Preclinical Evidence That PD1 Blockade Cooperates with Cancer Vaccine TEGVAX to Elicit Regression of Established Tumors

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Abstract

Biomarker studies have shown that expression of the T-cell coregulatory ligand PDL1 on tumor cells correlates with clinical responsiveness to the PD1 antibody nivolumab. Here, we report the findings of a preclinical cancer vaccine study demonstrating vaccine-dependent PDL1 upregulation in the tumor microenvironment. We formulated an IFNγ-inducing cancer vaccine called TEGVAX that combined GM-CSF and multiple Toll-like receptor agonists to increase the number of activated dendritic cells. Treatment of established tumors with TEGVAX retarded tumor growth in a manner associated with enhanced systemic antitumor immunity. Unexpectedly, TEGVAX also upregulated PDL1 expression in the tumor microenvironment, possibly explaining why tumors were not eliminated completely. In support of this likelihood, PDL1 upregulation in this setting relied upon IFNγ-expressing tumor-infiltrating CD4+ and CD8+ T cells and administration of a PD1-blocking antibody with TEGVAX elicited complete regression of established tumors. Taken together, our findings provide a mechanistic rationale to combine IFNγ-inducing cancer vaccines with immune checkpoint blockade.

Introduction

A major advance in clinical immunotherapy has been the blockade of inhibitory immune receptors and their ligands, collectively termed immune checkpoints. Specifically, blockade of CTLA4 with ipilimumab and PD1 with nivolumab both demonstrated clinical efficacy in a number of different advanced malignancies (1, 2). Colocalization of PDL1 and tumor-infiltrating lymphocytes (TIL) that expressed IFNγ in the clinical specimens together with the induction of PDL1 on tumor cell lines in response to IFNγ have led to the idea that expression of this inhibitory ligand represents an adaptive response to “threat” by tumor-specific T cells in the microenvironment (3). This adaptive immune resistance mechanism has been hypothesized as an important means of the developing cancer cells to evade the cytotoxic antitumor TILs. Thus, PDL1 expression, which correlated with clinical response to nivolumab (4), may signify the presence of antitumor immune responses while low expression may signify an absence of antitumor immunity. In accord with this notion, poorly immunogenic tumors, such as B16, have minimal PDL1 expression (5).

These findings point toward combinatorial therapy that brings together checkpoint blockade with a driver of tumor-specific immunity in patients with cancer who have an ineffective antitumor immune response. The most straightforward approach to accomplish this would be to combine cancer vaccines that can increase the tumor-specific Th1 IFNγ-secreting lymphocytes with anti-PD1 blockade (6).

With an extensive history of safety as well as expression of diverse, unbiased tumor antigens, lethally irradiated tumor cell vaccines engineered to secrete GM-CSF have the potential to be combined with immune checkpoint blockade antibodies in patients (7, 8). Local granulocyte macrophage colony-stimulating factor (GM-CSF) can mobilize and recruit myeloid precursors into dendritic cells (DC), but this cytokine does not intrinsically induce DC activation (9). Thus, one major limitation of GM-CSF–secreting tumor vaccine (GM-vaccine) has been its limited capacity to activate antigen-presenting cells (APC) necessary for optimal tumor antigen presentation in the afferent arm of the immune system (10). Toward improving the efficacy of antigen-based cancer vaccines by stimulating plasmacytoid DC (pDC), Ali and colleagues formulated TLR9 agonist with tumor lysates and GM-CSF to induce improved in vivo efficacy (11). To further optimize this combinatorial method, we developed a strategy that incorporates GM-CSF, cell-based vaccine with unbiased tumor antigens, and multiple Toll-like receptor (TLR) agonists (11–17) that can activate both the conventional/classical (cDC) and the pDC in the innate immune system. We formulated....

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glucopyranosyl lipid A (GLA, a TLR4 agonist) and resiquimod (R848, a TLR7/8 agonist)—two agents found to be safe in patients—with a tumor cell–based vaccine to create TLR agonist enhanced GM-vaccine (TEGVAX) and studied its anti-tumor effects in an established, palpable B16 treatment model, which is resistant to most previously tested strategies of active immunotherapy (18–20).

We first demonstrated that TEGVAX significantly enhanced DC activation, tumor-specific CTL activity, and in vivo anti-tumor responses in the systemic treatment of palpable B16 melanoma. However, no mice were cured, and we observed that this vaccination/treatment induced upregulation of PDL1 in tumors in an IFNγ-dependent manner. Addition of PD1 blockade to this vaccine resulted in regression of a significant proportion of tumor-bearing animals.

Materials and Methods

Mice, cells, and reagents
Six- to 8-week-old female C57BL/6, Balb/c, and C3H/HeOuJ mice (The Jackson Laboratory) were housed according to the Johns Hopkins Hospital (JHH) Animal Care Committee. C57BL/6 MyD88−/−TRIF−/−, and C57BL/6 (Cg) Rag2−/− mice were obtained from Dr. Franck Housseau (JHH, Baltimore, MD). B16 and B16 GM-vaccine cells were cultured in RPMI-1640 media containing 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). In PDL1 experiments, B16 were cultured with serum-free media. CD11c+ cells were isolated by anti-mouse CD11c microBeads (MACS, Miltenyi Biotec). CD4-depleting GK1.5 antibody and CD8-depleting 2.43 (Bio X Cell) at 1.0 mg/mL and R848 at 0.2 mg/mL were prepared in RPMI-1640 media containing 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). In PDL1 experiments, B16 were cultured with serum-free media. CD11c+ cells were isolated by anti-mouse CD11c microBeads (MACS, Miltenyi Biotec). CD4-depleting GK1.5 antibody and CD8-depleting 2.43 (Bio X Cell) at 200 µg per dose were injected intraperitoneally every 2 days. Hybridoma expressing blocking anti-PD1 antibody (clone G4) was obtained from Dr. Charles Drake (JHH).

Vaccine preparation
GLA at 1.0 mg/mL and R848 at 0.2 mg/mL were prepared in 10% (w/v) squalene oil-in-water emulsion vehicle (Immune Design). GLA/R848 dissolved in emulsion was incubated with lethally irradiated (150 Gy) GM-vaccine cells at 4°C for 0.5 to 2 hours and washed four times with PBS. This GM-vaccine formulation with GLA and R848 was labeled as TEGVAX. Control GM-vaccines were treated with emulsions and washed without adjuvants. In some cases, GLA and R848 were absorbed into GM-vaccine cells with Lipofungin and washed four times to remove nonabsorbed TLR agonists and transfectants.

Tumor treatment assay
C57BL/6 mice were injected with 1–5 × 10^4 B16 cells in the footpads. Once palpable tumor developed (5–10 days), 100 µL of 10^6 B16 GM-vaccine or B16 TEGVAX was injected subcutaneously into the contralateral limb. For all these experiments, 5 to 10 mice were used per group. All the experiments were repeated at least five times. Daily tumor measurements were initiated once all three dimensions reached anywhere from 0.5 to 4 mm and the relative tumor volume was calculated by the formula: Length (mm) × Width (mm) × Height (mm) × 0.5326 × 0.01 (21). C3H/HeOuJ mice and Balb/c mice were used with SCCFVII/SF cells and CT26 cells, respectively, with comparable methods (22). In brief, CT26 TEGVAX consists of irradiated (150 Gy) 1 × 10^6 CT26 and allogeneic 1 × 10^6 B78H1 GM-CSF with absorbed GLA at 1 mg/mL and R848 at 0.2 mg/mL as described above. SCCFVII TEGVAX was prepared from GM-CSF–secreting SCCFVII cells with GLA/R848 absorbed as above (23). For all the vaccines, GM-CSF expression level ranged from 50 to 500 ng/10^6 cells/24 hours. For the PD1 blockade experiments, 100 µg/mice/injection of anti-PD1 (G4) was injected intraperitoneally twice a week once the tumor was palpable in conjunction with vaccine treatments. In some experiments, 100 µg/mice/injection of IFNγ-neutralizing antibody (XMG1.2 – Bio X Cell) was injected intraperitoneally twice per week.

DC activation assay
Draining lymph nodes (DLN) from tumor-bearing or naive mice were harvested 3 to 7 days postvaccine treatments and digested in media containing DNase I and Liberase Blendzyme 2 (20,000 Mandl U/mL; Roche). DC-enriched populations were obtained by depleting CD3+ and CD19+, and gated for CD11c+ and B220+. These were evaluated by a multicolored FACS analysis using CD80, CD86, CD40, and MHCI antibodies (BD Biosciences).

In vivo CTL assay
Splenocytes were labeled with 0.5 µmol/L, and 5 µmol/L carboxyfluorescein succinimidyl ester (CFSE-Molecular Probes). The 5 µmol/L CFSE-labeled cells were pulsed with 10 µg/mL P15E (KSPWFTTL) peptide. The 0.5 µmol/L CFSE-labeled cells were pulsed with β-gal (TYPARWGL) peptide. Mice were injected intravenously with a 1:1 mixture of these cells, and the splenocytes were isolated after 24 hours and analyzed by flow cytometry. Antigen-specific killing was calculated using the following formula: (1−% of CFSElow/% of CFSEp-gal) × 100.

Histological analysis and immunohistochemistry
Necrosis foci quantitation was performed on Mayer hematoxylin and eosin–stained slides (Sigma). Necrosis foci in 10 separate regions under ×20 magnification were quantitated in a blinded manner under the guidance from Dr. Janice Taube (JHH), a board certified dermatopathologist. For immunohistochemistry (IHC), 10 µm thick frozen sections were fixed with acetone, and blocked with 1% BSA for 30 minutes at room temperature. For paraffin embedded tissue, the sections were fixed in 4% paraformaldehyde before 1% BSA blocking, αCD4, αCD8, αCD86 FITC conjugates, and αCD45 and αPDL1 primary antibodies were incubated for 1 hour at 4°C. Alexa Fluor 568 conjugate antibody was used as secondary antibody in some cases. 4',6-diamidino-2-phenylindole (DAPI) was used as the nuclear counterstain. Positive cells in 10 randomly selected fields at ×40 magnification were quantitated in a blinded manner. The microscope was Nikon, Eclipse E800. The camera was Nikon, DS-Qi1Mc. The software was NIS-Element AR 3.0.

Cytokine analysis
CD3-depleted DC was cultured with Golgistop (BD) protein transport monensin and lipopolysaccharide (LPS) 0.1 µg/mL for 5 hours and stained for anti-mouse CD11c, CD86, CD80, and MHCII. After gating for pDC and cDC populations (see figure...
Figure 1. TEGVAX that combined GM-CSF and multiple TLR agonists (GLA and R848) increased DC trafficking and activation, and this vaccine induced an antitumor response. All the experiments were replicated at least three times. A, IHC of endogenous CD11c⁺ DCs at the site of vaccine injection comparing GM-vaccine versus irradiated B16. Bar, 200 μm. B, GM-CSF increased the trafficking of endogenous DC to the DLN. Mice were simultaneously injected with paramagnetic iron oxide–labeled GM-vaccine and vaccine in the footpad. The mice were imaged with microMRI at the draining popliteal lymph node site 3 days after injection, and the endogenously cell-to-cell–labeled APCs were quantitated in the primary DLN (10). Pixel counts on DLN were quantitated in each mouse (N = 3) to compare the GM-vaccine and vaccine treatment (**, P < 0.05). C, TEGVAX increased CD86 and CD80 activation molecules in vivo on both pDCs and cDCs from the DLN in comparison with GM-vaccine 3 days after vaccine injection. pDC was gated as CD11chighMHCIIhighB220high and cDC was gated as CD11chighMHCIIhighB220low. TEGVAX-treated mice also had significantly increased IL12, TNFα, and IFNα–positive CD11c⁺ cells in comparison with GM-vaccine or the control treated groups. TEGVAX-treated group also had increased IFNγ-secreting CD8³ T cells in the DLN. DLN were depleted with CD3 before intracellular staining for CD11c⁺ cells in some cases. FSC, forward scatter gating; SSC, side scatter gating. D, TEGVAX induced significant in vivo antitumor response against palpable B16 tumor in comparison with GM-vaccine. The presence of emulsion vehicles did not affect these individual antitumor responses. All these in vivo treatment experiments are representative of at least three to five separate experiments, and each group in all experiments had 10 mice per group. The antitumor response of TEGVAX was abrogated in MyD88⁻⁻/TRIF⁻⁻ double knockout mice as well as Rag2⁻⁻ null mice (**, P < 0.01).
intracellular cytokine analysis (IL12, IFNα, and TNFα) were performed after membrane permeabilization with Cytokit (BD). For the lymphocyte analysis, harvested splenocytes or DLN cells were stimulated with 1 mmol/L/mL phorbol 12-myristate 13-acetate and 1 mg/mL ionomycin and Golgi-stop. Suspension cells were stained for anti-mouse CD3, CD4, CD8, and IFNγ with isotype controls.

**MRI imaging**
Vaccine cells were labeled with 50 μg/mL PEG-phospholipid–encapsulated ferromagnetic iron oxide (WFION) with an edge length of 22 nm (24). A total of 10⁶ labeled vaccine cells in 40 μL were injected into the footpad and imaged on 11.7 Tesla Bruker MRI using birdcage volume resonator using T₂-weighted multigradient echo sequence. Image parameters were: FOV = 2.2 × 2.2 cm, Matrix size = 256 × 192, TR = 500 ms, TE = 3.1 ms, slice thickness = 0.5 mm, and 12 slices. For quantification, regions of interest (ROI) were drawn on the lymph nodes and adjacent muscle tissue as internal controls. For each ROI for every slice, pixel intensity histogram was plotted with the lowest pixel intensity in the control muscle tissue as the threshold and quantitated as described previously (10).

**Statistical analysis**
We used a paired t-test to calculate two-tailed P value to estimate statistical significance of differences between two treatment groups using Excel software.

**Results**
TEGVAX increased DC activation as well as IFNγ-secreting T cells, but still failed to induce regression of established tumor

We first formulated TEGVAX that can secrete DC-recruiting GM-CSF and activate these DCs via multiple TLR agonists to prime tumor-specific T cells. GM-vaccine indeed increased the abundance of CD11c⁺ DCs at the site of vaccine injection.

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**Figure 2.** Antitumor responses from TEGVAX were found in multiple tumor models. A, TEGVAX induced antitumor response in palpable CT26 colon carcinoma and palpable SCCFVII tongue carcinoma models compared with controls (**, P < 0.01). CT26-GM and SCCFVII-GM vaccine was prepared as described previously (43, 44). C3H/HeOUJ mice treated with TEGVAX also demonstrated antitumor response (**, P < 0.01). B, palpable B16 bearing mice were treated with multiple injections of vaccines and TLR agonists on day 7, day 14, and day 21 (arrow: ***, P < 0.01).
(Fig. 1A). Using the magnetovaccination MRI method to quantitate the number of endogenous APCs with endocytosed surrogate tumor antigen (ferromagnetic label) from the vaccine cells, we found that GM-vaccine–treated mice had significantly increased APC trafficking to the DLN (Fig. 1B; ref. 25).

We then tested whether GLA and R848 formulated in GM-vaccine cells could increase the number of activated DCs in the DLN of nontumor-bearing mice. Compared with GM-vaccine,

Figure 3. TEGVAX treatment increased tumor necrosis and TILs in the tumor microenvironment. A, hematoxylin & eosin staining of treated B16 tumor tissue showed that TEGVAX-treated tumor showed increased number of necrotic foci (circled areas; magnification, ×20). B, necrotic foci were quantified in 10 separate regions under ×20 magnification in tumor tissue treated with TEGVAX and GM-vaccine (**, *P < 0.01). C, TEGVAX-treated B16 melanoma had increased infiltration of CD4 and CD8 T cells in the tumor microenvironment. D, the tumor-infiltrating CD4 and CD8 T cells were counted in 10 randomly chosen fields with immunofluorescent microscope. Bar, 200 μm. TEGVAX-treated B16 tumors had significantly increased infiltrating lymphocytes in comparison with GM-vaccine– and GLA/R848-treated tumors (†, *P < 0.05). All the experiments were replicated at least three times.
TEGVAX was able to significantly enhance the activation phenotype of pDCs and cDCs in vivo (Fig. 1C). The cytokine profiles from these CD11c⁺ cells from the CD3-depleted DLN also demonstrated increased amounts of IL12, IFNα, and TNFα that can skew the T-cell repertoire toward Th1 responses (Fig. 1C). When we gated for CD3⁺ cells, there were more activated IFNγ-producing T cells in mice treated with TEGVAX (Fig. 1C).

We tested TEGVAX with established B16 tumors in a therapeutic model of palpable disease. As shown in Fig. 1D, mice that received TEGVAX displayed lower tumor growth rate. Both GM-vaccine alone and TLR agonists alone had some modest slowing of the tumor growth, but the combination treatment produced the best in vivo antitumor response. To assess whether T cells were important for the antitumor response of TEGVAX, we performed vaccine treatment assays with Rag2⁻/⁻ mice and this antitumor response was abrogated in these mice (Fig. 1D). We also determined that the antitumor effect of TEGVAX was completely abrogated in MyD88/TRIF double knockout mice, ensuring that TLR signaling was critical for its efficacy (Fig. 1D). To assess whether this antitumor response can apply to different tumor types and murine strains, we also tested TEGVAX in the SCCFVII squamous cell carcinoma model in C3H mice as well as CT26 colon carcinoma model in Balb/c mice with comparable results, (Fig. 2A). We also performed these treatment assays with multiple TEGVAX treatments in the B16 model to improve this in vivo response, but no tumor regression was noted despite the decreased growth rate of the tumor (Fig. 2B).

TEGVAX increased lymphocytic infiltration into the tumor microenvironment as well as tumor-specific CTL responses

When we examined the tumor microenvironment of these treated mice, increased regions of focal necrosis were found with TEGVAX treatment in comparison with those treated with GM-vaccine or controls (Fig. 3A and B). Immunohistochemistry with CD4 and CD8 antibodies showed that TEGVAX-treated tumor had significantly increased infiltrating CD4 and CD8 cells within the tumor tissue in comparison with the vaccine controls (Fig. 3 and DC).

Figure 4. TEGVAX-treated mice had increased number of tumor-specific CTLs. In vivo CTL assays with P15E peptides were performed in B16 tumor bearing mice treated with vaccines. All the experiments were replicated at least three times. Assays were performed on day 25 after vaccine treatment from each of the treated groups (**, P < 0.01).
On the basis of the expression of the immunodominant p15E antigen in B16 tumors, we performed in vivo CTL assays to quantify the level of p15E-specific IFNy-secreting CTLs. At days 21 to 31, there was a clear separation in the growth rate of TEGVAX from other control groups, there were increased number of p15E-specific CTLs in the spleen of the mice treated with TEGVAX in comparison with GM-vaccine or other control mice (Fig. 4). However, despite these robust afferent immune responses, viable tumor was present in all the treated mice.

**TEGVAX treatment increased IFNγ+-dependent PDL1 upregulation by tumor cells**

On the basis of the adaptive resistance hypothesis (3), we sought to determine whether the antitumor activity of TEGVAX could be dampened by the induction of PDL1 in the tumor cells from the IFNy-secreting tumor-specific TILs in the tumor microenvironment. As described by others, we found that B16 cells treated in vitro with IFNy can induce the expression of PDL1 (Fig. 5A). When we examined the TIL from TEGVAX-treated mice, we noted an increased level of IFNy-secreting CD4 and CD8 in the tumor microenvironment (Fig. 5B). We also noted colocalization of PDL1 and CD8 T cells in the tumor microenvironment (Supplementary Fig. S1). To test whether these increased Th1 and CTL responses in vivo after TEGVAX administration are associated with the upregulation of PDL1 on the tumor cells, we stained the tumor tissue and found that TEGVAX significantly increased the expression of PDL1 in comparison with GM-vaccine or vehicle-treated mice (Fig. 5C). This increased PDL1 expression in the tumor microenvironment was significantly diminished when TEGVAX-treated animals were treated with IFNy-neutralizing antibody in vivo. (Fig. 5D).

**Anti-PD1 antibody with TEGVAX can induce regression of established B16 tumors to reverse the adaptive immune resistance mechanism**

On the basis of these results, we hypothesized that adding PD1 pathway blockade to TEGVAX treatment might unleash the potent TEGVAX-dependent T-cell responses to mediate a stronger antitumor effect, a finding with important clinical relevance. When the blocking anti-PD1 antibody treatment was combined with TEGVAX, established B16 tumors were noted to regress in more than 50% of the mice treated, whereas TEGVAX-treated mice could not induce tumor regression (Fig. 6A and B). In these mice that responded with the combined treatment, vitiligo was noted as showed in Fig. 6C. Mice treated with anti-PD1 alone had essentially minimal antitumor response in comparison with the untreated group. The poor response to anti-PD1 alone is likely due to the fact that B16 growth does not induce enough of an endogenous antitumor response to be significantly enhanced by PD1 pathway blockade. Mice treated with combined anti-PD1/TEGVAX that showed regressions of tumor were followed for another month (day 70) and no tumor developed. When these mice were challenged with B16 tumor at another site, no tumor developed (data not shown). These experiments were replicated 5 times with more than 50 mice per group with comparable results. Figure 6D summarizes the cumulative data of the tumor volume of the TEGVAX/anti-PD1 group that is statistically significant smaller than the other groups.

When PD1 blockade was combined with GM-vaccine alone, there was a modest antitumor response that approximated the growth rate seen with TEGVAX group, but none of these mice demonstrated regression as seen with TEGVAX/anti-PD1-treated groups (Fig. 6E). Control experiments with neutralizing IFNy antibody abrogated the antitumor response of TEGVAX/anti-PD1, which is consistent with adaptive immune resistance mechanism (Fig. 6F).

**Discussion**

Cancer immunotherapy strategies have been limited due to multiple resistance mechanisms, but two clinical studies demonstrated that immune checkpoint inhibitors can improve clinical responses in multiple tumor types (1, 2). Our findings provide in vivo mechanistic demonstration that vaccines should be coupled with anti-PD1 blockade in future clinical trials. In our B16 model, PD1 induction by IFNy-producing CTLs was critical in the efficacy of the anti-PD1 blockade (26–28). PD1 blockade by itself did not have significant antitumor response (Fig. 6), and only when combined with PD1-inducing TEGVAX did the tumor regress. This apparent discrepancy with the success of anti-PD1 clinical trial as a monotherapy underscores the critical role of adaptive immune resistance mechanism. Clinical responses were tightly correlated with the colocalized expression of PDL1 on the tumor and TILs, suggestive of endogenous antitumor immunity held in check. Our B16 tumors had minimal baseline expression of PDL1 (Fig. 5C) and the lack of in vivo response with anti-PD1 alone implicates a limited endogenous antitumor immune response that cannot be reversed. Only the induction of sufficient tumor-specific CTLs with immune checkpoint molecule blockade did we observe regression in vivo. In support of our mechanistic findings, Spranger and colleagues recently showed tight correlation between CD8+ TILs associated with PD1 in patients with melanoma, and CD8+-dependent PDL1 induction in preclinical models (29).

Figure 5. TEGVAX induced T-cell-driven IFNy-dependent upregulation of PDL1 expression in the tumor microenvironment. A, B16 melanoma cells were cultured in serum-free media with or without IFNy. Cells were stained with anti-murine PDL1 conjugates, and its mean fluorescent intensity (MFI) was measured using flow cytometry. B, tumor infiltrating T cells were harvested from the treated mice and intracellular staining of IFNy from both CD4 and CD8 populations were performed. MFI of CD4+ IFNy+ and CD8+ IFNy+ were quantitated in each of the treated groups (*, P < 0.05). C, treated tumor tissues were harvested and stained with rat anti-mouse PDL1 antibodies and anti-rat Cy3 conjugates to assess PDL1-positive cells. Bar, 200 μm. Blinded quantitation was performed in ten different fields at ×40 magnification (**, P < 0.01). D, tumor tissue from mice treated with GM-vaccine, TEGVAX, or TEGVAX administered with neutralizing IFNy antibody was harvested and stained for PDL1 expression. Bar, 200 μm. All the experiments were replicated at least three times (**, P < 0.01).
The objective responses in patients with advanced cancer with anti-PD1 monotherapy ranged from 18% to 28%, which implies that there is much room for clinical improvements with the addition of IFNγ-producing vaccines. Toward this clinical goal, we formulated a GM-CSF–secreting tumor cell vaccine with GLA (TLR4) and R848 (TLR7/8) agonists and demonstrated significant augmentation of in vivo antitumor response in comparison with GVAX alone or TLR agonists alone in 3 different murine models. We showed that these adjuvants increased the number of activated pDCs as well as classical/conventional DCs and their antitumor effects were MyD88/TRIF dependent. These in vivo antitumor responses were T-cell–dependent and associated with an elevation in p15E-specific T cells in the B16. Despite this antitumor response, the lack of tumor regression was associated with PDL1 induction in the tumor microenvironment.

Figure 6. TEGVAX with anti-PD1 treatment induced regression of established B16 tumors. All the experiments were replicated at least five times. A, tumor growth rate of established B16 tumor treated with various combinations of vaccines and blocking anti-PD1 antibody (left). Vaccines and anti-PD1 treatments were performed weekly. B, the tumor measurements of individual mice in each of the treated groups (right). C, treated mice with regressed tumor demonstrated autoimmune phenotype. The mouse on the right is one of the mice treated with TEGVAX/anti-PD1 and the mouse on the left is one of the mice treated with GM-vaccine. Arrow points to site of vitiligo. D, cumulative volume measurements of five experiments comparing TEGVAX/anti-PD1 treatment with controls demonstrate significantly consistent antitumor responses. A total of 50 mice per group were included in this analysis (N = 50/group). The mean volumes with SDs from five different tumor treatment assays were combined. The P values were generated using paired Student t test, comparing volume measurements with SD with the adjacent rows. E, TEGVAX + anti-PD1 treatment has greater antitumor response in comparison with GM-vaccine + anti-PD1 treatment in vivo. F, neutralization of IFNγ abrogates the improved antitumor response of combinatorial TEGVAX and anti-PD1 treatment. All the experiments above were replicated at least three times. Each treatment group had 10 mice/group.
test the adaptive immune resistance mechanism (3, 5). TEGVAX was combined with anti-PD1 blockade, and we observed regression of established B16 tumors as shown in Fig 6. These treatment assays with neutralizing IFNγ antibody suppressed the TEGVAX-dependent upregulation of PD-L1 in the tumor microenvironment (Fig 5D), and the regression of tumor seen with TEGVAX/anti-PD1 blockade was abrogated (Fig 6E). It should be noted that there is no published clinical data that have combined vaccines with immune checkpoint blockade.

Previous reports showed that the combination of anti-PD1, anti-CTLA4, and vaccine may promote antitumor responses, but their in vivo assay involved initiating treatment on non-palpable, nonestablished B16 tumor (30, 31). Both groups showed that anti-PD1 and cellular vaccines can be combined to induce an antitumor response in vivo, but these reports utilized a nonpalpable tumor inoculation method, which may not model clinical scenarios with established tumor, and they did not examine the upregulation of PD-L1 expression on the tumor cells. Our therapeutic assay was much more stringent in that we initiated treatment 7 to 10 days after B16 inoculation, at which point an organized immune inhibitory tumor microenvironment can be established, which cannot be treated with known formulations of vaccines. We saw no outgrowth of initially palpable B16 tumors that regressed even 2 months after the initiation of treatments, and a secondary challenge did not show any tumor growth (Fig 6A and B). Moreover, we did not need to combine anti-PD1 with other immune checkpoint blockade antibodies to demonstrate regression of established tumor. Addition of other immune checkpoint blockades to further improve the cure rates in our system remains an open question. Duraiswamy and colleagues showed that the addition of anti-CTLA4 with anti-PD1 and vaccine can also induce partial regression of established colon and ovarian carcinoma model (32). Our report, however, demonstrate the mechanistic link between IFNγ-producing TILs that can upregulate PD-L1 in the tumor microenvironment to render this poorly immunogenic B16 tumor amenable to anti-PD1 treatment in a stringent model of adaptive immune resistance. Optimizing the various combinations of translatable blocking checkpoint molecules (anti-CTLA4, anti-LAG3, anti-TIM3) can be performed with a bulky B16 tumor in the future, but our data clearly demonstrate that an important starting point for a combinatorial regimen is IFNγ producing vaccine with anti-PD1.

It should be reiterated that all the components of TEGVAX/anti-PD1 have been tested in patients to be relatively safe. With its demonstration as a potent antitumor agent by itself, TEGVAX in combination with anti-PD1 antibody has high translational potential for clinical trials. GLA and R848 were initially selected because these adjuvants significantly induced enhanced anti-tumor cytokine profiles compared with other TLR agonists, and both have been tested in patients to be safe (17, 33–35). We previously demonstrated an in vivo response with intratumoral injection using GM-vaccine formulated with LPS, but now we report a much more potent response with TEGVAX, a therapeutic vaccine administered distant from the site of established tumor (22). The mechanisms that underlie the intratumoral injection in comparison with the systemic injection in these treatment models are different, but cumulatively, these experiments demonstrate that TLR agonists formulated with a cellular vaccine injected intratumorally or systemically are unlikely to have a procarcinogenic effect (17, 36–39).

Immune evasion mechanisms of the established tumor are multifactorial, and we have not addressed the role of stromal tissue (40, 41) or myeloid cells (MDSC or macrophages; refs. 42, 43) in their response to the combination of TEGVAX and anti-PD1 blockade. Cumulatively, however, this report demonstrates that TEGVAX that can significantly augment the priming of tumor-specific T cells, even for established tumors, and it is an excellent candidate to be a component in a combinatorial therapy with anti–PD1-blocking antibody or other forms of immune checkpoint blockade in patients with cancer.

Disclosure of Potential Conflicts of Interest
H. Levitsky is a global head, cancer immunotherapy experimental medicine, in F. Hoffmann La Roche. D. Pardoll has ownership interest (including patents) in Aduro. Y.J. Kim has a commercial research grant from Aduro BioTech. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Fu, L.-J. Malm, Y.J. Kim
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