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4-1BB ligand enhances tumor-specific immunity of poxvirus vaccines

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Abstract

Purpose—Recombinant poxvirus vaccines have been explored as tumor vaccines. The immunogenicity of these vaccines can be enhanced by co-expressing costimulatory molecules and tumor-associated antigens. While the B7-CD28 interaction has been most comprehensively investigated, other costimulatory molecules utilize different signaling pathways and might provide further cooperation in T cell priming and survival. 4-1BB (CD137) is a TNF family member and is critical for activation and long-term maintenance of primed T-cells. This study was conducted to determine if a poxvirus expressing the ligand for 4-1BB (4-1BBL) could further improve the immune and therapeutic responses of a previously reported poxvirus vaccine expressing a triad of costimulatory molecules (B7.1, ICAM-1, and LFA-3).

Experimental Design—A recombinant vaccinia virus expressing 4-1BBL was generated and characterized in an *in vitro* infection system. This vaccine was then used alone or in combination with a vaccinia virus expressing CEA, B7.1, ICAM-1, and LFA-3 in CEA-transgenic mice bearing established MC38 tumors. Tumor growth and immune responses against CEA and other tumor-associated antigens were determined. The level of anti-apoptotic proteins in responding T cells was determined by flow cytometry on tetramer selected T cells.

Results—The combination of 4-1BBL with B7.1-based poxvirus vaccination resulted in significantly enhanced therapeutic effects against CEA-expressing tumors in a CEA transgenic mouse model. This was associated with an increased level of CEA-specific CD4⁺ and CD8⁺ T cell responses, induction of antigen spreading to p53 and gp70, increased accumulation of CEA-specific T cells in the tumor microenvironment, and increased expression of bcl-XL and bcl-2 in CD4⁺ and CD8⁺ T cells in vaccinated mice.

Conclusion—4-1BBL cooperates with B7 in enhancing anti-tumor and immunologic responses using a recombinant poxvirus vaccine model. The inclusion of costimulatory molecules targeting distinct T cell signaling pathways provides a mechanism for enhancing the therapeutic effectiveness of tumor vaccines.

Keywords

vaccinia; vaccination; TRICOM; CEA; 4-1BBL; costimulation

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Introduction

The magnitude of antigen specific T-cell activation has been a focus of vaccine therapy for cancer. The interaction of the costimulatory molecule B7 with its ligand CD28 has been the most comprehensively investigated with several studies demonstrating potent anti-tumor immunity when B7.1 is co-expressed with tumor-associate antigens. This suggests that engagement of costimulatory molecules normally expressed on antigen-presenting cells (APCs) with cognate ligands on T cells could be an important factor in tumor eradication (1). There have been few studies evaluating the effects of combining costimulatory molecules that target distinct signaling pathways within naïve T cells.

The TNF/TNFR family is a large group of related molecules that can act as costimulatory molecules for activation and maintenance of T-cells (2). In this family, 4-1BBL is a well characterized costimulatory molecule expressed by APC, including B cells, macrophages, and DCs (3). APC-expressed 4-1BBL binds to 4-1BB (CD137), a member of the TNFR superfamily expressed on monocytes, neutrophils, DC, NK cells and recently activated T cells. 4-1BB signaling has profound effects on T cells, including activation of both CD4⁺ and CD8⁺ T cells, increased levels of cytokine secretion (4,5), enhanced clonal expansion (4,6,7) and increased survival (6–8). Recent studies have noted strong anti-tumor effects mediated by 4-1BB stimulation using agonistic anti-4-1BB antibodies (9–12) and adenoviruses expressing 4-1BBL (13–15). Previous reports suggested that 4-1BB was superior to B7.1/CD28 signaling for stimulation of CD8⁺ T cell expansion (16) and that 4-1BB might activate T cells in the absence of B7.1/CD28 interactions (17–19) or APC stimulation (5). Other reports, however, have demonstrated that combining B7.1/CD28 stimulation and 4-1BB signaling was required for optimal induction of CD25 and bcl-XL expression in CD8⁺ T cells (5), increased CTL activity (20) and protective anti-tumor immunity (21).

We have previously demonstrated that the addition of ICAM-1 and LFA-3 to replication-competent poxviruses expressing B7.1 improves antigen-specific therapeutic responses in murine models, especially when low levels of Signal 1 are provided (22). In this study we describe the additional benefit of expressing 4-1BBL with a triad of costimulatory molecules, including B7.1, ICAM-1, and LFA-3 (designated TRICOM) in a carcinoembryonic antigen (CEA) transgenic mouse model. A recombinant vaccinia virus containing 4-1BBL transgene was generated (rV-4-1BBL) and resulted in enhanced T cell proliferation and survival in co-culture *in vitro* assays of virus-infected DC and naïve T cells. The rV-4-1BBL vaccine induced non-specific tumor growth arrest in an established murine tumor treatment model. When rV-4-1BBL was combined with a vaccinia virus expressing CEA and TRICOM a significant enhancement in responses were observed with a single immunization against both 4 and 7 day established CEA-bearing tumors in CEA-transgenic mice. This was associated with an increased level of CEA-specific CD4⁺ T cell proliferation, Th1 cytokine release, improved CD8⁺ T cell cytotoxicity and avidity, and induction of an antigen cascade against other tumor-associated antigens, i.e. p53 and gp70. In addition, the use of 4-1BBL with TRICOM resulted in increased expression of the anti-apoptotic proteins bcl-XL and bcl-2 in both CD4⁺ and CD8⁺ T cells and accumulation of CEA-specific T cells within the tumor microenvironment. These results have implications for the rational design of more potent vaccines in the treatment of established human tumors.

Materials and Methods

Animals and cell lines

Female C57BL/6 mice transgenic for human CEA were obtained from a breeding pair, provided by Dr. John Thompson (Institute of Immunobiology, University of Freiburg, Germany). The generation and characterization of the CEA-transgenic mouse has been previously described

(23,24). Female C57BL/6 and BALB/c mice were obtained from the National Cancer Institute-Frederick Cancer Research Animal Facility (Frederick, MD). Mice were housed and maintained under pathogen-free conditions in microisolator cages until used for experiments at 6–8 weeks of age.

Cell lines were obtained from ATCC (Rockville, MD); BSC-1 and CV-1 cells derived from African green monkey kidney cells; HeLa cells derived from human cervical carcinoma cells; and thymidine kinase gene-deficient 143B (143B-TK⁻) cells derived from a human sarcoma cell line (thymidine kinase gene-deficient). Murine colon adenocarcinoma MC38 expressing human CEA (designated MC38-CEA⁺) were used for *in vivo* therapy study after washing in PBS (25). All cell lines were maintained in DMEM containing 10% FCS, 10 mM L-glutamine, 100 U/mL streptomycin and 100 U/mL penicillin (complete media, reagents from Gibco BRL, Grand Island, NY).

Recombinant vaccinia viruses

The construction of recombinant vaccinia viruses has been described previously and was applied with slight modifications (26). For the rV-4-1BBL virus, 4-1BBL was amplified by PCR from plasmid pDC201 containing the *4-1BBL* gene (ATTC, Manassas, VA) and cloned into the *KpnI* site of the recombinant vaccinia pSC65 plasmid (a generous gift from Dr. Bernard Moss, NIH, Bethesda, MD) under control of the vaccinia synthetic early/late promoter. The plasmid also contained the selectable marker *LacZ* under control of the vaccinia P7.5 promoter. The pSC65 plasmid containing *4-1BBL* gene was co-transfected with wild type vaccinia virus (ATCC) infected CV-1 cells using lipofectamine (Invitrogen, Carlsbad, CA) according to standard protocols. An empty pSC65 plasmid was transfected to construct the recombinant vaccinia virus expressing only *LacZ* as a negative control (designated rV-LacZ). Infected cells were collected and thymidine kinase disrupted virus was selected by infecting 143B-TK⁻ cells in the presence of 5-bromodeoxyuridine (BrdU, Sigma, St. Louis, MO). Recombinant vaccinia virus expressing 4-1BBL (designated rV-4-1BBL) was selected as described previously (26). Viral stocks were propagated in HeLa cells and purified on sucrose gradient by ultracentrifugation. *4-1BBL* gene expression was confirmed by PCR and protein expression was confirmed by Western blot analysis of BSC-1 cells infected with rV-4-1BBL. To confirm cell surface expression of 4-1BBL, bone marrow-derived immature DCs were infected with rV-4-1BBL (or control rV-LacZ) at MOI = 1 for 16 hrs. Cells were stained with PE-conjugated anti-4-1BBL (rat IgG2a; eBioscience, San Diego, CA), and analyzed by flow cytometry using CellQuest software.

The recombinant vaccinia virus containing the murine *B7.1*, *ICAM-1*, and *LFA-3* genes in combination with the human *CEA* gene (designated rV-CEA/TRICOM) has been described (22). The recombinant fowlpox virus containing the gene for murine *GM-CSF* (designated rF-GM-CSF) has been described (27). Non-recombinant vaccinia virus (Wyeth strain) was designated V-WT. Therion Biologics Corp. (Cambridge, MA) kindly provided these viruses.

Dendritic cell preparation

Dendritic cells (DCs) were prepared from bone marrow cells of C57BL/6 or BALB/c mice according to Inaba *et al* (28) with some modifications. Briefly, cells from femurs and tibiae were enriched by plastic adherence and differentiated into DCs by culture in complete RPMI media supplemented with recombinant murine GM-CSF (rmGM-CSF) and rmIL-4 (Peprotech, Rocky Hill, NJ) both at 25 ng/mL for 7 days. Media was replaced with fresh complete RPMI containing the same supplements every other day. To mature DCs, LPS (1 µg/mL) was added for 24 hours. To characterize DCs, cells were stained with fluorescent-conjugated mAb (all from BD PharMingen; San Diego, CA) for class I, class II, CD11c, CD80, and CD86, and then

analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton-Dickinson, Mountain View, CA).

T-cell costimulation and apoptosis assays

Splenocytes from C57BL/6 mice were purified for T cells using the Pan T-cell Isolation Kit MicroBeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer instructions. The purity of T cells was validated by flow cytometry and >97 % of the cells were CD3⁺. T cells (3×10^5 /well) were cultured with anti-CD3 mAb (0.25–1 µg/mL) and DCs (3×10^4 /well) in 10 % FCS-RPMI 1640 in 96-well plates for 48 hrs. For the assay, DCs were infected with rV-4-1BBL or rV-LacZ at MOI = 1 for 16 hours, and then γ -irradiated at 300 Gy. ³H-thymidine (0.2 µCi/well) was added to the wells for the last 18 hours and harvested using a MicroBeta liquid scintillation counter (Wallac, Gaithersburg, MD). For the allo-reactive T-cell proliferation assay, T cells derived from C57BL/6 mice were cultured with DCs derived from BALB/c mice. Cytokine release by T cells was evaluated by harvesting culture supernatants 48 hrs after stimulation. The supernatants were analyzed for murine IFN- γ and TNF- α using the Cytometric Bead Array kit (BD PharMingen, San Diego, Ca), and for IL-12 (p40) using OptEIATM ELISA kit (BD PharMingen) according to the manufacturer's instructions.

To analyze T-cell apoptosis, T cells (3×10^5 /well) were cultured with DCs (3×10^4 /well) for 72 hours. T cell viability was determined by Trypan blue exclusion before and after 72 hours of culture. T cells were harvested from the plates after culture, stained with APC-conjugated anti-CD3 mAb, FITC-conjugated Annexin V and PE-PI using the apoptosis detection kit (R&D system, Inc., Minneapolis, MN) following Fc receptor-blocking with anti-CD16/CD32 mAb. Cells were analyzed after gating the CD3⁺ fraction.

Tumor treatment studies

CEA-transgenic mice were implanted s.c. with MC38-CEA⁺ tumor cells (3×10^5 cells/mouse) in the right flank. Mice were vaccinated s.c. with V-WT, rV-4-1BBL, and/or rV-CEA/TRICOM on day 4 after tumor implantation. Viruses were injected at 10^8 pfu/mouse admixed with 10^7 pfu/mouse of rF-GM-CSF. Tumor size was measured using calipers 1–2 times a week. Tumors were measured as follows: Volume (mm³) = Length x Width. Mice were sacrificed when either size (length or width) of tumors exceeded 20 mm.

T cell responses

To evaluate antigen-specific CD4⁺ T-cell responses, splenic T cells were tested for proliferation in response to protein or peptide antigens as previously described (29). Briefly, pooled splenic T cells (1.5×10^5 cells/well) were cultured in 96-well flat-bottomed plates with irradiated naive syngeneic splenocytes as APCs (5×10^5 cells/well) and human CEA protein (Aspen Bio, Littleton, CO) at the indicated concentration. As a positive control, cells were stimulated with Concanavalin A (2.5 µg/mL, Sigma-Aldrich). T cells and APCs were cultured with media only as a negative (background) control. Cells were cultured for 5 days, ³H-thymidine (1 µCi/well) added to the wells for the last 18–24 hrs, and cells were harvested using a Tomtec cell harvester (Wallac Inc., Gaithersburg, MD). The incorporated radioactivity was measured using a MicroBeta liquid scintillation counter (Wallac, Gaithersburg, MD). Data are depicted both as total CPM and after the mean proliferation of negative control responses were subtracted from that in response to CEA protein. These experiments were repeated twice and the data shown as the mean Δ cpm $\pm$ standard deviation (SD).

To evaluate CD8⁺ T-cell responses specific for tumor antigens, splenocytes from vaccinated animals were pooled and dispersed into single-cell suspensions, and stimulated with the H-2D^b-restricted peptide CEA_{526–533} (10 µg/mL, EAQNTTYL) (24,30), the H-2D^b-restricted peptide p53_{232–240} (2 µg/mL, KYMCNSSCM) (31) or the H-2K^b-restricted peptide

p15E₆₀₄₋₆₁₁ (1 µg/mL, KSPWFTTL, referred as gp70 peptide) (32). Six days later, bulk lymphocytes were separated by centrifugation through a Ficoll-Hypaque gradient. Using purified lymphocytes, tumor-killing activity was tested as described previously (29). Briefly, recovered lymphocytes, ⁵¹Cr-labeled target cells (EL-4, 5x10³ cells/well), and each peptide were incubated for 5 hours (96-well U-bottom plates), and radioactivity in supernatants measured using a gamma-counter (Corba Autogamma, Packard Instruments, Downers Grove, IL). As control peptides, VSV-N₅₂₋₅₉ (RGYVYQGL) (33) was used for H-2D^b-restricted peptides, and ovalbumin₂₅₇₋₂₆₄ (SIINFEKL) (34) was used for H-2K^b-restricted peptides. The percentage of tumor lysis was calculated as follows: % tumor lysis = [(experimental cpm – spontaneous cpm) / (maximum cpm – spontaneous cpm)] x 100. Non-specific ⁵¹Cr-release in response to each control peptide was subtracted from that induced by the appropriate tumor antigen peptide. These experiments were repeated twice and the data is shown as the mean ± SD.

CD8⁺ T cell cytokine production in response to defined tumor antigen peptides was tested from recovered lymphocytes separated as described above. Recovered lymphocytes (5x10⁵ cells/well) were re-stimulated with fresh irradiated naïve splenocytes as APCs (5x10⁶ cells/well) and with each peptide (10 µg/mL of CEA peptide, 2 µg/mL of p53 peptide, or 1 µg/mL of gp70 peptide). Twenty-four hours later, the supernatant fluid was collected and analyzed for murine IFN-γ and TNF-α using the Cytometric Bead Array kit (BD PharMingen). Non-specific cytokine production in response to each control peptide was subtracted from that induced by the appropriate tumor antigen peptide.

Tetramer analysis

To analyze T-cell populations in vaccinated mice, tumors, spleens and peripheral blood were harvested. Tumors and spleens were mechanically dispersed into single-cell suspensions. Peripheral blood was collected into 4% citrated PBS. Red blood cells were removed from these cell samples. The following antibodies were purchased from BD PharMingen and used for analysis: FITC- or APC-conjugated anti-CD3e (hamster IgG1), CyChrome-conjugated anti-CD4 mAb (rat IgG2b), CyChrome-conjugated anti-CD8 mAb (rat IgG2a), and appropriate isotype control antibodies. To evaluate the generation of CEA-specific CTLs in vaccinated mice, cells were stained with FITC-conjugated anti-CD3e, CyChrome-conjugated anti-CD8 mAb and PE-conjugated CEA₅₂₆₋₅₃₃/H-2D^b-tetramer (referred to as CEA-tetramer) obtained from Beckman Coulter (Fullerton, CA). To evaluate the generation of gp70-specific CTL in mice, cells were stained with FITC-conjugated anti-CD3e, CyChrome-conjugated anti-CD8 mAb and PE-conjugated p15E₆₀₄₋₆₁₁/H-2K^b-tetramer (referred as gp70-tetramer) obtained from the NIH Tetramer Facility (32). Cell samples from blood and spleens of naïve mice were tested as a control for this tetramer assay, and 2–3 % of these cells were positive in the CD8⁺CEA-tetramer⁺ fraction or the CD8⁺gp70-tetramer⁺ fraction after gating on CD3⁺ T cells. Immunofluorescence staining was performed after Fc receptor-blocking with anti-CD16/CD32 mAbs (30 min, on ice). Cells were incubated with antibodies or tetramer for 30 min on ice in the dark, and then washed three times with 1% BSA-PBS 3 times. In some experiments, cells were intracellularly stained with anti-bcl-XL (IgG2a, Biosource International Inc., Camarillo, CA) followed by staining with FITC-conjugated anti-mouse IgG2a (BD PharMingen), or FITC-conjugated bcl-2 mAb (hamster IgG, BD PharMingen) after permeabilization using Cytofix/Ctoperm kit (BD PharMingen) according to the manufacturer's instructions. The immunofluorescence was compared with the appropriate isotype-matched controls and analyzed with Cellquest software using a FACSCalibur cytometer (Becton-Dickinson).

Statistical analysis

Significant differences were evaluated by Analysis of Variance (ANOVA) with repeated measures using Statview 4.1 (Abacus Concepts Inc, Berkeley, CA) or GraphPad PRISM

software. Significant differences in dot distribution or mean fluorescence intensity of flow cytometry analysis data were determined using the Kolmogorov-Smirnov test. For graphical representation of data, y-axis error bars indicate the SD of the data for each point on the graph.

Results

Characterization of rV-4-1BBL

We constructed a recombinant vaccinia virus expressing the full-length murine *4-1BBL* gene. To confirm 4-1BBL protein expression, immature bone marrow-derived DCs were infected with rV-4-1BBL at MOI = 1 for 16 hours. More than 30% of infected DCs expressed 4-1BBL on their surfaces by flow cytometry analysis (Fig. 1A). There was a dose-dependent increase in the percentage of 4-1BBL-positive cells with 60% of DCs expressing 4-1BBL at an MOI of 4 (data not shown). The expression of 4-1BBL was not noted on uninfected or control rV-LacZ-infected DCs.

The functional activity of 4-1BBL expressed by rV-4-1BBL was evaluated in T cell costimulation assays in which purified splenic T cells were cultured with virus-infected DCs in presence of anti-CD3 mAb for 48 hours. As shown in Figure 1B, when T cells were cultured with uninfected DCs (open bars) there was a modest increase in T cell proliferation. In agreement with previous reports, control rV-LacZ-infected DCs (gray bars) inhibited T cell proliferation (35–36). In contrast to control vaccinia virus infection, T cells stimulated by rV-4-1BBL-infected DCs exhibited a significant increase in proliferation ($P < 0.001$; Fig. 1B, solid bars). There was a corresponding increase in Th1 cytokine production, including IL-12, IFN- γ , and TNF- α , in all DC-T cell cultures, but the highest levels were observed in rV-4-1BBL-exposed T cell cultures (Fig. 1C). We also observed enhanced allogeneic T-cell proliferation in response to rV-4-1BBL exposure (Fig. 1D).

4-1BB signaling promotes T cell survival by blocking apoptosis (6–8). Therefore, we determined the number of apoptotic T cells after stimulation by rV-4-1BBL-infected DCs. As shown in Figure 1E, T cells stimulated with rV-LacZ-infected DCs induced T cell apoptosis and necrosis as compared to T cells stimulated with uninfected DCs. T cell apoptosis, however, was significantly blocked when T cells were stimulated with rV-4-1BBL-infected DCs, consistent with previous studies (4–8). These results demonstrate that rV-4-1BBL expressed functional 4-1BBL capable of stimulating naïve T cells and promoting their survival.

Vaccine therapy of CEA⁺ tumors with rV-4-1BBL in combination with rV-CEA/TRICOM

In previous studies a recombinant vaccinia virus expressing CEA, B7.1, ICAM-1, and LFA-3 (designated rV-CEA/TRICOM) demonstrated protection against challenge with CEA-expressing tumor cells and significantly delayed the growth of established tumors when mice were vaccinated in a prime-boost approach using four immunizations (37–39). In order to determine if 4-1BBL would enhance therapeutic responses, human CEA-transgenic mice were implanted with MC38-CEA⁺ tumors on day 0 and all mice demonstrated progressive tumor growth with palpable lesions by day 4 (mean tumor size $22.1 \pm 6.2 \text{ mm}^3$). There was no discernable effect on tumor growth when mice were treated s.c. with PBS vehicle or V-WT on day 4 (Fig. 2A) or 7 (Fig. 2B) after tumor challenge. In contrast, when mice were vaccinated s.c. with a single dose of rV-4-1BBL, tumor growth was modestly suppressed when the vaccine was given at day 4 ($p = 0.017$), but not at day 7 ($p = 0.14$). Tumor growth was significantly suppressed when mice were vaccinated s.c. with rV-CEA/TRICOM on day 4 ($p = 0.04$) or 7 ($P = 0.01$) compared to V-WT control mice. There was a highly significant decrease in tumor growth when mice received a single vaccination of rV-4-1BBL and rV-CEA/TRICOM on day 4 ($P = 0.0001$) or 7 ($p = 0.003$), with 2 of 15 mice being rendered tumor free (Fig. 2). These

results suggest that 4-1BBL significantly enhances the therapeutic effects of vaccination with poxviruses expressing TRICOM.

Analysis of CEA-specific T-cell immune responses

To explore possible mechanisms associated with the therapeutic responses observed with the combination of rV-4-1BBL and rV-CEA/TRICOM vaccination, CEA-transgenic mice with established tumors were vaccinated according to the same schedule performed in Figure 2. These mice were sacrificed, and splenocytes were assayed for T-cell immune responses on day 18 after tumor implantation. Figure 3A shows that vaccination with rV-4-1BBL alone (closed triangles) induced significant T cell proliferation, even in the absence of CEA protein, indicating non-specific proliferation persisting 18 days post vaccination and is in agreement with Nam et al., that 4-1BBL expression acts as a general immune stimulator (40). After subtracting non-specific proliferation CEA-specific CD4⁺ T cell responses were significantly increased only when mice were vaccinated with both rV-4-1BBL and rV-CEA/TRICOM (Fig. 3A, right panel, closed squares; $P < 0.003$ at 0.78 $\mu\text{g/mL}$).

In contrast to CD4⁺ T cell responses, CEA-specific CTL activity was markedly increased only in the groups vaccinated with rV-4-1BBL or the combination of rV-4-1BBL and rV-CEA/TRICOM (Fig. 3B, $P < 0.002$ at E:T ratio = 10:1). The greatest CTL activity was observed in the group receiving combination vaccines where significant activity was observed at lower E:T ratios compared with the group receiving rV-4-1BBL alone (Fig. 3B, closed squares; $P < 0.01$ at 1.25:1). Furthermore, the group receiving combination therapy showed the highest cytokine production from CD8⁺ T cells exposed to CEA peptide (Fig. 3C). These results indicate that administration of rV-4-1BBL alone induces non-specific T-cell responses in the CEA transgenic model system, but the combination of rV-4-1BBL and rV-CEA/TRICOM results in synergistic enhancement of CD4⁺ T cell proliferation and improved functional CD8⁺ CTL responses.

Increased anti-apoptotic molecule expression in T cells of mice vaccinated with rV-4-1BBL and rV-CEA/TRICOM

4-1BB signaling promotes the survival of T cells via NF- κ B mediated increases in expression of anti-apoptotic genes, such as *bcl-XL* and *bcl-2* (6–8). Thus, CD4⁺ and CD8⁺ T cells from MC38-CEA⁺ tumor-implanted CEA-transgenic mice treated with the vaccine regimen described above were assayed for intracellular expression of *bcl-XL* and *bcl-2* on samples taken 18 days after tumor implantation. Table 1 displays the results for T cells derived from peripheral blood and the spleen. Bcl-XL expression was markedly increased by rV-4-1BBL in both CD4⁺ and CD8⁺ T cells, but rV-4-1BBL did not increase *bcl-XL* expression in the CEA-tetramer⁺ CD8⁺ T cell population from the spleen, although expression was slightly higher in peripheral blood CD8⁺ T cells. When rV-CEA/TRICOM was used in combination with rV-4-1BBL, however, there was a significant increase in *bcl-XL* expression on all T cells, including the CEA-tetramer⁺ CD8⁺ T cell population. Bcl-2 expression was only slightly increased in peripheral blood T cells but not in splenic T cells following rV-4-1BBL vaccination. However, the combination of rV-4-1BBL and rV-CEA/TRICOM significantly enhanced *bcl-2* expression on T cells from both blood and spleen (Table 1). These results suggest that T cells were resistant to apoptosis after activation by rV-4-1BBL and rV-CEA/TRICOM and this was mediated by *bcl-XL* and *bcl-2*.

Induction of tumor antigen cascade in mice vaccinated with rV-4-1BBL and rV-CEA/TRICOM

Since effective anti-tumor responses have correlated with antigen spreading in several systems, we evaluated vaccinated mice for the appearance of T cells specific for other tumor-associated antigens present in the tumor cell line, in particular wild-type p53 and the endogenous retroviral *env* epitope gp70. T cell responses against these antigens have been previously associated with

anti-tumor efficacy of CEA⁺ tumors following vaccination with poxviruses expressing CEA (41–42). Figure 4 demonstrates p53- (Fig. 4A) and gp70-specific (Fig. 4B) CTL activities were noticeably induced by rV-4-1BBL, and these activities could be significantly enhanced by the addition of rV-CEA/TRICOM. In addition, IFN- γ production from CD8⁺ T cells, particularly in response to gp70, was increased in mice vaccinated with the combination of rV-4-1BBL and rV-CEA/TRICOM (Fig. 4C). Interestingly, CD8⁺ T cells did not produce TNF- α in response to p53, but did in response to gp70, and the combination vaccine group showed the highest production (Fig. 4C). These results demonstrate that mice vaccinated with the combination of rV-4-1BBL and rV-CEA/TRICOM exhibit antigen spreading that includes p53- and gp70-specific CD8⁺ T cell responses.

Localization of tumor antigen-specific CD8⁺ T cells in mice vaccinated with rV-4-1BBL and rV-CEA/TRICOM

To further analyze the CD8⁺ T cell response to vaccination with the combination of rV-4-1BBL and rV-CEA/TRICOM, we examined the level of tetramer-specific CD8⁺ T cells in the local tumor sites. MC38-CEA⁺ tumor-implanted CEA-transgenic mice were treated with the vaccine regimen according to the same schedule performed in Figure 2. On day 18 after tumor implantation, tumor-infiltrating cells and peripheral blood cells were harvested and tested for CEA- and gp70-tetramer binding using flow cytometry, as described in Materials and Methods. As seen in Figure 5A, CEA-specific CD8⁺ T cells were markedly increased in the at tumor sites in mice receiving the combination regimen. A similar increase in gp70-specific T cells was found at the tumor site only when mice received the combination regimen (Fig. 5B). These results suggest that vaccine therapy with rV-4-1BBL and rV-CEA/TRICOM can induce and then recruit both CEA- and gp70-specific CD8⁺ T cells to the site of established tumors.

Discussion

4-1BBL-4-1BB costimulation activates T cells through increased cytokine production (4,5), enhanced clonal expansion (4,6,7) and induction of anti-apoptotic molecule expression (6–8). Here, we confirmed and extended these observations to a therapeutic poxvirus vaccine system *in vitro* utilizing DCs infected with rV-4-1BBL and *in vivo* utilizing T cells from tumor-bearing mice vaccinated with rV-4-1BBL. These results are important since poxviruses are currently in Phase III clinical trials and additional methods of increasing the potency of such vaccines would have immediate translational potential. The selection of appropriate immune modulatory molecules will likely allow the selective manipulation of immune responses. Thus, the combination of B7.1 and 4-1BBL seemed logical since these molecules signal through distinct pathways and likely promote initial priming and later expansion of antigen exposed T cells. These results are also significant in that the expression of 4-1BBL by vaccinia virus was able to overcome the inhibition of T cell priming observed after infection of DC by control vaccinia virus (35–36).

Another interesting finding was the high level of non-specific T cell activation and associated therapeutic responses observed against CEA⁺ tumors after vaccination with rV-4-1BBL alone (Fig. 2, 3 and 4). This is consistent with the strong anti-tumor efficacy of 4-1BB stimulation using agonistic anti-4-1 BB antibodies and in studies of adenoviruses expressing 4-1BBL (9–12). Since 4-1BB signaling has demonstrated activation of CD28-negative T cells, it has been suggested that 4-1BB may utilize alternative pathways for achieving T cell stimulation *in vivo* (17–19). Furthermore, 4-1BB has been able to activate T cells in the absence of APCs (5). The expression of 4-1BB is found on other immune cells, most notably NK cells, which may explain the non-specific effects observed in our model.

The anti-tumor activity and T cell immune responses induced by rV-4-1BBL were significantly enhanced by the addition of rV-CEA/TRICOM when compared to vaccination with individual

vaccines. Tumor growth was strongly inhibited in mice treated with the combination, and 2 of 15 mice vaccinated with the combination had complete regression of established tumors after a single vaccination. This was especially significant since CEA⁺ tumors were established in CEA transgenic mice and differs from previous reports where at least three booster vaccinations were required for therapeutic activity (22). The activation of both CD4⁺ and CD8⁺ T cells was not surprising and we hypothesized that 4-1BBL would enhance therapeutic responses, at least in part by promoting the survival of CEA-specific T cells. This was confirmed as expression of both bcl-XL and bcl-2 was significantly enhanced in vaccinated mice, particularly when rV-4-1BBL was administered in combination with rV-CEA/TRICOM. Tumor-specific T cells overexpressing bcl-2 have been shown to maintain target cell recognition for prolonged periods of time and mediates improved anti-tumor efficacy *in vivo* when bcl-2 levels are increased (43). Thus, it is likely that enhanced bcl-XL and bcl-2 expression by the combination vaccine regimen contributed to longer survival of antigen-specific T cells and subsequent anti-tumor responses.

The induction of an antigen cascade has also been associated with stronger anti-tumor responses against established tumors (41–42). The MC38 tumor line used in our model expresses CEA and is known to express other putative antigens, including wild-type p53 and gp70. Mice vaccinated with rV-4-1BBL developed p53-specific T cell responses, which were increased only slightly by the combination of rV-4-1BBL and rV-CEA/TRICOM (Fig. 4). There was also an increase in gp70-specific CD8⁺ T cells in mice receiving rV-4-1BBL and rV-CEA/TRICOM, although this effect was most pronounced in mice receiving the combination regimen. We also found an increased number of gp70-specific T cells within established tumors in mice vaccinated with the combination vaccine regimen. The gp70-specific T cells released high levels of IFN- and TNF- α , whereas p53-specific T cells appeared to induce only IFN- production (Fig. 4C). The reason for this difference is not clear but may relate to differences in peptide binding affinity between the prokaryotic gp70 and the self-p53 epitopes as has been reported in other model antigen systems (44). We also found an increased number of gp70-specific T cells within established tumors in mice vaccinated with the combination vaccine regimen. These observations may be related to cross presentation of tumor antigens following an initial NK cell response induced by 4-1BBL vaccination and we are actively exploring this possibility. While it is possible that the generation of T cells directed against other antigens may induce autoimmunity we did not specifically evaluate the antigen cascade in non-tumor bearing mice or examine normal tissues for evidence of T cell infiltration. We consider the possibility of significant unlikely, however, since the mice appeared healthy and previous studies with the TRICOM vectors in mice failed to demonstrate autoimmunity (45). In any event, the initiation of such a cascade suggests that the combination regimen may be especially powerful in initiating anti-tumor immunity.

The effectiveness of vaccination against established tumors depends on the ability of vaccine-primed T cells to traffic to sites of tumor growth. We evaluated in our model by using a K^b-restricted CEA peptide tetramer to identify CD8⁺ T cells in the tumor microenvironment. There was a significant increase in tetramer-binding CD8⁺ T cells at the tumor site of mice vaccinated with the combination of rV-4-1BBL and rV-CEA/TRICOM (Fig. 5). This was accompanied by a similar increase in gp70-specific T cells. Thus, our results support the conclusion that combination vaccination with rV-4-1BBL and rV-CEA/TRICOM improves anti-tumor efficacy through enhanced survival of antigen-specific T cells which are recruited into local tumor sites. This approach also initiates an antigen cascade against other tumor-associated antigens although the mechanism for this cascade needs to be better defined. This has practical implications for the design of tumor vaccines since the tumor microenvironment may significantly inhibit the recruitment and function of tumor-reactive T cells in established tumors (46). Collectively, these results support the use of novel combinations of specific

antigens and immunomodulatory molecules as a general strategy for improving the effectiveness of tumor vaccines.

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Figure 1.

Characterization of rV-4-1BBL. (A) 4-1BBL expression on DCs. Immature bone marrow-derived DCs were infected with recombinant vaccinia viruses at 1 MOI for 16 hours and stained with anti-4-1BBL mAb (solid line). Gray line (shaded), uninfected DCs; gray dotted line, DCs infected with rV-LacZ. (B) Autologous T cells were cultured with the indicated dose of anti-CD3 alone (dotted bar), in the presence of uninfected DC (open bar), DC infected with rV-LacZ (gray bar) or rV-4-1BB (black bar) and standard thymidine incorporation proliferation assay performed. (C) Cytokine production measured by standard ELISA from T cells exposed to DCs, as in (B). (D) H-2K^b T cells were cultured with the indicated dose of anti-CD3 alone (dotted bar), in the presence of uninfected H-2K^d-derived DC (open bar), DC infected with rV-LacZ (gray bar) or rV-4-1BB (black bar) and standard thymidine incorporation proliferation assay performed. (E) rV-4-1BBL prevents apoptosis and necrosis of T cells. Uninfected DCs, rV-4-1BBL-infected DCs or rV-LacZ-infected DCs were irradiated and cultured with T cells

for 72 hrs. T cells were stained with Annexin V and PI, and analyzed by flow cytometry. All results are representative of 3 independent experiments. The SDs are based on the mean of triplicate wells for data in B-D (*P < 0.05, **P < 0.001).

Figure 2. rV-4-1BBL admixed with rV-CEA/TRICOM enhances therapeutic responses against CEA⁺ tumors. CEA-transgenic mice were implanted subcutaneously (s.c.) with MC38-CEA⁺ tumors on day 0 (15 per group). Four days (A) or 7 days (B) after tumor implantation, indicated vaccines were administered together with rF-GM-CSF. Control mice were treated s.c. with PBS vehicle or V-WT (1×10^8 pfu). Experimental mice were vaccinated s.c. with rV-4-1BBL (5×10^7 pfu) admixed with V-WT (5×10^7 pfu), rV-CEA/TRICOM admixed with V-WT (5×10^7 pfu), or rV-4-1BBL (5×10^7 pfu) and rV-CEA/TRICOM (5×10^7 pfu). Tumor volume was monitored 2 times a week. Data are a compilation of 2 independent experiments.

Figure 3.

Vaccination induces T cell responses in CEA-transgenic mice. Mice were implanted s.c. with MC38-CEA⁺ tumors on day 0 and vaccinated with V-WT, rV-4-1BBL and/or rV-CEA/TRICOM admixed with rF-GM-CSF on day 4 as described in Figure 2. Splenic lymphocytes were used for *in vitro* assays 18 days after tumor implantation (5 per group, pooled). (A) CEA-specific CD4⁺ T cell proliferation was measured by thymidine incorporation assay with indicated doses of CEA protein. Right: data depicted as Δcpm; SDs are based on the mean of triplicate wells. (B) CEA-specific CTL activity (10 μg/mL CEA peptide) was measured by standard chromium release assay. SDs are based on the mean of triplicate wells. Open circles, PBS-treated group; open triangles, V-WT-vaccinated group; closed triangles, rV-4-1BBL-

vaccinated group; open squares, rV-CEA/TRICOM-vaccinated group; closed squares, combination rV-4-1BBL and rV-CEA/TRICOM. (C) IFN- γ production by CD8⁺ T cells exposed to increasing doses of CEA peptide. (D) TNF- α production specific for CEA peptide from CD8⁺ T cells exposed to increasing doses of CEA peptide (Open bars, cytokine production in response to control peptide (10 μ g/mL); gray bars, cytokine production in response to CEA peptide (10 μ g/mL). In right graph, data is depicted as Δ pg/mL.

Figure 4.

rV-4-1BBL and rV-CEA/TRICOM induces antigen cascade. CEA-transgenic mice were implanted s.c. with MC38-CEA⁺ tumors on day 0 and vaccinated with V-WT, rV-4-1BBL and/or rV-CEA/TRICOM admixed with rF-GM-CSF on day 4 as described in Figure 2. Splenic lymphocytes were used for *in vitro* assays 18 days after tumor implantation (5 per group, pooled). (A) p53-specific CTL activity (2 μg/mL p53 peptide) with SDs based on the mean of triplicate wells. (B) gp70-specific CTL activity (1 μg/mL gp70 peptide) with SDs based on the mean of triplicate wells. Open circles, PBS-treated group; open triangles, V-WT-vaccinated group; closed triangles, rV-4-1BBL-vaccinated group; open squares, rV-CEA/TRICOM-vaccinated group; closed squares, combination rV-CEA/TRICOM and rV-4-1BBL. (C) Cytokine production specific for p53 or gp70 peptide from CD8⁺ T cells.

Figure 5. rV-4-1BBL and rV-CEA/TRICOM induces tumor-antigen-specific CD8⁺ T cells that localize to sites of established tumors. CEA-transgenic mice were implanted s.c. with MC38-CEA⁺ tumors on day 0 and vaccinated with V-WT, rV-4-1BBL and/or rV-CEA/TRICOM admixed with rF-GM-CSF on day 4 as described in Figure 2. Mice were sacrificed and used on day 18 after tumor implantation (5 per group, pooled). Tumors were harvested and mechanically dispersed into single-cell suspensions. Cells were stained with anti-CD3 mAb, anti-CD8 mAb and CEA- or gp70-tetramer. (A) CEA-specific and (B) gp70-specific T cells from a single tumor are shown after the indicated vaccine was given. The number indicates % CD3⁺CD8⁺ tetramer-binding cells in CD3⁺ cells.

Table 1

Increase of anti-apoptotic molecule expression on T cells of tumor-bearing mice vaccinated with rV-4-1BBL and/or rV-CEA/TRICOM

CD3 ⁺ cells	Mean fluorescence intensity				
	PBS	V-WT	rV-4-1BBL	rV-CEA/ TRICOM	rV-CEA/ TRICOM + rV-4-1BBL
Bcl-X_L expression					
PBC					
CD8 ⁺ Tetramer ⁺	858.38	923.71	1238.91	903.99	1222.68 *
CD8 ⁺	88.19	83.05	105.45	86.43	112.56 *
CD4 ⁺	80.78	70.49	93.46	102.87	127.14 *
SPC					
CD8 ⁺ Tetramer ⁺	192.90	204.45	192.90	492.42	441.60
CD8 ⁺	63.29	59.01	67.45	63.66	72.41 *
CD4 ⁺	54.16	62.42	65.99	62.07	111.56 *
Bcl-2 expression					
PBC					
CD8 ⁺ Tetramer ⁺	130.93	209.73	206.92	181.05	230.43 *
CD8 ⁺	51.12	45.53	51.12	52.49	59.28 *
CD4 ⁺	48.80	47.17	52.32	68.08	67.51
SPC					
CD8 ⁺ Tetramer ⁺	44.87	59.79	44.87	57.53	138.02 *
CD8 ⁺	33.39	36.14	29.34	27.85	48.34 *
CD4 ⁺	37.29	32.93	28.97	30.76	33.58 *

CEA-transgenic mice were implanted s.c. with MC38-CEA⁺ tumors on day 0. These mice were vaccinated with V-WT, rV-4-1BBL and/or rV-CEA/TRICOM admixed with rF-GM-CSF on day 4 as described in Figure 2. The mice were sacrificed, and the peripheral blood cells and spleen cells were used for this assay on day 18 after tumor implantation (n=5/group, pooled). Cells were stained with anti-CD3 mAb, anti-CD4 or CD8 mAb, and/or CEA-tetramer, and then intracellularly with anti-Bcl-X_L mAb or anti-Bcl-2 mAb.

Bold number, Significant increase ($P < 0.01$) as compared with V-WT-vaccinated group.

* Significant increase ($P < 0.01$) as compared with both rV-4-1BBL-vaccinated group and rV-CEA/TRICOM-vaccinated group.