (E7)-based vaccination in TC-1 cervical cancer model and a self-TAA (survivin)-based vaccination in 3LL lung carcinoma model.

Engagement of 4-1BB with its ligand, 4-1BBL, expressed by activated APCs results in T-cell activation, clonal expansion, survival, and establishment and maintenance of long-term memory (5, 22, 34). Therefore, 4-1BB signaling has extensively been exploited for improving the efficacy of various immunotherapeutic approaches against cancer and infections. Signaling via 4-1BB using an agonistic antibody was shown to enhance the CD8⁺ T-cell response as well as broaden their repertoire to subdominant influenza epitopes (35). Importantly, 4-1BB stimulation was shown to be sufficient for the generation of primary CD8⁺ T-cell responses (33). Consistent with these findings, we showed that vaccination with the SA-4-1BBL and E7<sub>49-57</sub> peptide generated effective primary and memory CD8⁺ T-cell responses in naive mice as well as in mice challenged with TC-1 tumors expressing E7 antigen, and these responses were more pronounced than those generated using TLR agonists MPL and CpG. Unlike MPL and CpG that primarily target dendritic cells, the better efficacy of SA-4-1BBL may be due to its direct effects on the function of both dendritic cells and T cells. Following vaccination, SA-4-1BBL may first interact with constitutively expressed 4-1BB on dendritic cells and stimulate these cells for antigen uptake and up-regulation of various immunostimulatory molecules for the generation of primary CD8⁺ T-cell responses. At the second stage, SA-4-1BBL may interact with 4-1BB up-regulated on the surface of antigen-experienced CD8⁺ T cells for expansion, survival, and establishment of long-term memory. This notion is consistent with our data showing the robust function of soluble SA-4-1BBL in directly activating dendritic cells and T cells. Therefore, the inability of TLR agonists to directly activate and prolong survival of antigen-specific CD8⁺ T cells (5) may explain the better activity of SA-4-1BBL that potentiates both primary and memory responses.

Immunization with a single dose of SA-4-1BBL and a synthetic peptide representing the dominant CD8⁺ T-cell epitope for E7 resulted in the eradication of 10-day established tumors with tumor-free survival in 75% of mice. In addition, vaccination after surgical removal of tumors protected 90% of mice from recurrences. The therapeutic efficacy was associated with a strong peptide-specific in vivo killing response and a high frequency of CD8⁺ T cells expressing the signature cytokine IFN-γ for the Th1 response. TC-1 tumors were shown to induce anergy in CD8⁺ T cells as a means of immune evasion (29, 36). Consistent with this finding, we showed gradual decrease of peptide-specific in vivo killing responses as a function of tumor size. Importantly, vaccination with SA-4-1BBL and E7<sub>49-57</sub> peptide resulted in the recovery of peptide-specific in vivo killing responses in animals with large tumor burdens. Our results are consistent with previous studies showing that signaling via 4-1BB receptor using an agonistic antibody prevents and reverses established anergy of CD8⁺ T cells in the P815 mastocytoma tumor and bone marrow transplantation models (36).

Importantly, vaccination with SA-4-1BBL and E7<sub>49-57</sub> peptide was more effective than TLR agonists LPS/MPL and CpG, two benchmark adjuvants used in various preclinical and clinical vaccine settings (2), as well as an agonistic 4-1BB antibody in eradicating the TC-1 tumors. The better therapeutic efficacy was associated with the ability of SA-4-1BBL to induce better CD8⁺ T-cell primary, recall, and memory responses as well as their infiltration into the tumor. It has recently been shown that TC-1 tumors may exploit the regulatory function of CD4⁺CD25⁺FoxP3⁺ Treg cells for immune evasion (37). Important in this context are the recent findings of den Haan and colleagues showing that vaccination with LPS or poly(I:C) not only activate the immune system but simultaneously induces Ag-specific, interleukin-10-producing Treg cells that strongly suppress CD8⁺ T-cell responses (38). In addition, plasmacytoid dendritic cells activated by CpG induce the conversion of CD4⁺CD25⁺ T cells into CD4⁺CD25⁺ regulatory T cells (39), and CpG can induce CD19⁺ splenic dendritic cells to acquire potent T-cell suppressive function through the production of indoleamine 2,3-dioxygenase (40). Therefore, the better efficacy of SA-4-1BBL-based therapeutic cancer vaccine in the present study may not only be due to its ability to generate an effective tumor-specific CD8⁺ T-cell response but also curb the regulatory immunity, such as reversal of CD8⁺ T-cell anergy and modulation of regulatory T-cell functions.

The development of potent adjuvants without adverse toxicity is crucial to the success of therapeutic vaccines. Our findings support...
the notion that select natural ligands to costimulatory molecules may have the potential to serve as effective adjuvants as components of therapeutic vaccines, provided that soluble, active forms of these molecules are generated. The robust effect of SA-4-1BBL in modulating innate, adaptive, and regulatory immunity with therapeutic consequences in the absence of detectable toxicity is consistent with this notion. Our data further show the utility of streptavidin as a fusion partner for the generation of costimulatory molecules with potent immunologic activities in soluble forms (18). Testing this novel form of SA-4-1BBL in clinical trials will assess its therapeutic potential, and if effective, this molecule may serve as safe and effective alternatives to TLR agonists and agonistic anti-

Disclosure of Potential Conflicts of Interest

SA-4-1BBL is licensed from University of Louisville by ApoImmune, for which H. Shirwan serves as CSO and H. Shirwan and E.S. Yolcu have significant equity interest. E.S. Yolcu: Ownership interest, ApoImmune. H. Shirwan: Employment, ownership interest, and consultant/advisory board, ApoImmune. The other authors disclosed no potential conflicts of interest.

Acknowledgments

Received 8/18/08; revised 3/6/09; accepted 3/11/09.

Grant support: NIH grants R43 CA 109866, R41 CA121665, KLCRP, and KSTC-145-402-22 (H. Shirwan), R43 AI071618 (H. Shirwan and E.S. Yolcu), and JBCC Research Fellowship (R.K. Sharma).

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We thank O. Grimany for technical help with recombinant proteins.

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