Systemic Immunotherapy of Non-Muscle Invasive Mouse Bladder Cancer with Avelumab, an Anti-PD-L1 Immune Checkpoint Inhibitor

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Abstract

Bacillus Calmette-Guerin (BCG) is the standard of care for intravesical therapy for carcinoma in situ and non-muscle invasive, nonmetastatic human urothelial carcinoma. Although the responsiveness to this immunotherapeutic is believed to be linked with (i) a high number of somatic mutations and (ii) a large number of tumor-infiltrating lymphocytes, recent findings of the roles that inhibitory immune receptors and their ligands play in tumor evasion may provide insights into the limitations of the effectiveness of BCG and offer new targets for immune-based therapy. In this study, an aggressive, bioluminescent orthotopic bladder cancer model, MB49 tumor cells transfected with luciferase (MB49luc), was used to study the antitumor effects of avelumab, an antibody to PD-L1. MB49luc murine tumor cells form multifocal tumors on the mucosal wall of the bladder reminiscent of non-muscle invasive, nonmetastatic urothelial carcinomas. MB49luc bladder tumors are highly positive for the expression of PD-L1, and avelumab administration induced significant (P < 0.05) antitumor effects. These antitumor effects were more dependent on the presence of CD4 than CD8 T cells, as determined by in vivo immune cell depletions. The findings suggest that in this bladder tumor model, interruption of the immune-suppressive PD-1/PD-L1 complex releases a local adaptive immune response that, in turn, reduces tumor growth. This bladder tumor model can be used to further identify host antitumor immune mechanisms and evaluate combinations of immune-based therapies for carcinoma in situ and non-muscle invasive, nonmetastatic urothelial carcinoma, to provide the rationale for subsequent clinical studies.

Introduction

Emerging experimental evidence and clinical evidence have coalesced around the interrelationships between mutational heterogeneity of different forms of cancer and the roles that immune cell checkpoint blockade might play in cancer immunotherapy (1, 2). Coincident with a better understanding of the roles of inhibitory immune receptors and their ligands, collectively termed checkpoint inhibitors, has been an appreciation that many human and mouse tumors are not blind to host immunity, but either express weak neo–tumor associated antigens or overexpress self-antigens or fetal antigens that can be recognized by T cells (2–5). Although investigators realized that a large subset of patients had evidence of immune cell infiltrates within their tumor microenvironment, it was data highlighting CTLA-4 blockage with ipilimumab that provided the crucial connection between an interruption of an immune cell checkpoint pathway and significant antitumor responses (6, 7). Studies have also focused on other checkpoint inhibitors, particularly the programmed death-1 (PD-1) receptor that interacts with distinct ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273; ref.8). PD-L1 is expressed on a wide variety of human and mouse tumor cells and some immune cell populations, whereas PD-L2 is mainly found on antigen-presenting cells (9, 10). PD-L1 expression on tumors and tumor-infiltrating lymphocytes expressing IFNγ colocalize in many tumors. With the accompanying T-cell–associated IFNγ production, PD-L1 expression is increased on the tumor cell surface (11). Enhanced PD-L1 expression on tumor cells dampens tumor immunity by inducing antigen-specific T-cell apoptosis or anergy (12). Interruption, or blockade, of the PD-1/PD-L1 interaction by the administration of an antibody to PD-1 or PD-L1 can overcome immune resistance in preclinical models and has been correlated with positive clinical outcomes in patients diagnosed with metastatic bladder cancer (13–15). One such agent is avelumab, which is a fully human IgG1 anti-PD-L1 antibody currently being studied clinically in multiple tumor types.

For the last four decades, the standard of care for carcinoma in situ and non-muscle invasive, nonmetastatic urothelial carcinoma has been immune-based: the intravesical instillation of attenuated Bacillus Calmette–Guerin (BCG; refs.16, 17). The mechanism of BCG action remains elusive, yet most investigators believe that the influx of immune cells is a crucial component (18). Approximately 30% to 45% of patients fail to respond initially to BCG or relapse within 5 years of treatment (19). Thus,
with the local production of IFNγ by invading immune cells, the question arises as to whether the PD-1/PD-L1 axis might contribute to unresponsiveness or relapse following BCG therapy. Increasing PD-L1 expression predicts localized bladder cancer stage progression independent of tumor grade, and PD-L1 levels are highest in carcinoma in situ and within granulomata of bladder tissues of patients who failed BCG therapy (19–21). Therefore, the presence of PD-L1 could conceivably play a role in abrogating host immune–related responses and result in bladder cancer progression, which infers a biologic role for the PD-1/PD-L1 interaction as a new immunotherapeutic target.

MB49 is a murine transitional cell bladder carcinoma line that forms tumors when injected subcutaneously or orthotopically into mouse bladders. The murine orthotopic bladder tumor model provides an opportunity to study the immune-related events involved in the use of immune cell checkpoint inhibitors for the treatment of carcinoma in situ and non-muscle invasive, nonmetastatic urothelial carcinoma and to establish scientific rationale for combining immune cell checkpoint inhibitors with other potential forms of therapy. Findings from the present study clearly show that the successful targeting of PD-L1 on MB49 bladder tumors with a PD-L1 antibody, avelumab, results in significant antitumor effects that are associated with the expansion/generation of an adaptive immune response.

Materials and Methods

Animals and cell lines

Female C57BL/6 mice were purchased from The Jackson Laboratory or Charles River Laboratories. F5 mice that are transgenic (Tg) for nucleoprotein of influenza virus A/NT/60/68 (H3N2-A56NMDAMC574;NP68)-specific, H-2Dq-restricted T-cell receptor were obtained from Taconic Farms. All mice were housed in microisolator cages in pathogen-free conditions. Mice used for the in vivo antitumor studies were 16 to 18 weeks old at the start of study. Animal care was in compliance with the Guide for Care and Use of Laboratory Animals (National Research Council).

The MB49 parental cell line (murine transitional cell carcinoma) was kindly provided by Dr. Peter Pinto (Urologic Oncology Branch, CCR, NCI, NIH). Cells were grown, batch frozen, and used in the experiments described. The MB49 LucSH+ cells (MB49 tec) were generated within our laboratory. All MB49 lines were grown in DMEM with 10% heat-inactivated FBS supplemented with 20 ng/mL of HuIgG1 or avelumab in 1 mL/well. After 5 days of in vitro T-cell activation supernatants were collected and stored at −20°C, and IFNγ concentrations were later determined using a standard ELISA kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Sample optical densities (ODs) at 450 nm were measured using a Synergy HT plate reader (Bio-Tek).

Murine tumor models

Subcutaneous tumor injections were carried out by inoculating C57BL/6 mice with 1 × 10⁵ MB49 parental cells on the right shaved flank. Tumor growth was measured with calipers, and 8 days after inoculation, mice were assigned to treatment groups. Treatments began on day 9, tumors were measured 2x/week throughout the study, and tumor volume was calculated as: Volume = (width)² × (length)/2. Mice were euthanized when the tumor size was greater than 1.5 cm³.

Intravesical instillation of orthotopic MB49 tec bladder tumors was carried out as previously described (22). Mice were anesthetized [ketamine (15 mg/kg) and xylazine (75 mg/kg)] and catheterized using Teflon catheters (SurFlash Polyurethane I.V. catheter 24G x 3/4, SR FF2419; Terumo Medical products). Orthotopic bladder tumors were established by instilling 7.5 × 10⁵ MB49 tec cells for 45 minutes into bladders that had been pretreated with 100 µL of poly-L-lysine (0.1 µg/mL) solution [PLL, molecular weight (MW) 70,000 to 150,000; Sigma-Aldrich]. Tumor take was confirmed by in vivo imaging 7 to 10 days later, at which time mice were placed into groups with equal tumor burden prior to treatment.

Immune cell subset depletion

Immune cell subset depletions were carried out by administering anti-CD4 (100 µg GK1.5) or anti-CD8 (100 µg 2.43) T-cell-depletion antibodies each day for 4 consecutive days and thereafter with weekly injections. Natural killer (NK)-cell depletion was achieved by weekly administration of 25 µL rabbit anti-asialoGM1 (Cedarlane Laboratories). The administration of the depleting antibodies was begun 1 week prior to the intravesical tumor cell instillation. Depletions were confirmed with flow cytometric analyses for CD4 and CD8 cells and NK lysis by YAC-1 targets using splenocytes from representative mice (Supplementary Fig. S1).

Treatments

Avelumab, a fully human anti–PD-L1 (IgG1), and an isotype-control antibody, were kindly provided by EMD Serono. Avelumab binds to both human and mouse PD-L1 with high affinity, Kd = 0.3 and 1.0 nmol/L, respectively. Both were diluted in Dulbecco’s phosphate buffered saline (DPBS) prior to injection. Tumor-bearing mice were treated with 400 µg per 100 µL and injected i.p. three times, 3 days apart. Because avelumab is a human IgG1, three injections had to be compressed within a 7- to 9-day window (i.e., days 9, 12, and 15 after tumor inoculation) to avoid the onset of neutralizing mouse anti-human Ig (data not shown).

BCG (1.0 mg; TICE, Organon USA, Inc.) was administered in a volume of 100 µL instilled by catheterization for 1 hour, three times at weekly intervals starting on day 8 after tumor instillation.
Kidneys, etc.) were removed for gross examination. Bladder tissue was embedded in optimal cutting temperature (OCT) compound and processed by routine histologic methods for hematoxylin and eosin (H&E). It has been reported that instillation of a higher number of MB49 tumor cells (i.e., $10^6$) into mouse bladders previously traumatized with TNP values were compared on an individual treatment group basis and analyzed with respect to region (bladder/tumor interface, tumor) and size (cells/micron squared; i.e., TNP cells/μm$^2$). The bladder/tumor interface was defined as 50 μm in either direction from the bladder/tumor border. Higher magnification images were taken on a Leica DMi4000 microscope with LAS AF (Leica Microsystems Inc.).

**Flow cytometry assays**

MB49 cells were seeded in T75 flasks in complete DMEM-supplemented medium and treated with 10 ng/mL IFNγ 24 hours later. After 48 hours, cells were harvested and stained for the expression of PD-L1 using avelumab or isotype-control antibodies (0.2 pg/10^6 cells) with a PE-conjugated, goat anti-human IgG secondary antibody (0.5 μg/10^6 cells; Jackson ImmunoResearch). Data were acquired with a BD FACS Calibur flow cytometer connected to a Macintosh computer running BD Cellquest software. Data analysis was conducted using FlowJo software (FLOWJO, LLC).

**Statistical analysis**

GraphPad Prism software (GraphPad Prism 6.01 for Windows, GraphPad Software, Inc.) was used to perform all statistical analyses. Details of appropriate statistical analyses are found within each figure legend. Differences were significant when the P value was less than 0.05.

**Results**

Interaction between avelumab and mouse PD-L1

Initial studies determined the extent to which avelumab, a human IgG1, interacts with mouse PD-L1. Consistent with the binding affinity studies (see Materials and Methods), avelumab, not the human isotype-control antibody, bound to PD-L1 on the surface of the MB49 cells, a murine transitional cell carcinoma of the bladder. Constitutive PD-L1 expression on the surface of the MB49 tumors was high (approximately 96% positive, mean fluorescence intensity (MFI) = 9.5) and was easily upregulated (approximately 99% positive, MFI = 59.5) with the in vitro addition of 10 units IFNγ/mL (Supplementary Fig. S2A). PD-L1 blockade can increase T-cell activation (21). Indeed, the addition of avelumab to an in vitro bioassay of BMDCs and peptide-pulsed T cells from F5 TCR Tg mice increased IFNγ production, indicating the ability of avelumab to not only recognize PD-L1, but also to interrupt its signaling (Supplementary Fig. S2B).

Antitumor effects of avelumab

Syngeneic C57BL/6 mice bearing subcutaneous MB49 tumors (40–60 mm$^3$) were treated with avelumab, isotype-control antibody, or diluent on days 9, 12, and 15 after tumor inoculation (Fig. 1A–C). Measurement of individual tumors clearly showed a slowing of tumor growth in the avelumab-treated mice (Fig. 1C). By day 36 after tumor implantation, there was a significant ($P < 0.01$) reduction in the average tumor volume of the avelumab-treated mice (Fig. 1D). Reduction in MB49 tumor growth in the mice treated with avelumab was durable and led to a statistically significant ($P < 0.05$) improvement in percent survival (Fig. 1E). Splenocytes isolated from the control and avelumab-treated mice were analyzed for the presence of T cells recognizing two possible tumor-associated antigens—the transmembrane...
Avelumab treatment significantly \( P < 0.05 \) inhibited the tumor-bearing mice. Indeed, on day 8 following MB49\(^{\text{Luc}}\) instillation, tumor burden, based on imaging and radiance, was confirmed, and mice were divided into two treatment groups: i.p. administration of avelumab or the isotype-control antibody was administered. Antibody treatments were carried out on days 9, 12, and 15 after tumor instillation. When the mice were imaged on day 21, a clear reduction in the bioluminescence signal (i.e., radiance) was present in the bladders of mice treated with avelumab (Fig. 2B). On day 29 after tumor instillation, mice were euthanized and individual tumor burdens were determined by bladder weights of the two treatment groups and compared with that from naive, non–tumor-bearing mice (average bladder weight: 22.0 ± 1.7 mg; Fig. 2C). Mice treated with the isotype-control antibody had considerable tumor burden in that the average bladder weight was more than 7-fold that of the naive, non–tumor-bearing mice (161.3 ± 56.1 vs. 22.0 ± 1.7 mg). Avelumab treatment significantly \( P < 0.05 \) inhibited the
growth of MB49\textsuperscript{Luc} bladder tumors: the average bladder weight in that treatment group, 34.1 ± 4.5 mg, was not different than the bladder weights from naïve, untreated mice (Fig. 2C). In a separate study, the reduction of MB49\textsuperscript{Luc} bladder tumor burden with avelumab administration also significantly (P < 0.01) improved percent survival when compared with isotype-control–treated mice (Fig. 2D).

Because the current standard of care for carcinoma in situ and non-muscle invasive nonmetastatic urothelial carcinoma is the intravesical instillation of BCG, it was important to compare systemic avelumab versus BCG therapy in mice bearing orthotopic MB49\textsuperscript{Luc} bladder tumors. Once again, tumor burden was assessed by changes in the amount of bioluminescence and overall increase in individual bladder weights. By day 21 after tumor instillation, there was a decrease in intravesical bioluminescence in the groups of mice treated with BCG alone, avelumab alone, or avelumab combined with BCG (Fig. 3A). On day 29, bladders taken from control-treated [i.e., isotype-control, i.p./saline intravesical (i.ves.)] mice instilled with the MB49\textsuperscript{Luc} tumor cells had developed tumor burden in that their overall bladder weights were approximately 10-fold that of naïve, untreated B6 mice (225.6 ± 79.1 vs. 22.0 ± 1.7 mg; Fig. 3B). Bladder instillation of BCG (1.0 mg) on days 7, 14, and 22 after tumor instillation reduced tumor growth (average bladder weight, 61.6 ± 13.6 mg), which was somewhat surprising because in previous studies, intravesical BCG instillation had little to no effect on bladder tumor burden (22). Such variability could be a consequence of leakage/voiding following intravesical BCG dosing and treatment duration as well as lot-to-lot differences in BCG bioactivity. In contrast, the antitumor effects of systemically administered avelumab were highly reproducible with an average bladder weight just 25% higher than that of naïve mice (27.6 ± 2.4 vs. 22.0 ± 1.7 mg; NS; Fig. 3B). The combination of systemic avelumab plus intravesical BCG instillation produced no
additional reduction in tumor burden over that of avelumab alone (average bladder weight 30.6 ± 1.8; Fig. 3B). Avelumab therapy was also better tolerated as no overt toxicity was observed, whereas mice receiving BCG either alone or in combination often exhibited hunched habitus or ruffled fur, changes that could be regarded as transient grade 1/2 toxicities.

Immune components of avelumab therapy

Bladders from mice of the four treatment groups shown in Fig. 3 were removed, OCT-embedded, and analyzed for the presence of CD4 and CD8 immune cell infiltrates at the bladder/tumor interface and within the tumor mass based on immunohistochemical staining (Fig. 4; Supplementary Fig. S4 for higher magnification). For the control mice treated with the isotype-control antibody (i.p.) and saline (i.ves.) 29 days after tumor installation, the normal bladder architecture was often severely disrupted, which made identification of the bladder/tumor interface difficult. Nevertheless, in those evaluable isotype-control–treated mice, there was a discernible gradient of CD4 T cells between the bladder and tumor with appreciable accumulation at the bladder/tumor interface (Fig. 4A). No such gradient was found in mice treated with avelumab (Fig. 4B and C). Although intravesical BCG treatment did reduce tumor burden, all mice had enlarged bladders at the study endpoint, suggesting a weak ongoing antitumor response. Indeed, relatively low numbers of CD4 and CD8 T cells were found either at the bladder/tumor interface or within the tumor mass (Fig. 4D–F). Avelumab treatment of 10 mice with bladder tumors resulted in complete tumor regression in 8 mice, confirming by histopathology. In the remaining 2 mice, small tumors remained, possibly indicating an active antitumor response with increased numbers of CD4 and CD8 T-cell infiltrates located both at the bladder/tumor interface and within the tumor mass (Fig. 4G–I). In mice treated with a combination of avelumab and intravesical BCG, increased numbers of CD4 and CD8 T cells were found within the tumor mass (Fig. 4J–L). It should be noted that these analyses were done at the study end, and results were significantly affected by different tumor burdens among the treatment groups. In future studies, the immune cell changes within the bladder, bladder/tumor interface, and the tumor mass will be determined during a time interval encompassing an active, ongoing antitumor immune response. Staining for PD-L1 expression revealed the presence of high PD-L1 expression levels in the bladder tumor masses whether or not they were being treated with avelumab (Fig. 5A–D). Interestingly, PD-L1 expression was lower in the bladders and at the bladder/tumor interface in those mice in which avelumab treatment successfully reduced tumor growth (Fig. 5C).

In a separate experiment, immune cell subsets were depleted using specific antibodies for CD4, CD8, or NK cells prior to avelumab administration to mice bearing orthotopic bladder tumors. As shown in Fig. 6A, avelumab was highly effective in reducing the growth of bladder tumors in immune intact mice. However, in mice depleted of either CD4 or CD8 cells, avelumab treatment was much less effective in controlling bladder tumor burden with tumor breakthrough occurring in a higher frequency in mice depleted of CD4 T cells. One can surmise that CD4 depletion would impede CD8 priming due to the lack of T-helper (Th1) cytokines. But the fact that some antitumor efficacy of avelumab was maintained in those mice depleted of CD8 T cells suggests a role for CD4 T cells in tumor growth control. These data were consistent with the immunohistochemical staining showing intratumoral localization of CD4 and CD8 T cells in mice treated with avelumab (Fig. 4G and H). Avelumab mediates antibody-dependent cell-mediated cytotoxicity in humans, but not in mice (P. Kim, Laboratory of Tumor Immunology and...
Biology, Center for Cancer Research, NCI; personal communication). Consistent with that finding was that NK-cell depletion had little or no adverse effects on the ability of avelumab to induce a significant reduction in the growth of orthotopic bladder tumors. It was of interest to determine whether successful treatment of bladder tumors by avelumab also induced protective, long-term immune memory. In a separate study, 17 of 38 mice instilled with MB49<sup>Luc</sup> tumor cells and subsequently administered avelumab had a complete resolution of their bladder tumors (based on imaging). Those 17 mice remained tumor free for 36 days after the initial tumor instillation, at which time 10 mice received an intravesical rechallenge with 7.5 × 10<sup>4</sup> MB49 tumor cells. At the same time, a group of naïve mice received MB49 tumor cells via bladder instillation, and all developed large bladder tumors by day 29 (Fig. 6B). However, the mice that were rendered tumor free after avelumab treatment were protected against the tumor growth.

Figure 4.
Immunohistochemical detection of CD4 and CD8 immune cell subsets in the bladders of mice treated with avelumab, BCG, or avelumab plus BCG. C57BL/6 mice (n = 10) bearing orthotopic MB49<sup>Luc</sup> tumors were treated with DPBS/saline, DPBS/BCG (1.0 mg), avelumab (3 × 400 μg)/saline, or avelumab/BCG, as described in Fig. 3. Mice were euthanized on day 29, and individual bladders from DPBS/saline (A, B), DPBS/BCG (D, E), avelumab/saline (G, H), and avelumab/BCG (J, K) were stained for CD4 (A, D, G, J) and CD8 (B, E, H, K) T cells as described in Materials and Methods. Bar = 600 μm. Plots C, F, I, and L represent the number of CD4 and CD8 T cells enumerated at the bladder/tumor interface and within the tumor mass as described in Materials and Methods. Data are from a representative experiment that was repeated with similar results. T, tumor.
rechallenge (Fig. 6B), suggesting that avelumab treatment generated a strong protective immunologic memory response against the MB49 tumors.

Discussion

Bladder cancer, the fifth most common cancer in the United States, has a high prevalence, along with melanoma and lung cancer, for somatic mutations, presumably due to DNA damage as a result of long-term exposure to extrinsic mutagens, such as smoking and carcinogenic chemicals (25, 26). Coincident with those mutations, investigators have found a large number of tumor-infiltrating lymphocytes in bladder cancer (27–29). Indeed, immune-directed therapy utilizing intravesical BCG instillation has been the standard of care for intravesical therapy for carcinoma in situ and non-muscle invasive, nonmetastatic urothelial carcinoma for the past four decades (16–19). Repeated BCG instillations have led to early accumulation of granulocytes followed by macrophages and lymphocytes, predominantly CD4 T cells, in the patients’ bladders (30, 31). Investigators have shown that BCG treatment induces a strong Th1 cytokine response, yet the ability of BCG to mediate antitumor effects has been largely attributed to NK-cell activation (32, 33). In the present experimental murine model, some of the characteristics found in patients diagnosed with carcinoma in situ and non-muscle invasive, nonmetastatic urothelial carcinoma were recapitulated: (i) MB49 luc tumor cell instillation resulted in a large number of infiltrating lymphocytes in the bladder, at the bladder/tumor interface, and within the tumor mass. (ii) Mice treated with BCG had more CD4 and CD8 T cells at the bladder/tumor interface and intratumorally, yet the potency of BCG to affect tumor reduction was minimal. Those findings present multiple possibilities to explain the limitations of BCG immunotherapy: (i) The immune cell infiltrate is broad-based, the tumor-associated antigen-specific component is a minor component. (ii) There is an antigen-specific antitumor T-cell response, but it is dampened by PD-1/PD-L1 upregulation in response to release of high concentrations of Th1 cytokines (i.e., IFNγ) by NK cells in the tumor microenvironment. The latter would explain the association between PD-1/PD-L1 overexpression with tumor grade and postoperative prognosis in human bladder cancers (28). It would also explain why antibody blockade of the PD-1/PD-L1 immunosuppressive pathway would benefit patients diagnosed with metastatic bladder cancer by releasing resident tumor-specific host immune cells, thus controlling tumor growth (34). Findings in the present study support the argument that this same approach, PD-1/PD-L1 disengagement, might benefit patients diagnosed with carcinoma in situ and non-muscle invasive, nonmetastatic urothelial carcinoma who either failed or relapsed following frontline BCG therapy.

Murine MB49 bladder tumor cells are constitutively highly positive for PD-L1, which was further upregulated following IFNγ treatment in vitro. High PD-L1 expression was maintained in vivo following the orthotopic instillation of MB49 cells that formed multifocal non-muscle invasive tumors on the mucosal bladder epithelium. Systemic administration of avelumab, an antibody to PD-L1, significantly (P < 0.05) reduced tumor burden, resulting in a statistically significant (P < 0.01), durable improvement in long-term survival. Mechanistic studies clearly showed the need for an intact immune system, with depletion of either CD4 or CD8 T
cells abrogating the antitumor effects of avelumab treatment. Depletion of NK cells had no adverse effect on the antitumor properties of avelumab. Moreover, avelumab therapy also induced strong T-cell memory responses that protected mice from subsequent tumor challenge.

A plausible working hypothesis is that intravesical or s.c. inoculation of MB49 tumor cells is accompanied by sufficient tumor cell disruption, resulting in the release of tumor-associated antigens inducing a host immune response. Two possible tumor-associated antigens are the envelope protein p15E antigen associated with the active component of endogenous retroviruses and/ or immune recognition of the HY antigens presented by the MB49 tumor cells implanted into female hosts. Release of either/bOTH of those tumor-associated antigens would provide appropriate signals that support the influx of immune cells seen in the bladder, at the bladder/tumor interface, and within the tumor mass of untreated mice. Although a low, but measurable splenic CTL activity directed against the p15E epitope was found in untreated mice, it had very little effect in impeding tumor growth. In either MB49 model, the presence of antitumor T cells in the tumor microenvironment of untreated and isotype Ig control–treated mice provided some short-term control on tumor growth. By day 20 [s.c. model], immunosuppressive actions in the tumor microenvironment overrode any immune control, resulting in exponential tumor growth. The PD-1/PD-L1 axis seems to be a potent component and contributor to local immunosuppression, as blockade of that axis by avelumab suppressed both subcutaneous and orthotopic MB49 tumor growth, leading to increased overall survival. These observations argue that the disruption of the PD-1/PD-L1 immunosuppressive pathway is followed by expansion of antigen-specific T cells and control over tumor growth. Support for that hypothesis comes from the increase in p15E-specific cytolysis in avelumab-treated mice, the loss of avelumab antitumor potency commensurate with either CD4 or CD8 T-cell depletions, and the generation of immunologic memory that protects tumor-free mice from subsequent tumor challenge.

The potent antitumor effects reported in the present study coupled with those from early clinical trials in patients diagnosed with metastatic bladder cancer have generated intense interest in developing a new paradigm in the immune-based treatment of bladder cancer (13–15). Although the PD-1/PD-L1 axis seems to be a crucial target for the treatment of metastatic bladder cancer,
others have suggested that a focus on the immune-related events that occur prior to the spontaneous initiation of a tumor-specific response would be worthwhile in understanding and designing novel immune adjuvants to expand the immunotherapeutic approach (35). There still remains an intriguing dichotomy with respect to PD-L1 tumor expression. Although its expression is related to tumor escape via immune suppression, the targeting of those tumors with the highest expression of PD-L1 levels seems to correlate with better antitumor responses. Perhaps the most direct explanation is that higher PD-L1 expression is related to more immune cell accumulation in the tumor microenvironment. These phenomena might be tied to the innate immune system and the events that trigger spontaneous generation of resident T cells. Because the innate immune system is closely tied to proinflammatory events, it would be interesting to examine the relative amount of local tissue inflammatory responses when tumor cells are instilled into the bladder. It is conceivable that a more robust inflammatory response generated at the mucosal surface of the bladder may facilitate T-cell priming, leading to a more potent antitumor response to subsequent anti–PD-L1 therapy. Indeed, as shown in Supplementary Fig. S3B, simple intravesical instillation of the MB49 cells seems to upregulate PD-L1 expression levels within the different mucosal and submucosal layers of the bladder.

The orthotopic intravesical murine bladder tumor model has several characteristics, such as non-muscle invasive bladder tumor growth and responsiveness to anti–PD-L1 immune-based therapies, which argue for it to be suitable as an experimental model to study immune-related factors involved in non-muscle invasive, nonmetastatic bladder cancer. Use of the model might provide important insights into the cellular mechanisms involved in the nonresponders to immune cell checkpoint immunotherapy, identification of effective immune-based combinations that would be expected to provide a more robust antitumor response than with immune cell checkpoint inhibitor monotherapy, and the PD-L1 function within a tumor microenvironment devoid of T cells. Such preclinical studies may provide the rationale for localized bladder cancer clinical studies that combine immune cell checkpoint inhibitors with other forms of immunotherapy, chemotherapy, radiotherapy, and small-molecule inhibitors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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