Introduction

The role of toll-like receptor (TLR)-mediated inflammation in the tumor microenvironment is a complex process whose role in carcinogenesis is still unclear. Various tumor models dissecting the downstream MyD88 and NF-kB signaling pathways initially showed procarcinogenic role of TLR signaling (1–3). However, Salcedo and colleagues have shown antitumor role of MyD88 signaling, whereas Garrett and colleagues have noted carcinogenic effect of MyD88 signaling in Rag2−/− mice (4, 5). TLR signaling in the hematopoietic compartment, however, has been shown to elicit antitumor responses, which have translated into multiple clinical trials (6, 7). In the context of infection, TLR4 agonists have been shown to render dendritic cell (DC) activation immunogenic whereas lack of TLR4 signaling can lead to tolerance (8). Medzhitov showed phagocytosed microbial antigens can be more efficiently presented in the presence of lipopolysaccharide (LPS; ref. 9). Cumulative, one valid strategy is to increase TLR stimulation directed toward increasing the number of activated antigen-presenting cells (APC) within the tumor microenvironment in vivo (10–12).

This study focuses on intratumoral injection as a direct means of modifying the tumor microenvironment to enhance both innate and adaptive antitumor immunity. To minimize potential TLR4 signaling on the tumor cells and to target the APCs in the tumor microenvironment, we evaluated intratumoral delivery of cellular vectors loaded with TLR4 agonist. The implication from studies of Medzhitov is that colocalization of TLR4 agonist and antigen can potentially enhance antitumor response when given as part of a combinatorial cellular vector with tumor antigens (9). An excellent candidate as a cellular vector for localized TLR agonist delivery is GVAX. The paracrine granulocyte

Abstract

Purpose: Because toll-like receptor (TLR) agonists have been well characterized as dendritic cell (DC) activators, we hypothesized that the admixture of TLR4 agonist into a cellular vector could improve the antitumor response in vivo.

Experimental Design: Granulocyte macrophage colony stimulating factor secreting whole cell tumor cell vector (GVAX) was formulated with lipopolysaccharide (LPS), a TLR4 agonist, and its intratumoral therapeutic efficacy was tested in three different murine models. We utilized immunohistochemistry, fluorescence-activated cell sorting, enzyme-linked immunosorbent spot (ELISPOT), and in vivo CTL analysis to assess both local innate immune responses within the tumor tissue as well as the downstream generation of antitumor T-cell responses.

Results: Intratumoral treatment of LPS-absorbed GVAX showed efficacy in improving an antitumor response in vivo in comparison with GVAX alone. Improved antitumor efficacy of this novel admixture was not present in TLR4 signaling impaired mice. In the CT26 model, 40% to 60% of the mice showed regression of the transplanted tumor. When rechallenged with CT26 tumor cells, these mice proved to be immunized against the tumor. Tumors treated with TLR4 agonist–absorbed GVAX showed increased infiltration of CD4 and CD8 T cells as well as increased numbers of CD86+ cells in the tumor tissue. Draining lymph nodes from the treated mice had enhanced number of activated CD86+, MHCII+, and CD80+ DCs in comparison with GVAX alone and mock-treated groups. ELISPOT assay and in vivo CTL assay showed increased numbers of CTLs specific for the AH1 tumor antigen in mice treated with LPS-absorbed GVAX.

Conclusion: TLR4 on antigen-presenting cells in the tumor microenvironment may be targeted by using cell-based vectors for improved antitumor response in vivo. Clin Cancer Res; 17(12); 3984–92. ©2011 AACR.
Translational Relevance

Use of toll-like receptor (TLR) agonists as adjuvants for advanced malignancies in patients is still controversial. One limitation is that TLR signaling can have mixed results in the tumor microenvironment. To target TLR4 signaling in antigen-presenting cells within the tumor microenvironment, we have formulated TLR4 agonist with a safe cellular vaccine, GVAX, which is currently undergoing clinical trials for multiple solid tumors. The following preclinical studies, therefore, provide scientific rationale for potential modification of a well-studied tumor vaccine platform to improve its clinical response in solid tumors amenable for intratumoral injections such as head and neck tumors.

macrophage colony stimulating factor (GM-CSF) delivery afforded by GVAX cells dramatically increases the numbers of myeloid cells, including DCs, macrophages, and granulocytes, at the site of injection (13–15). GVAX has been found to be safe from numerous phase I trials but was recently found to have uncertain efficacy in a phase III trial for advanced hormone refractory prostate cancer (16). As a cellular vector, however, GVAX may offer a starting platform to immunize the patients with multiple tumor antigens and deliver apoptotic and cellular debris loaded with TLR4 agonists to activate APCs in the tumor microenvironment as part of a combinatorial therapy.

To activate the locoregional APCs, LPS was formulated with GVAX cells, and this novel combinatorial regimen was injected intratumorally and studied for antitumor efficacy in several murine models.

Methods

Murine tumor cell lines

The SCCFVII/SF head and neck squamous carcinoma, B16-F0 melanoma, and B16-F0 transduced to secrete GM-CSF cell lines were cultured in RPMI 1640 with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 U/mL). CT26 was cultured similarly with MEM nonessential amino acids (Sigma), 1 mmol/L sodium pyruvate, and L-glutamine. CT26 were cultured in the same media as CT26 with the addition of Hygromycin B (1 g/L; Roche).

Mice

Adult (>50 days of age) female C57BL/6, BALB/C, C3H/HeOuJ, and C3H/HeJ mice were purchased from Jackson Laboratory and housed according to the JHU Animal Care and Use Committee. C57BL/6 MyD88–/– and C57BL/6 MyD88–/–TRIF–/– mice were obtained from Dr. Franck Housseau (Johns Hopkins University).

TLR4 agonist formulation with GVAX

Lipolectamine (120 μg/mL), LPS from Escherichia coli 026:B6 (10–50 μg/mL), and LPS-BODIPY (Molecular Probes) were combined into liposome complex. Cells for LPS formulation were seeded at a density of 5 × 10^5 cells were incubated with the Lipolectamine-LPS complex for 6 hours, washed 5 times prior to injection. To quantitate LPS incorporated into cells, Limulus amebocyte lysate (LAL) assay (Cambrex) was done as directed by the manufacturer. LPS (23 EU/ng) concentrations ranging from 0 to 30 EU/mL were used as standards. Prior to lethal irradiation, LPS-absorbed GVAX was cultured to ensure cell growth. Annexin staining verified no evidence of apoptosis after formulation (data not shown).

In vivo vaccine treatment assay

C57BL/6 mice were injected s.c in the right flank with 5 × 10^6 B16-F0 cells. Three to 5 days later, 10^6 lethally irradiated (150 Gy) B16 GM-CSF (GVAX), 10^6 lethally irradiated (150 Gy) LPS-formulated GVAX or LPS-absorbed 293T cells were injected intratumorally. In some cases, the vaccines were injected in the contralateral limb from the tumor-inoculated limb. C3H/HeOuJ mice and BALB/c mice were used with SCCFVII/SF cells and CT26 cells, respectively with comparable methods.

Immunohistochemistry

Frozen CT26 tumor were cut in 10 μm thickness and blocked with 1% bovine serum albumin 30 minutes at RT. Anti-mouse CD45 (eBioscience) and anti-mouse CD4–fluorescein isothiocyanate (FITC), CD8–FITC, and CD19–FITC antibodies (BD Pharmingen) incubated for 1 hour were used. Anti-CD45–Cy3 (Invitrogen) was used as secondary in some cases. 4',6-diamidino-2-phenylindole was used as counterstain for 10 minutes. Cells in randomly selected fields at 40× magnification were counted by using Nikon Eclipse F800 microscope and developed with Nikon DS-Qi1mc camera. NIS-Element AR 3.0 was the software used for these experiments.

DC activation assay

Spleens and draining lymph nodes (DLN) from tumor-challenged mice were harvested 5 days post-GVAX and TEGVAX treatment. Crushed spleens were digested in media containing DNase I (Roche) and Liberase Blendzyme 2 (20,000 Mandl U/mL; Roche). DC-enriched populations were obtained by depleting CD3ε and CD19ε, and gated for CD11cε and B220ε. These were evaluated by a multicolored fluorescence-activated cell sorting analysis by using CD80, CD86, and MHCII antibodies from BD Biosciences.

ELISPOT assay

Enzyme-linked immunosorbent spot (ELISPOT) plates (MultiScreen HTS filter plate; Millipore) were coated with a mouse IFN-γ Ab (MabTech) for 24 hours and 4T1 breast cancer cells were pulsed with 10 μg/mL of AH1 peptide overnight. A total of 10^6 CD8 cells from spleen and lymph nodes were plated in triplicates to be cocultured with pulsed or unpulsed 10^5 4T1 cells or stimulated with 1
μmol/L of phorbol 12-myristate 13-acetate and 10 ng/mL of Ionomycin as positive controls. On day 3, biotinylated anti-mouse IFN-γ Ab (MabTech) and Strepaavidin–horseradish peroxidase for ELISPOT (BD) were added. AEC Substrate Reagent Set for ELISPOT (BD) was used to develop spots and analyzed by using an ELISPOT Plate Reader (Immunospot).

In vivo CTL assay
A CellTrace CFSE Cell Proliferation Kit (Molecular Probes) was used to test the cytolytic activity of CTLs in CT26 tumor–challenged mice treated with or without TEGVAX. Splenocytes were processed and pulsed with either β-gal or AH1 peptide at a concentration of 10 μg/mL for 90 minutes. The β-gal population was carboxyfluorescein succinimidyl ester (CFSE)-labeled low (0.5 μmol/L) and the AH1 population was CFSE-labeled high (5 μmol/L). After 10 minutes, both CFSE-labeled cells were injected into the mice at 10^7 cells/mouse. Twenty-four hours post-injection, splenic cells were harvested and analyzed for detection of ratios of CFSE-labeled cells (17). Peptide-specific killing was calculated by using the formula (1% of CFSE peptide/% of CFSE no peptide) × 100.

Statistical analysis
We used paired t test to calculate 2-tailed P value to estimate statistical significance of differences between 2 treatment groups by using Excel software. Kaplan–Meier curves were generated by using GraphPad Prism software and analyzed with log-rank test. Statistically, significant P values are labeled in the figures and the legends with asterisks.

Results

TLR4 agonist formulation with GVAX
To enhance locoregional innate immune cell activation and minimize systemic toxicity of TLR4 stimulation and minimize TLR4 stimulation on tumor cells, LPS was formulated into GVAX (TEGVAX–TLR agonist–enhanced GVAX). We used a commercially available vector, Lipo-fectamine, to optimize absorption of LPS into GVAX cells prior to lethal irradiation. LPS-BODIPY fluorophore was used to show that 25 μg/mL of LPS for 6 hours resulted in 99.4% of the cells to be labeled (Fig. 1). LAL assay (Cambrex) was then used to show that 4.73 ± 0.2 ng of LPS was absorbed into 5 × 10^5 cells by using the Lipofectamine method that optimized LPS formulation (data not shown). For each of the in vivo murine tumor experiments, aliquots of TEGVAX were tested by using LAL assay and murine GM-CSF ELISA assay to ensure a comparable amount of LPS and GM-CSF in the TEGVAX formulation. The typical GM-CSF secreted ranged from 50 to 200 ng/mL/10^6 cells/24 h.

TEGVAX can induce in vivo antitumor response in multiple murine models
We initially tested the efficacy of intratumoral TEGVAX injection in a B16 murine model, whereby TEGVAX was delivered intratumorally 3 to 5 days after tumor inoculation in a therapeutic model. One rationale for intratumoral injection was to ensure that locoregional APCs that circulate between the tumor and the DLNs were targeted. These timepoints of treatment relative to initial tumor implantation were selected because we previously showed that anergic and tolerant tumor-specific T cells were present as early as 3 days after B16 injection (18). As shown in Figure 2, intratumoral TEGVAX administration decreased the growth rate of the B16 tumor in comparison with intratumoral injection of GVAX alone, which translated to survival curve differences (Fig. 2A). However, all the mice treated with TEGVAX eventually developed large tumors. We injected equimolar amount of LPS intratumorally as in the TEGVAX treatment group and these mice were no different than mice treated with GVAX or PBS (Supplementary Fig. S1). Equimolar LPS...
Figure 2. TEGVAX can induce an antitumor response in vivo. A, B16 inoculated 5TBL6; B, SCCFVII/SF inoculated C3H/HeOuJ; and C, CT26 inoculated BALB/c mice were treated with appropriate PBS, GVAX, or TEGVAX intratumorally typically from 3 to 5 days after the tumor injection. In vivo tumor progression in the TEGVAX group in all the murine models studied was statistically slower than those in the GVAX group (*, P < 0.05; left). A total of 10 to 20 mice per group were used, and all the experiments replicated 3 times. For the B16 and the SCCFVII/SF models, there were no differences between GVAX-treated group and the PBS-treated group (Supplementary Fig. S1 and data not shown). In the CT26 model, 40% to 60% of the mice in the TEGVAX group experienced a regression of their palpable tumor to undetectable levels by 3 weeks. Equimolar LPS formulated into 293T cells also showed no difference in growth rate. Mice showing regression of tumor were rechallenged with CT26 tumor (2 × 10⁶) and no tumor growth were noted. In all 3 murine models, the reduced tumor growth rate correlated with enhanced survival for mice in the TEGVAX group compared with those in the GVAX group (**, P < 0.01; right).

To verify that the antitumor response noted above were because of the TLR4 signaling, B16 tumor was treated with TEGVAX and GVAX in mice with MyD88⁻/⁻ and MyD88⁺/⁻ TRIF⁻/⁻ genotypes (Fig. 3A). Small differences noted early between the GVAX group and PBS groups eventually disappeared at later timepoints in MyD88⁻/⁻ mice. MyD88 and TRIF are essential intracellular mediators of TLR4 signaling, and the complete absence of these downstream TLR4 signaling mediators also abrogated the enhanced in vivo antitumor response noted in wild-type mice. For the SCCFVII model, the experiment was carried out in C3H/HeJ mice that lack functional TLR4, and, once again, the antitumor effect of TEGVAX was abrogated (Fig. 3B).
TEGVAX induces enhanced T-cell infiltration and APC maturation in the tumor

To examine the potential mechanism of the in vivo antitumor responses from TEGVAX treatment, the tumor tissue was harvested and analyzed for lymphocytic infiltration. As shown in Figure 4, tumor treated with TEGVAX had quantitatively increased CD4 and CD8 infiltration in comparison with the control and the GVAX-treated tumors. Moreover, given our hypothesis that TLR4 agonist stimulates the locoregional APCs to mature, we probed the tissue with CD86 and noted quantitative enhancement of CD86+ cells in the tumor treated with TEGVAX. We found no significant differences in the infiltration of F4+ macrophages between the treatment groups (data not shown).

TEGVAX augments DC activation in the locally DLNs

The immunostaining data from Figure 4 were consistent with the hypothesis that TLR4 agonist–absorbed cell vaccines can increase the number of activated locoregional DCs. Given that the LPS absorbed into GVAX is probably phagocytosed into the infiltrating DCs as cellular debris and micelles with tumor antigens, we predicted that there would be increased number of activated locoregional DC population with TEGVAX treatment. To test this, we purified DCs from the DLNs from tumor-bearing mice treated with either PBS, GVAX, or TEGVAX and gated for the conventional DC population with B220 and CD11c. Multi-parametric staining of DC activation marker, CD86, CD80, and MHCII from these gated cells, shows that DCs from the TEGVAX-treated group has greater population of activated phenotype (Fig. 5).

Tumor-specific cytotoxic T cells are expanded in mice treated with TEGVAX

CT26 model is associated with a well-characterized immunodominant CT26 antigen, AH1 that can facilitate quantitation of tumor-specific cytotoxic T cells. To test whether the in vivo antitumor responses for TEGVAX that activates locoregional DCs can increase the downstream population of tumor-specific cytotoxic effector cells, ELISPOT assays were conducted (19). T cells from the DLN and spleen were harvested and IFN-γ producing cytotoxic T cells screened in the presence of MHC class I Ld restricted AH1 peptides pulsed with APC in the ELISPOT assay. Although minimal AH1-specific T cells were detected on days 3 to 5 after treatment with TEGVAX (data not shown), by day 8, there were statistically significant AH1-specific T cells in the TEGVAX group in both the DLNs and the spleen as shown in Figure 6A. In vivo CTL assays also showed enhanced cytotoxic T-cell priming for the AH1 peptide in the TEGVAX-treated group in comparison with the control groups (Fig. 6B). The "average killing" value calculated between TEGVAX and GVAX only reached statistical significance at $P < 0.07$, but each of the experiments showed consistent trend for enhanced number of AH1-specific T cells in the TEGVAX-treated groups. In vivo CTL assays with
p15E-specific T cell from the B16 model also showed enhanced p15E-specific T cells in the TEGVAX group (data not shown).

**Discussion**

In this article, we tested the efficacy of intratumoral injection of TEGVAX, a novel TLR4 agonist–formulated GVAX, whereby we were able to absorb TLR4 agonist into GVAX cells. We showed that TLR4 agonist–absorbed GVAX has significantly improved antitumor response in 3 different therapeutic murine models, including SCCFVII/SF, B16, and CT26. For the CT26 model, TEGVAX injected intratumorally was able to prevent tumor growth in 40% to 60% of the mice, whereas GVAX or LPS injection alone had no such effect.

By adding a potent TLR4 agonist into the tumor microenvironment via a cellular vector with our TEGVAX reagent, we showed that the growth rate of the inoculated tumor is significantly blunted. We believe that it is not only the addition of TLR4 agonist, but also its novel delivery absorbed into lethally irradiated vaccine cells with tumor antigens as vectors. Equimolar LPS injection alone did not have an *in vivo* response. Injection of equimolar LPS directly into the tumor separately from GVAX and control experiments by using LPS absorbed into 293T cells also did not produce the antitumor response in our mice models (Fig. 2 and Supplementary Fig. S1). The effect of TLR4 agonist in the tumor microenvironment is a complex process that has produced conflicting *in vivo* responses. Recent reports showed that TLR4 expressed on HNSCC cell lines could promote carcinogenesis (3). However, expression of TLR4 in HNSCC primary tumor is heterogeneous and it is unclear in whether TLR4 signaling is a critical carcinogenic signaling *in vivo*. Others have shown that TLR4 stimulation can break CD8+ T-cell tolerance and eradicate established tumors (17, 20). MyD88 signaling, which is downstream to TLR4, has been shown to be procarcinogenic in some animal models (5). However, our results are consistent with a recently presented hypothesis developed by Medzhitov and others that TLR4 agonists
The lack of antitumor response in GVAX treatment group may stem from possible upregulation of myeloid derived suppressor cells that has been associated with GM-CSF signaling in the tumor microenvironment (21). Regardless, TEGVAX-treated groups had greater number of activated DCs in comparison with the GVAX or the PBS-treated groups to potentially induce tumor-specific CTL response in our experimental models.

Activated APCs can prime tumor-specific T cells as downstream effectors, so we examined the tumor tissue from each of the treated and the control groups for CD4 and CD8 T-cell infiltration by using immunohistochemistry. Quantitatively, TEGVAX-treated tumors showed greater infiltration of both CD4 and CD8 T cells in comparison with GVAX- or PBS-treated tumors. Whether the increase in CD4+ cells is also associated with any changes in Treg or Th17 cells are still being investigated. Regardless of this, there appeared to be a positive correlation between T-cell infiltration and the overall tumor response in vivo. We stained for natural killer cells in the tumor tissue but did not see any qualitative differences (data not shown).

We quantitated the tumor-specific, Ld-restricted, AH1-specific, IFN-γ–secreting CTLs from each of the control and treated groups in the CT26 model, and both ELISPOT and in vivo CTL assay showed that TEGVAX-treated mice had increased the number of AH1-specific CTLs. These results strongly suggest that one downstream consequence of TEGVAX treatment is the upregulation of antitumor cytotoxic IFN-γ–producing T cells in both the DLNs and the spleen that can slow the rate of the tumor growth.

Consistent with this mechanistic picture is an experiment, whereby TEGVAX injected on the contralateral limb from the tumor growth site had comparable in vivo antitumor response as intratumoral injection as shown in Supplementary Fig. S2. Both intratumoral and systemic treatments were lymphocyte dependent as shown by the abrogation of these effects in Rag2-/- mice. Although intratumoral injection can elicit both innate and adaptive immune response, injection of TEGVAX on the contralateral limb predominantly increases the priming of naive T cells into vaccine-specific T cells.

In terms of the architecture of the tumor microenvironment, TEGVAX-treated tumor had broad infiltration of CD86+ cells. Although both PBS and GVAX-treated tumor had localized aggregates of lymphocytes and APCs, TEGVAX-treated tumors had diffuse infiltration of CD4+, CD8+, and CD86+ cells (see arrow in Fig. 4). These ectopic lymphoid aggregates have been described in pancreatic cancer specimens from patients treated with GVAX (ref. 22; Jaffee and Zhang, personal communication). The significance of these aggregates is unclear, but there seems to be a negative correlation between these aggregates and the tumor response in vivo.

Given that one limitation of GVAX is limited number of activated APCs, development of TEGVAX is a step toward an efficacious combinatorial immunotherapeutic reagent for advanced cancer patients. GVAX has been found to be safe in multiple phase I trials, and preclinical in vivo efficacy absorbed into micelles can induce an upregulation of antigen presenting machinery in the phagolysosome by the APCs (9).

With TEGVAX, our strategy was to dress syngeneic cells as "foreign" cells with strong danger signals that can activate APCs in the tumor microenvironment. LPS formulated into cellular vaccines may be preferentially targeted toward the locoregional APCs such as DCs. We hypothesized that apoptotic cellular debris with TLR4 agonist from the injected TEGVAX cells would be phagocytosed by the local APCs such that TLR4 signaling on the tumor cells would be minimized. Hence, this would explain the consistent lack of in vivo antitumor response in the mouse groups treated with LPS alone or with LPS and GVAX without the absorption formulation. When we examined the draining LN, TEGVAX treatment was associated with an increased number of activated DCs as defined by high expression of CD86+, CD80+, and MHC class II+ cells in comparison with GVAX-treated groups as shown in Figure 5. GVAX-treated groups also had increased number of activated DCs but less than TEGVAX-treated DCs, which is consistent with the immunohistochemical data from Figure 4. GM-CSF from GVAX can also activate DC cells that explain the increased number of activated DC in GVAX group in comparison with the control group treated with PBS alone.

Figure 5. TEGVAX treatment increases activated DCs in the DLNs. A total of 10 to 20 CT26 tumor–bearing BALB/c mice were treated with PBS (not shown), GVAX, or TEGVAX peritumorally 3 days after the tumor injection. DCs were isolated from the DLN 5 to 8 days after treatment by enzymatic digestion. B220–CD11c– conventional DCs were gated and CD86, MHCII, and CD80 staining were analyzed as shown. TEGVAX had an increased number of CD86+MHCII+ and CD80+MHCII+ DCs in the DLN in comparison with GVAX-treated group. These experiments were replicated more than 3 times. The results from these other experiments are depicted in Supplementary Fig. S4.
of TEGVAX, as shown in this article, warrants further investigation. Of greatest concern is the use of TLR4 agonist therapy in cancer patients. Goto and colleagues had previously injected 10 μg of LPS into cancer patients with tolerable side effects that were not greater than WHO grade 3 (23). Extrapolating from previous trials with GVAX in cancer patients in which 10^8–9 GVAX cells were used, this fact that the multiple clinical cancer vaccines have been typically mixed with TLR4 agonists. The MAGE-A3 ASCI lung cancer vaccine for NSC lung cancer and Cervarix for cervical cancer contains monophosphoryl lipid A, a TLR4 agonist (7). Bacillus Calmette Guerin, another TLR4 agonist, was found to be important for low-volume melanoma disease (24). Our article, however, points out the importance of TLR4 agonist formulation in terms of in vivo efficacy. Simple mixing of cellular vaccine and adjuvants may be a suboptimal method to integrate the power of danger signals into the combinatorial vaccine. Future studies will also compare different TLR4 agonists that have been tested in patients with comparable formulations.

In conclusion, we report a novel formulation of GVAX tumor vaccines with improved antitumor efficacy with intratumoral injection in several murine models. Intratumoral injection of vaccine is potentially feasible in select cases of accessible tumors such as head and neck and skin malignancies. Moreover, intratumoral injection has important translational implications for neoadjuvant therapy prior to surgery in patients with a high risk of relapse. Intratumoral injection prior to resection could generate systemic antitumor immune responses that could eliminate the micrometastasis undetectable at the time of surgery, which are the sources of tumor relapse.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Intratumoral Administration of TLR4 Agonist Absorbed into a Cellular Vector Improves Antitumor Responses

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