Priority Brief

Cancer Immunology Research

Vaccines Combined with Immune Checkpoint Antibodies Promote Cytotoxic T-cell Activity and Tumor Eradication

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Abstract

We demonstrate that a poly(lactide-co-glycolide) (PLG) cancer vaccine can be used in combination with immune checkpoint antibodies, anti–CTLA-4 or anti–PD-1, to enhance cytotoxic T-cell (CTL) activity and induce the regression of solid B16 tumors in mice. Combination therapy obviated the need for vaccine boosting and significantly skewed intratumoral reactions toward CTL activity, resulting in the regression of B16 tumors up to 50 mm^2 in size and 75% survival rates. These data suggest that combining material-based cancer vaccines with checkpoint antibodies has the potential to mediate tumor regression in humans. *Cancer Immunol Res; 4(2); 95–100. ©2015 AACR.*

Introduction

Cancer accounts for almost 15% of all deaths worldwide and has produced consistent death rates for over 50 years (1). However, recent advances in immunotherapy generate antitumor cytotoxic T cells (CTL) that may dramatically affect patient outcomes (2, 3). The T-cell receptor complex (TCR) recognizes tumor-derived peptides bound to surface major histocompatibility complexes (MHC) to produce specific and systemic tumor cell targeting. Cancer vaccine protocols load antigen-presenting cells (APC) with tumor-associated antigens and activate them to costimulate and expand antitumor CTL immunity (3). Clinically, vaccines have demonstrated the ability to augment T-cell reactions, and in 2010 the FDA approved the first solid tumor vaccine, Provenge (sipuleucel-T; Dendreon), for the treatment of advanced prostate cancer (3, 4). Importantly, immunizations typically do not produce tumor shrinkage, which limits survival benefits (4). This may be due, in part, to tumor-mediated signaling of CTLA-4 and PD-1, which dampens T-cell activity (2). CTLA-4 suppresses T-cell activation by blocking T-cell costimulation during TCR ligation, whereas PD-1 engagement by PD-L1 and PD-L2 (expressed by tumor cells or co-opted resident cells) limits T-cell activity within tumors by promoting anergy, death, or exhaustion (2, 5, 6). In addition, both CTLA-4 and PD-1 are expressed by regulatory T cells (Treg), which may reside within tumors or lymphoid tissues to further suppress T-cell activation (2, 6, 7). This knowledge of tumor and T-cell biology is the basis for antibody therapies that specifically block these immunosuppressive checkpoints. Recently, the anti–CTLA-4 antibody ipilimumab has demonstrated clinical efficacy, being the first agent to significantly prolong the overall survival of patients with inoperable stage III/IV melanoma (8, 9). Antibodies targeting the PD-1 pathway have recently entered the clinic and are showing dramatic effects in subsets of patients in a variety of cancer types (6). Despite these successes, most treated patients still succumb to progressive disease, indicating that vaccines or antibody therapies alone are insufficient to effect complete tumor cell killing (3, 4, 6, 9).

Approaches that utilize therapeutic vaccines to produce CTLs, while inhibiting their suppression via checkpoint antibodies, should be synergistic in amplifying T-cell immunity and effecting tumor shrinkage. We recently developed a biomaterial-based, therapeutic vaccine that has unprecedented effectiveness in maintaining sustained T-cell responses and produces tumor regression in preclinical models of melanoma and other cancers (10, 11). To test the effects of immune checkpoint blockade antibodies (anti–CTLA-4 and anti–PD-1) and cancer vaccines in combination, we utilized a therapeutic B16-F10 melanoma model.

Materials and Methods

Cell lines

B16-F10 melanoma cells were obtained from the ATCC (catalog CRL-6475) in 2010 and 2012. Upon receipt, the cells were cultured to passage 3, aliquotted and frozen in liquid nitrogen. For tumor experiments, B16-F10 cells were thawed and cultured in DMEM (Life Technologies, Inc.), containing 10% fetal bovine serum (Life Technologies, Inc.), 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere, and early-passage cells (between 4 and 9) were utilized for experiments.

Mice

C57BL/6 mice (6–8-week-old female; The Jackson Laboratory) were cared for in accordance with the American Association for the

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Accreditation of Laboratory Animal Care International regulations. Experiments were all approved by the Harvard University Institutional Animal Care and Use Committee.

PLG vaccine fabrication

A 85:15, 120-kD copolymer of D,L-lactide and glycolide (PLG; Alkermes) was utilized in a gas-foaming process to form porous PLG matrices (10). In brief, PLG microspheres encapsulating GM-CSF were first made using standard double emulsion (10). To incorporate tumor lysates into PLG scaffolds, biopsies of B16-F10 tumors that had grown subcutaneously (s.c.) in the backs of C57BL/6J mice (The Jackson Laboratory) were digested in collagenase (250 U/mL; Worthington) and suspended at a concentration equivalent to 10^7 cells per mL after filtration through 40-μm cell strainers. The tumor cell suspension was subjected to 4 cycles of rapid freeze in liquid nitrogen and thaw (37°C) and then centrifuged at 400 rpm for 10 minutes. The supernatant (1 mL) containing tumor lysates was collected and lyophilized. To incorporate CpG-oligodeoxynucleotides (ODNs) into PLG scaffolds, CpG-ODN 1826, 5′-tcc atg acg ttc ctg acg tt-3′ (Invivogen) was first condensed with poly(ethylenimine) (PEI, *M*n ∼60,000; Sigma Aldrich) by dropping CpG-ODN 1826 solutions into a PEI solution, while vortexing the mixture (11). The charge ratio between PEI and CpG-ODN (NH3+:PO4−) was kept constant at 7 during condensation. The condensate solutions were then vortexed with 60 μL of 50% (wt/vol) sucrose solution, lyophilized and mixed with dry sucrose to a final weight of 150 mg.

PLG microspheres were then mixed with the sucrose containing PEI-CpG-ODN condensate and tumor lysate and compression molded. The resulting disk was allowed to equilibrate within a high-pressure CO_2 environment, and a rapid reduction in pressure causes the polymer particles to expand and fuse into an interconnected structure. The sucrose was leached from the scaffolds by immersion in water, yielding scaffolds that were 80% to 90% porous (10).

Vaccine assays

For therapeutic vaccination, animals were challenged with an s.c. injection of 10^5 B16-F10 melanoma cells (ATCC) in the back of the neck. At day 3, a subset of mice were treated with 100 μg of either anti–CTLA-4 (9D9) or anti–PD-1 (RMP1-14; Bioxcell), or both antibodies and treatment was repeated every 3 days for 30 days. At day 9 after tumor challenge, PLG vaccines with melanoma tumor lysates and GM-CSF in combination with CpG-ODN were implanted s.c. into the lower left flank of C57BL/6J mice. Animals were monitored for the onset of tumor growth (approximately 1 mm^3) and sacrificed for humane reasons when tumors grew to 20 to 25 mm (longest diameter).

T-cell infiltration and activation

PLG vaccines were excised at day 14, and the ingrown tissue was digested into single-cell suspensions using a collagenase solution (Worthington; 250 U/mL) that was agitated at 37°C for 45 minutes. CTLA-4 and PD-1 antibody therapy for these experiments were administered at the same time points as the vaccination experiments and terminated with vaccine explantation. The cell suspensions were then poured through a 40-μm cell strainer to isolate cells from scaffold particles, and the cells were pelleted and washed with cold PBS and counted using a Z2 coulter counter (Beckman Coulter). The inguinal lymph nodes draining the vaccine sites were also excised and processed into single-cell suspensions. On the indicated days, B16-F10 tumors were also removed from mice and digested in 1 mg/mL collagenase II (250 U/mL; Worthington) and 0.1 mg/mL DNase for 1 hour at 37°C, and dissociated cells were filtered through a 40-μm filter. Negative T-cell separation was performed using a murine, pan T-cell separation kit (Miltenyi Biotec), which primarily removes innate immune cells and APCs along with debris and necrotic cells from suspension.

To assess T cells isolated from the vaccine site, draining lymph nodes, and tumors, isolated cells were directly stained with antibodies for phenotype characterization by fluorescence-activated cell sorting (FACS) analysis. PE-Cy7–conjugated CD3 stains were performed in conjunction with APC-conjugated CD8a (CD8 T cells), FITC-conjugated CD4 (CD4 T cells) and PE-conjugated FoxP3 (Tregs) and analyzed with flow cytometry. Tumor-infiltrating leukocytes were also costained FITC–anti-IFNγ, and PE-anti-CD107a for analysis of these activation markers. All antibodies and the FoxP3 antibody staining kit were obtained from eBioscience. Cells were gated according to single positive FITC, APC, and PE stainings, using isotype controls. The percentage of cells staining positive for each surface antigen was recorded.

Statistical analysis

All values in the present study were expressed as mean ± SD. Statistical significance of differences between the groups was analyzed by a two-tailed Student *t* test, and a *P* value of less than 0.05 was considered statistically significant.

Results

PLG matrices were fabricated, as described (10), to coordinate the recruitment and antitumor programming of dendritic cells via the controlled presentation of tumor lysates with GM-CSF and CpG-rich ODN (PLG vaccines). In mice bearing 3-day-old B16 melanoma tumors (5×10^5 cells), treatment with CTLA-4 and PD-1 antibodies alone has no effect on tumor progression and survival outcomes in these animals (Supplementary Fig. S1). Moreover, a single PLG vaccination modestly suppressed tumor progression but did not affect long-term survival in any mice bearing B16 melanoma tumors (Fig. 1). Strikingly, treatment with CTLA-4 and PD-1 antibodies at day 3 combined with a single PLG vaccination at day 9 significantly reduced the rate of tumor progression and resulted in long-term survival rates of 75% and 40%, respectively, with complete tumor regression in the surviving mice (Fig. 1A–C). Tumors were pretreated with antibody blockade prior to vaccination because this sequence likely reflects the clinical setting where these antibodies are becoming standards of care. If antibody administration is ceased after vaccination, the effects on tumor inhibition are lost (Supplementary Fig. S2), suggesting that blockade treatment significantly augments the subsequent T-cell responses induced by vaccination.

Indeed, combining blockade antibodies with PLG vaccination significantly skewed the tumor-infiltrating leukocyte (TIL) response toward active, cytotoxic T cells, relative to suppressive Tregs (Fig. 2 and Supplementary Fig. S2). This finding is consistent with the finding of tumor regression, as higher CD8/Treg ratios within tumors are indicative of effective vaccination (3, 9).

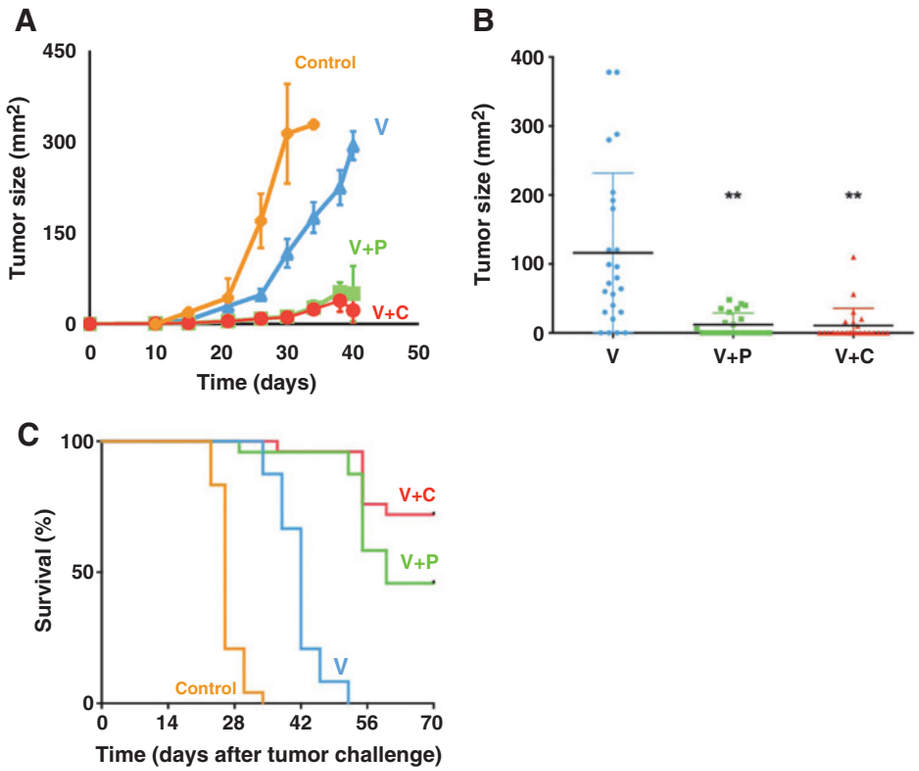

Figure 1.

Tumor protection induced by therapeutic PLG vaccination in combination with blockade antibodies. A, comparison of the tumor growth of untreated mice (control) bearing established melanoma tumors (inoculated with 5×10^5 B16-F10 cells and allowed to develop for 9 days) and mice treated after tumor challenge with PLG vaccines (V) or PLG vaccines in combination with antibodies to PD-1 (V+P) or CTLA-4 (V+C). B, dot plot of the tumor size in untreated mice (control), and mice treated with PLG vaccines (V) or PLG vaccines in combination with antibodies to PD-1 (V+P) or CTLA-4 (V+C) 30 days after tumor challenge. C, comparison of the survival of untreated mice (control) and mice treated with PLG vaccines (V) or PLG vaccines in combination with antibodies to PD-1 (V+P) or CTLA-4 (V+C) after tumor challenge. The antibody treatments were initiated on day 3 and injected i.p. every 3 days for 30 days after tumor challenge. Values in A ($n = 24$) represent mean and SEM. **, $P < 0.01$ as the vaccine compared with other experimental conditions (V vs. control; V vs. V+P; V vs. V+C) unless otherwise noted. This experiment was performed three times ($n = 8$; $N = 24$ in total) and results were pooled together.

PLG vaccination at day 9 after tumor challenge induced significant $CD3^+CD8^+$ T-cell infiltration into 20-day-old B16 tumors, resulting in approximately 3,000 cytotoxic T cells per mm^2 of tumor (Fig. 2A). The addition of anti–PD-1 treatment to vaccination provided a 3.7-fold increase in tumor-infiltrating $CD3^+$ $CD8^+$ T cells, whereas the addition of anti–CTLA-4 therapy produced over 24,000 $CD3^+CD8^+$ cytotoxic T cells per mm^2 of tumor (Fig. 2A). In contrast, these treatment groups had no effect on the numbers of tumor-resident $CD4^+FoxP3^+$ Tregs (Fig. 2B). The intratumoral ratio of $CD8^+$ effector T cells to Tregs at day 18 tripled with PD-1 antibody administration compared with vaccination alone (Fig. 2C). Combining anti–CTLA-4 with vaccination resulted in a 15-fold increase in the effector T-cell/Treg ratio compared with vaccination alone at day 18 (35.9 to 2.3; Fig. 2C). The same analysis was conducted at day 30 after tumor challenge, and only immunizations combined with anti–CTLA-4 were able to generate significant $CD8^+$/Treg ratios (approximately 6-fold increase; Supplementary Fig. S3), consistent with the long-term survival data. In addition, supplementing vaccination with PD-1 or CTLA-4 antibody therapy resulted in 3-fold and 8-fold increases in intratumoral, cytotoxic T-cell activation, respectively, as determined by CD107a and IFN-γ coexpression (Fig. 2D and E). The addition of checkpoint blockade enhanced not only the density of activated $CD8^+$ TILs but also the percentage of total $CD8^+$ T cells that were activated (Fig 2D and E), indicating that these treatments promoted T-cell cytotoxicity locally, within tumors.

Combining vaccination with both CTLA-4 and PD-1 antibodies initially suppressed tumor growth over single antibody combinations with PLG vaccines (Supplementary Fig. S4A). Tumor inhibition coincided with increased cytotoxic T-cell activity relative to vaccine augmentation with a single antibody (Supplementary Fig. S4B–S4D). However, this combined checkpoint inhibition with vaccination did not significantly affect long-term survival in comparison with the addition of one blockade (data not shown). These data suggest that combining PLG vaccines with either CTLA-4 or PD-1 antibodies can dramatically enhance the numbers of activated, effector T cells within tumors, which likely perform tumor cell killing to enhance survival in these animals.

CTLA-4 and PD-1 antibody treatment also enriched the vaccine site with $CD4^+$ and $CD8^+$ T cells at day 14 after implantation (Fig. 3A). CTLA-4 antibody treatment combined with vaccination significantly increased the local numbers of $CD8^+$ cytotoxic T cells

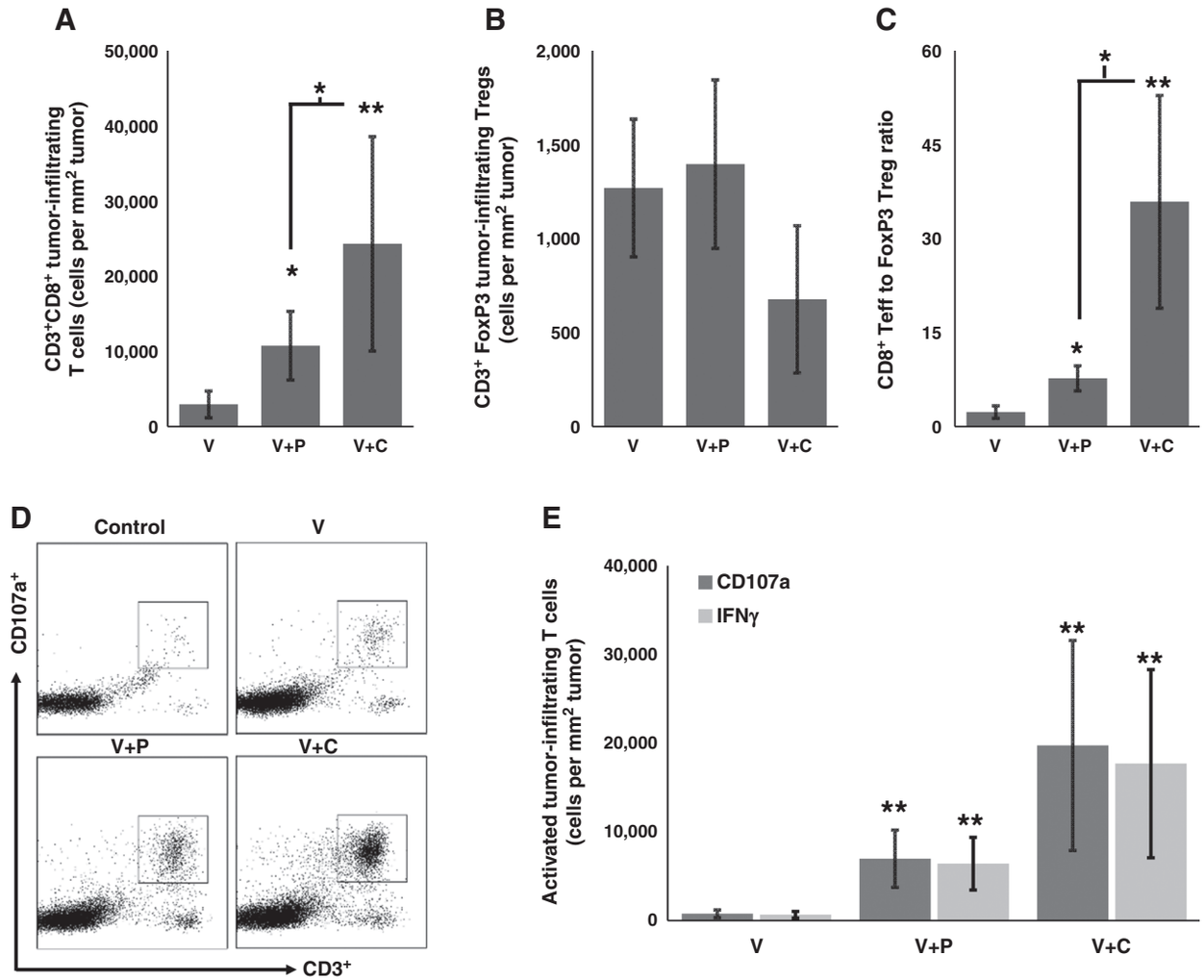

Figure 2.

Engineered PLG vaccine in combination with blockade antibodies enhances intratumoral effector T-cell activity. A, total number of $CD3^+CD8^+$ T cells and B, $CD3^+FoxP3^+$ Tregs isolated from the B16 tumors of untreated mice (control) and mice treated with PLG vaccines alone (Vax) or in combination with antibodies to PD-1 (+ PD-1) and CTLA-4 (+ CTLA4). C, ratio of $CD3^+CD8^+$ T cells to $CD3^+FoxP3^+$ Tregs isolated from the B16 tumors of untreated mice (control) and mice treated with PLG vaccines alone (Vax) or in combination with antibodies to PD-1 (+ PD-1) and CTLA-4 (+ CTLA-4). D, FACS plots representing tumor-infiltrating leukocytes in tumors of untreated mice (control) and mice treated with PLG vaccines alone (Vax) or in combination with antibodies to PD-1 (+ PD-1) and CTLA-4 (+ CTLA4). Single-cell suspensions were prepared from tumors at day 18 and stained for activated cytotoxic T-cell markers, CD8 and CD107a. Numbers in FACS plots indicate the percentage of the cell population positive for both markers. E, numbers of $CD8^+$, tumor-infiltrating T cells positive for either IFNγ or CD107a in blank matrices (control), PLG vaccines alone or vaccines in combination with antibodies to PD-1 and CTLA-4. The antibody treatments were administered i.p. as described for vaccination experiments until tumor excision at day 18 for T-cell infiltration analysis. Vaccination was initiated 9 days after tumor challenge. All cellular staining was performed following MACS cell separation of total cell suspensions extracted from tumors. Values in A–D ($n = 10$) represent mean and standard deviation. *, $P < 0.05$; **, $P < 0.01$, as compared with the vaccine alone (V vs. V+P; V vs. V+C) unless otherwise noted. This experiment was performed twice. Teff, T-effector cell.

by 5- and 10-fold over PD-1 addition, or vaccines alone (Fig. 3B). CTLA-4 blockade also enhanced Treg activity within vaccines relative to PD-1 combination or vaccination alone (Fig. 3C). Importantly, the cytotoxic T-cell to Treg ratio was approximately 3-fold greater with antibody addition in comparison to vaccine treatments alone (Fig. 3D). In addition, increased $CD8^+$ T-cell numbers and reduced $FoxP3^+$ Treg numbers were found within the vaccine-draining inguinal lymph nodes with combination treatment of CTLA-4 and vaccines, in contrast to PD-1 addition or vaccines alone (Supplementary Fig. S5).

Discussion

In summary, this study indicates that combination therapy of antibodies and PLG vaccines can enhance antitumor CTL activation and promote significant tumor regression. Single PLG vaccination is able to produce significant numbers of activated TILs, but these cells fail to shrink tumors unless this response is amplified by either CTLA-4 or PD-1 signaling. Combination therapy produced effector TIL numbers that comprised up to 25% of all tumor cells.

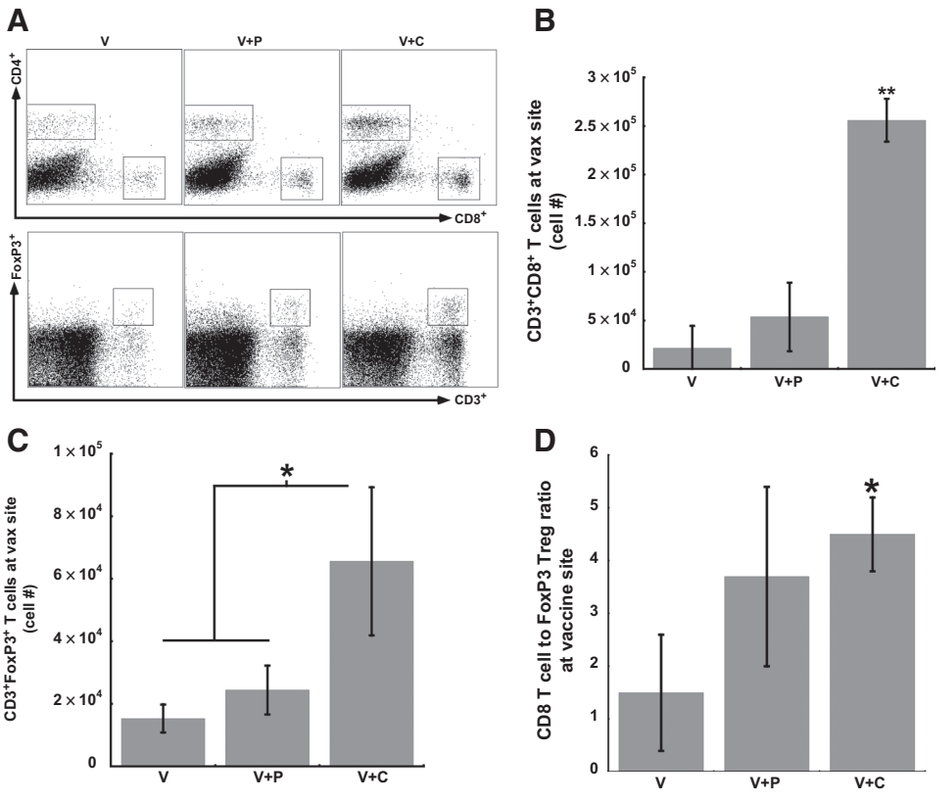

Figure 3.
Engineered PLG vaccine in combination with CTLA-4 maintains local T-cell activity at the vaccine site. The T-cell infiltrates into PLG vaccines were implanted in mice for 14 days. A, FACs histograms of $CD4^+$, $CD8^+$, and $CD4^+FoxP3^+$ T-cell infiltrates isolated from PLG implants in mice treated with PLG vaccines alone (V) or in combination with PD-1 antibody (V+P) or anti–CTLA-4 antibody (V+C). The numbers of $CD3^+CD8^+$ T cells (B), $CD4^+FoxP3^+$ Tregs (C), and the ratio of $CD3^+CD8^+$ effector T cells to $CD4^+FoxP3^+$ T cells (D) isolated from the vaccine site in mice treated with PLG vaccines alone (V) or in combination with PD-1 antibody (V+P) or anti–CTLA-4 antibody (V+C). The antibody treatments were administered i.p. as described for vaccination experiments until vaccine explantation. CD8/Treg ratio was calculated from cell numbers. Values represent mean and standard deviation ($n = 5$). *, $P < 0.05$; **, $P < 0.01$ as vaccine alone compared with all other experimental conditions (V vs. V+P; V vs. V+C) unless otherwise noted. This experiment was performed once.

In contrast to previous studies utilizing checkpoint blockade treatment alone or in combination with vaccines (12–14), antibody therapy administered with vaccination in this study had no effect on Treg populations at the vaccine site or in tumors. In contrast, a recent study demonstrated that combining anti–CTLA-4 with Gvax can deplete Treg numbers in a tumor microenvironment and this is dependent on the presence of tumor infiltrating, Fc receptor–expressing macrophages (14). Thus, observations of Treg depletion are associated with cellular and molecular changes in the tumor microenvironment that may be modulated by the vaccine strategy. Notably, the PLG vaccine used in this study has been optimized to control the presentation of GM-CSF and adjuvants, relative to other vaccine formulations (e.g., Gvax), in order to enhance effector T-cell activity and downregulate Tregs and other immunosuppressive mechanisms induced by GM-CSF (11, 15). Therefore, the PLG vaccine likely creates a tumor and vaccine microenvironment that responds to anti–CTLA-4 by preferentially enhancing effector T-cell generation and expansion over Tregs. Further, *in vitro* studies investigating the functional capacity and antigen specificity of effector T cells and Tregs should be performed to clarify their activation state and the mechanisms by which inhibitors synergize with PLG vaccination. Experiments in other, more immunosuppressive, tumor models may further elucidate the effects of blockade inhibitors on PLG vaccine–induced Tregs.

Interestingly, vaccination with anti–CTLA-4 and not anti–PD-1 increased $CD8^+$ effector T-cell activity at the vaccine sites and within lymph nodes, consistent with enhanced costimulation by resident APCs. Additionally, immunization supplemented with anti–PD-1 conferred significant effector T-cell activation only within tumors, and not at the scaffold site, suggesting that PD-1 activity predominates within the B16 tumor environment relative to the vaccine site. Further work, including an assessment of PD-1^+ and CTLA-4^+ T cells at the sites of vaccination and tumors, should be performed to elucidate the superior effect of CTLA-4 inhibition over PD-1. These data have significant clinical relevance, because the PLG system, designated WDvax, is currently in phase I testing for the treatment of metastatic melanoma (clinicaltrials.gov ID-NCT01753089), and suggest that vaccinations be utilized in combination with blockade antibodies.

Disclosure of Potential Conflicts of Interest

G. Dranoff reports receiving commercial research support from Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

The former Editor-in-Chief of this journal (Glenn Dranoff) is an author on this article. In keeping with the AACR's editorial policy, the peer review of this submission was managed by a senior member of *Cancer Immunology Research's* editorial team; a member of the AACR Publications Committee rendered the final decision concerning acceptability.

Authors' Contributions

Conception and design: O.A. Ali, G. Dranoff, D.J. Mooney
Development of methodology: O.A. Ali
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O.A. Ali, S.A. Lewin, D.J. Mooney

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O.A. Ali, S.A. Lewin, D.J. Mooney
Writing, review, and/or revision of the manuscript: O.A. Ali, G. Dranoff, D.J. Mooney
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): O.A. Ali, D.J. Mooney
Study supervision: D.J. Mooney

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