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

Amanda J. Vandever^{1,§}, Jonathan K. Fallon^{1,§}, Robert Tighe², Helen Sabzevari², Jeffrey Schlom^{1,¶}, and John W. Greiner^{1,¶}

¹Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland USA

²EMD Serono Research and Development Institute, Billerica, Massachusetts USA

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. While 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 (MB49^{luc}), was used to study the antitumor effects of avelumab, an antibody to PD-L1. MB49^{luc} murine tumor cells form multifocal tumors on the mucosal wall of the bladder reminiscent of non–muscle invasive, nonmetastatic urothelial carcinomas. MB49^{luc} 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.

Keywords

non–muscle invasive bladder cancer; immunotherapy; avelumab; anti-PD-L1

Corresponding author: Jeffrey Schlom, PhD, Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health; 10 Center Drive, Room 8B09, MSC 1750, Bethesda, MD 20892; ; Email: js141c@nih.gov. Phone: 301-496-4343; Fax: 301-496-2756

[§]These authors contributed equally to this work.

[¶]These authors contributed equally to this work.

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Introduction

Emerging experimental 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 blockade 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) (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 IFN- γ co-localize in many tumors. With the accompanying T cell-associated IFN- γ 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) (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 30–45% of patients fail to respond initially to BCG or relapse within 5 years of treatment (19). Thus, with the local production of IFN- γ 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 biological 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 (³⁶⁶ASNENMDAM³⁷⁴;NP68)-specific, H-2D^b-restricted T-cell receptor were obtained from Taconic Farms (Hudson, NY). 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^{luc}) were generated within our laboratory. All MB49 lines were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated fetal bovine serum supplemented with 1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, and penicillin/streptomycin (100 U/mL). MB49^{luc} growth medium also contained Zeocin (200 µg/ml). MB49^{luc} are parental MB49 cells transfected with a pSELECT-zeo-LucSh plasmid using Lipofectamine (InvivoGen, San Diego, CA) for luciferase expression detected by *in vivo* imaging.

In vitro F5 TCR.Tg T cell activation

Bone marrow-derived dendritic cells (BMDCs) were generated from adult female C57BL/6 mice following *in vitro* growth for 6 days in complete RPMI medium supplemented with 20ng/ml murine recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10ng/ml murine recombinant IL-4. Medium and non-adherent cells were discarded on days 2 and 4 and replaced with fresh medium containing GM-CSF/IL-4. On day 6, the non-adherent cells were collected, washed, and used for *in vitro* T cell activation studies. PD-L1 expression on these BMDCs was determined by cell surface staining with avelumab (data not shown).

BMDCs (50,000/well) were pulsed overnight with 10–1,000ng/ml NP68 peptide (ASNENMDAM, H-2D^b) or control HY peptide (WMHHNMDLI, H-2D^b) in 24-well plates. After 24 hours, splenic CD8α⁺ cells were purified from F5 TCR.Tg mice using negative selection magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Isolated F5 CD8α⁺ cells were added to the 24-well plates at 10,000 cells/well along with 10 µg/ml of HuIgG1 or avelumab in 1ml/well. After 5 days of *in vitro* T cell activation, supernatants were collected and stored at –20°C and IFN-γ

concentrations were later determined using a standard ELISA kit (Thermo Fisher Scientific, Grand Island, NY) according to the manufacturer's instructions. Sample optical densities (ODs) at 450nm were measured using a Synergy HT plate reader (Bio-Tek, Winooski, VT).

Murine tumor models

Subcutaneous tumor injections were carried out by inoculating C57BL/6 mice with 1×10^5 MB49 parental cells on the right shaved flank. Tumor growth was measured with calipers and 8 days post-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: $\text{Volume} = (\text{width})^2 \times (\text{length})/2$. Mice were euthanized when the tumor size was greater than 1.5 cm^3 .

Intravesical instillation of orthotopic MB49^{luc} bladder tumors was carried out as previously described (22). Mice were anesthetized [ketamine (15 mg/kg), xylazine (75 mg/kg)] and catheterized using Teflon catheters (SurFlash Polyurethane I.V. catheter 24G $\times$ 3/4, SR *FF2419, Terumo Medical products, Somerset, NJ). Orthotopic bladder tumors were established by instilling 7.5×10^4 MB49^{luc} cells for 45 minutes into bladders that had been pretreated with 100 μL of poly-L-lysine (0.1 $\mu\text{g}/\text{mL}$) solution (PLL, molecular weight (MW) 70,000 to 150,000) (Sigma-Aldrich, St. Louis, MO). Tumor-take was confirmed by *in vivo* imaging 7–10 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. NK cell depletion was achieved by weekly administration of 25 μL rabbit anti-asialo-GM1 (Cedarlane Laboratories, Burlington, NC). 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 of 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 (Billerica, MA). avelumab binds to both human and mouse PD-L1 with high affinity - $K_d = 0.3$ and 1.0 nM , 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. Since avelumab is a human IgG1, three injections had to be compressed within a 7–9 day window (i.e., days 9, 12, and 15 post-tumor inoculation) to avoid the onset of neutralizing mouse anti-human Ig (data not shown).

Bacillus Calmette-Guerin (1.0 mg) (TICE, BCG, Organon USA, Inc., Roseland, NJ) 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.

Bioluminescent imaging

Orthotopic bladder tumor growth was detected by IVIS Lumina (Caliper Life Sciences, Alameda, CA) In-vivo Imaging. The abdominal regions of the mice were shaved and the mice were injected i.p. with luciferin salt (15 mg/kg), 10 minutes before imaging. Temporary anesthesia was administered by Forane (Isoflurane, USP, Baxter, Deerfield, IL) inhalation at 2% for the duration of imaging. The acquired bioluminescence signals in a region of interest (ROI) over the tumor site were read out as total flux radiance (photons/sec/cm²/steradian [sr]) using the Living Image software, Version 4.1 (Caliper Life Sciences, Hanover, MD).

In vitro T cell functional assays

Spleens were isolated from MB49 subcutaneous tumor-bearing mice that had been treated with avelumab. Splenocytes were washed; red blood cells were lysed with ACK buffer and pulsed for 6–8 days with p15E peptide (10 µg/mL) in 10 mL of complete medium, consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 0.1 mM nonessential amino acids, 15 mM Hepes buffer (pH 7.4), 1 mM sodium pyruvate, and penicillin/streptomycin (100 U/ml). Splenocytes were harvested and used in cytotoxic T-cell lysis (CTL) and/or cytokine release assays.

For the cytokine release assay, splenocytes were plated in 24 well plates at 0.5×10^6 cells/well along with 5×10^6 irradiated (20 Gy) peptide-pulsed splenocytes from naive syngeneic mice. Peptide was added at 10, 1, or 0.1 µg/mL. Peptides included p15E (KSPWFTTL, H-2K^b) or control HY peptide (WMHHNMDLI, H-2D^b). Total volume within the wells was 2 ml. Supernatants were collected at 48 hours and IFN-γ concentration was measured by ELISA as per the manufacturer's protocol (Pierce Protein Research Products, Grand Island, NY).

For the CTL assay, splenocytes were plated in triplicate wells of round-bottom, 96-well plates along with EL-4 cells that had been labeled with Indium-111 and pulsed with p15E or control peptide VSV-NP (RGYVYQGL, H-2K^b). The plates were incubated overnight and a gamma counter was used to measure Indium-111 release. Resulting cytotoxicity was calculated as % specific lysis = [(experimental cpm - spontaneous cpm) / (total cpm - spontaneous cpm)] × 100. Triplicates are shown as mean ± SE.

Histology and immunohistochemistry

For pathological evaluation, tissues (i.e., bladder, lungs, liver, kidneys, etc.) were removed for gross examination. Bladder tissue was fixed in 10% neutral buffered formalin, embedded in paraffin blocks, and processed by routine histological methods for H&E staining. The remaining bladder tissue was embedded in optimal cutting temperature (OCT) compound for staining of CD3 (clone OKT3, AbD-Serotec, Raleigh, NC), CD4 (clone RM4-5, BD PharMingen, San Jose, CA), CD8α (clone 53-6.7, BD PharMingen, San Jose, CA) and PD-L1 (clone AF1019, R&D Systems, Minneapolis, MN) as well as H&E. It has been reported that instillation of a higher number of MB49^{luc} tumor cells (i.e., 10^6) into mouse bladders previously traumatized with silver nitrate resulted in lung metastases (23). Upon

examination of the H&E stained slides, a board certified pathologist found the urinary bladder pathology to be “carcinoma, transitional cell, malignant without metastasis” with no accompanying metastatic lesions in the lungs. Stained slides were scanned using the Leica Biosystems anatomic pathology scanner. The Aperio Image Scope analyses were completed using Positive Pixel Count (PPC, v8.1). which determined the Total Number of Positive Pixels ($N_{\text{total}} - N_{\text{negative}} = \text{TNP}$) for each corresponding graph. 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 microns in either direction from the bladder/tumor border. Higher magnification images were taken on a Leica DMI4000 microscope with LAS AF (Leica Microsystems Inc., Buffalo Grove, IL).

Flow cytometry analysis

MB49 cells were seeded in T75 flasks in complete DMEM supplemented medium and treated with 10ng/mL IFN- γ 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 $\mu\text{g}/10^6$ cells) with a PE-conjugated, goat anti-human IgG secondary antibody (0.5 $\mu\text{g}/10^6$ cells, Jackson Immunoresearch, West Grove, PA). Data was 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, Ashland, OR).

Statistical analysis

GraphPad Prism software (GraphPad Prism 6.01 for Windows, GraphPad Software, Inc., La Jolla, CA) 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 tumor cells 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 IFN- γ /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 IFN- γ 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 post-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 post-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 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 component of the retroviral envelope protein p15E and the Y antigens (termed HY) presented by the MB49 tumor cells implanted into female hosts. A low, but measurable p15E CTL (Fig. 1F) and IFN- γ (Fig. 1F, insert) release was found following the *in vitro* peptide stimulation of splenocytes from avelumab-treated mice. In agreement with previous findings (24), despite the expression of male minor histocompatibility (HY) antigens by the MB49 tumor cells, no HY-specific response was found in the tumor-bearing, control-treated female mice. Interestingly, control-treated mice also had a low, but measurable p15E-specific CTL (Supplementary Fig. S2C).

Targeting PD-L1 in an orthotopic murine model

PD-L1 levels were measured using immunohistochemical staining in bladders of mice following MB49^{luc} instillation (Supplementary Fig. S3). As expected, the intravesical instillation of the MB49^{luc} cells resulted in non-muscle invasive tumor formation on the mucosal surface of the bladder (Supplementary Fig. S3A). Immunohistochemical staining revealed strong PD-L1 staining on the tumor cell surface with accompanying staining in the transitional epithelium, lamina propria and submucosa, which may indicate a broad pro-inflammatory response to MB49^{luc} tumor cell instillation (Supplementary Fig. S3B). For comparison, some weak PD-L1 staining was found in the transitional epithelium of bladders from naïve mice (Supplementary Fig. S3C).

In an initial study, bladders of mice were instilled with MB49^{luc} tumor cells and after 8 days were grouped according to the amount of tumor-associated bioluminescence (Fig. 2A). Bioluminescence determined by intravital imaging is helpful in assessing early tumor burden (i.e., tumor take) and the amount of radiance (photons) can be used to select mice for each treatment group based on equivalent tumor burden (22). Indeed, on day 8 following MB49^{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 IgG (Fig. 2A). Based on ongoing clinical studies using 10–20 mg/kg, 400 μ g (20 mg/kg) avelumab or the isotype-control antibody was administered. Antibody treatments were carried out on days 9, 12, and 15 post-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 post-tumor instillation, mice were euthanized and individual tumor burdens were determined by bladder weights of the two treatment groups and compared with that from naïve, 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 naïve, 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^{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^{luc} bladder tumor burden with avelumab administration also significantly ($P < 0.01$) improved percent survival when compared with isotype control treated mice (Fig. 2D).

Since 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^{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 post-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^{luc} tumors 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 post-tumor instillation reduced tumor growth (average bladder weight (61.6 ± 13.6 mg), which was somewhat surprising since 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.), at 29 days post-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 discernable 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 for the CD8 T cells which were equally distributed between bladder/tumor interface and the tumor mass (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, confirmed by histopathology. In the remaining two 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 impacted 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 less 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 Figure 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 Th1 helper 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 4H). Avelumab mediates antibody-dependent cell-mediated cytotoxicity (ADCC) in humans, but not in mice (P. Kim, personal communication). Consistent with that finding was that natural killer (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^{luc} 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 7.5×10^4 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 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, i.e., smoking, carcinogenic chemicals, etc. (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 last four decades (16–19). Repeated BCG instillations have led to early accumulation of granulocytes followed by macrophages and lymphocytes, predominately CD4 T cells, in the patients' bladders (30, 31). Investigators have shown that BCG treatment induces a strong T-helper type (Th)1 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: (1) The immune cell infiltrate is broad-based, the tumor associated antigen-specific component is a minor component. (2) 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., IFN- γ) 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 argue 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 IFN- γ 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 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 cytotoxicity 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 has 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. While 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. Since 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^{luc} 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 argues 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 check point 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Boon T, Gajewski TF, Coulie PG. From defined human tumor antigens to effective immunization? *Immunol Today*. 1995; 16:334–336. [PubMed: 7576068]
2. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulski K, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature*. 2013; 499:214–218. [PubMed: 23770567]
3. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol*. 2011; 29:235–271. [PubMed: 21219185]
4. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, et al. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature*. 2007; 450:903–907. [PubMed: 18026089]
5. Valmori D, Scheibenbogen C, Dutoit V, Nagorsen D, Asemussen AM, Rubio-Godoy V, et al. Circulating tumor-reactive CD8 T cells in melanoma patients contain a CD45RA-CCR7 effectors subset exerting *ex vivo* tumor-specific cytolytic activity. *Cancer Res*. 2002; 62:1743–1750. [PubMed: 11912149]
6. Linsley PS, Brady W, Grosmaire L, Aruffo A, Damie NK, Ledbetter JA. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med*. 1991; 173:721–730. [PubMed: 1847722]
7. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med*. 2011; 364:2517–2526. [PubMed: 21639810]
8. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*. 2000; 192:1027–1034. [PubMed: 11015443]
9. Okazaki T, Honjo T. PD-1 and PD-1 ligands from discovery to clinical application. *Int Immunol*. 2007; 19:813–824. [PubMed: 17606980]
10. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity*. 2007; 27:111–122. [PubMed: 17629517]

11. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocyte lesions supports an adaptive resistance mechanism of tumor escape. *Sci Transl Med*. 2012;127ra37.
12. Dong H, Strome SE, Saloman DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*. 2002; 8:793–800. [PubMed: 12091876]
13. Hamid O, Robert C, Daud A, Hodi FS, Hwu W-J, Kefford R, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med*. 2013; 369:134–144. [PubMed: 23724846]
14. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012; 366:2443–2454.
15. Brahmer JR, Tykodi SS, Chow LQM, Hwu W-J, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med*. 2012; 366:2455–2465. [PubMed: 22658128]
16. Morales A, Eidinger D, Bruce AW. Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. *J Urol*. 1976; 116:180–183. [PubMed: 820877]
17. Lamm DL, van der Meijden PM, Morales A, Brosman SA, Catalona WJ, Herr HW, et al. Incidence and treatment of complications of bacillus Calmette-Guerin intravesical therapy in superficial bladder cancer. *J Urol*. 1992; 147:596–600. [PubMed: 1538436]
18. Patard JJ, Saint F, Velotti F, Abbou CC, Chopin DK. Immune response following intravesical Bacillus Calmette-Guerin instillation in superficial bladder cancer: a review. *Urol Res*. 1998; 26:155–159. [PubMed: 9694595]
19. Askeland EJ, Newton MR, O'Donnel MA, Luo Y. Bladder cancer immunotherapy: BCG and beyond. *Adv Urol*. 2012; 2012:1–13.
20. Inman BA, Sebo TJ, Frigole X, Dong H, Bergstraith EJ, Frank I, et al. PD-L1 (B7-H1) expression by urothelial carcinoma of the bladder and BCG-induced granulomata. *Cancer*. 2007; 109:1499–1505. [PubMed: 17340590]
21. Nakanishi J, Wada Y, Matsumoto K, Azuma M, Kikuchi K, Ueda S. Overexpression of B7-H1 (PD-L1) significantly associates with tumor grade and postoperative prognosis in human urothelial cancers. *Cancer Immunol Immunother*. 2007; 56:1173–1182.
22. Zaharoff DA, Hoffman BS, Hooper CJ Jr, Khurana KK, Hance KW, Rogers CJ, et al. Intravesical immunotherapy of superficial bladder cancer with chitosan/interleukin-12. *Cancer Res*. 2009; 69:6192–6199. [PubMed: 19638573]
23. Newton M, Askeland E, Andresen E, Chehval V, Wang X, Askeland R, et al. Anti-interleukin-10R1 monoclonal antibody in combination with bacillus Calmette-Guerin is protective against bladder cancer metastasis in a murine orthotopic tumour model and demonstrates systemic specific anti-tumour immunity. *Clin Exper Immunol*. 2014; 177:261–268. [PubMed: 24593764]
24. Halak B, Maguire H, Lattime E. Tumor-induced interleukin-10 inhibits type I immune response directed at a tumor antigen as well as a non-tumor antigen present at the tumor site. *Cancer Res*. 1999; 59:911–917. [PubMed: 10029084]
25. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics. *CA Cancer J Clin*. 2008; 58:71–96. [PubMed: 18287387]
26. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. *Nature*. 2013; 500:415–421. [PubMed: 23945592]
27. Tsujihashi H, Matsuda H, Uejima S, Akiyama T, Kurita T. Immunocompetence of tissue infiltrating lymphocytes in bladder tumors. *J Urol*. 1988; 140:890–894. [PubMed: 3262173]
28. Tsujihashi H, Matsuda H, Uejima S, Akiyama T, Kurita T. Immunoresponse of tissue infiltrating lymphocytes in bladder tumors. *J Urol*. 1989; 141:1467–1470. [PubMed: 2657115]
29. Lipponen PK, Eskelinen MJ, Jauhiainen K, Harju E, Terho R. Tumor infiltrating lymphocytes as an independent prognostic factor in transitional cell bladder cancer. *Eur J Cancer*. 1993; 29A:69–75. [PubMed: 1445749]
30. Shintani Y, Sawada Y, Inagaki T, Kohjimoto Y, Uekado Y, Shinka T. Intravesical instillation therapy with bacillus Calmette-Guerin for superficial bladder cancer: study of the mechanism of bacillus Calmette-Guerin immunotherapy. *Int J Urol*. 2007; 14:140–146. [PubMed: 17302571]

31. Bohle A, Brandau S. Immune mechanisms in bacillus Calmette-Guerin immunotherapy for superficial bladder cancer. *J Urol*. 2003; 170:964–969. [PubMed: 12913751]
32. Ratliff TL, Ritchey JK, Yuan JJ, Andriole GL, Catalona WJ. T-cell subsets required for intravesical BCG immunotherapy for bladder cancer. *J Urol*. 1993; 150:1018–1023. [PubMed: 8102183]
33. Gan YH, Zhang Y, Khoo HE, Esuvaranathan K. Antitumour immunity of Bacillus Calmette-Guerin and interferon alpha in murine bladder cancer. *Eur J Cancer*. 1999; 35:1123–1129. [PubMed: 10533458]
34. Powles T, Vogelzang NJ, Fine GD, Eder JP, Braiteh FS, Loriot Y, et al. Inhibition of PD-L1 by MPDL3280A and clinical activity in pts with metastatic urothelial cancer (UBC). 2014 ASCO Annual Meeting. *J Clin Oncol*. 2014; 32:5s. (suppl.; abstr 5011).
35. Woo S-R, Corrales L, Gajewski TF. Innate immune recognition of cancer. *Annu Rev Immunol*. 2015; 33:445–474. [PubMed: 25622193]

Synopsis

In an orthotopic model of non-muscle invasive bladder cancer, in which BCG had minimal activity, the systemic administration of the anti-PDL1 checkpoint inhibitor avelumab demonstrated durable anti-tumor responses and long-term survival mediated by CD4 and CD8 T cells.

Figure 1. PD-L1 as a target for avelumab treatment in C57BL/6 mice bearing MB49 subcutaneous tumors

(A–C) Growth of individual s.c. MB49 tumors in PBS-treated (A), human Ig isotype control (B) and avelumab-treated (C) C57BL/6 mice. Arrows indicate treatment days and n = number of tumor-free mice. Data are representative of 2 independent experiments. (D) Average tumor volumes from C57BL/6 mice ($n = 10$) bearing s.c. MB49 tumors shown in panels A–C. Mice were treated with PBS (solid circles), human Ig isotype control (open squares) or avelumab ($3 \times 400 \mu\text{g}$) (solid triangles) on days 9, 12 and 15 (arrows). **, $P < 0.01$ (one-way ANOVA with Tukey's test) vs. untreated and human Ig control treated groups. (E) Survival curves for the mice described in D. *, $P < 0.05$ (Log-rank test) avelumab treated (solid triangles) vs. isotype control Ig treated group (open squares). (F) Splenocytes were isolated from 3 mice in which avelumab treatment significantly reduced MB49 s.c. tumor growth in panel C. Splenocytes were stimulated *in vitro* in the presence of $10 \mu\text{g}/\text{ml}$ of either p15E (circles), HY (triangles) or VSV, a control peptide (squares). CTL killing was assayed as outlined in the Materials and Methods. Data are from a representative experiment that was repeated with similar results. Insert: IFN- γ production by peptide pulsed splenocytes measured as described in the Materials and Methods. Solid and open bars represent the amount of IFN- γ generated by p15E and HY-stimulated splenocytes, respectively, over a 3-day incubation period. Error bars in panels D and F indicate SEM.

Figure 2. Avelumab effects on the growth of orthotopic MB49^{Luc} tumors in B6 mice
 (A) Bioluminescence images and total flux radiance measurements of MB49^{Luc} bladder tumors on day 8 post tumor instillation. Mice received 400 μ g of either the isotype control Ig or avelumab on days 9, 12, and 15. (B) Day 21 bioluminescence images and radiance measurements of those mice. (C) Individual bladder weights of mice treated with the isotype control Ig (circles) or avelumab (triangles) that were euthanized on day 29. Bladder weights of naïve mice (squares, no tumor instillation) are also included. Solid line represents mean bladder weight for each group, *, $P < 0.05$ (one-way ANOVA, Tukey's test) isotype control vs. avelumab-treated mice and naïve. NS, not significant. (D) Percent survival curve showing the isotype control Ig (solid circles) or avelumab-treated mice (solid squares), **, $P < 0.01$ (Log-rank test). Data are from a representative experiment that was repeated with similar results.

Figure 3. Comparison of the antitumor effects of avelumab, BCG alone, or avelumab plus BCG (A) Bioluminescence images and total flux radiance measurements of MB49^{Luc} bladder tumors on day 21 post tumor instillation. C57BL/6 mice ($n = 10$) bearing orthotopic MB49^{Luc} tumors were treated with DPBS/saline, DPBS/BCG (1.0 mg), avelumab (3 $\times$ 400 μ g)/saline, or avelumab/BCG. BCG was administered intravesically on days 8, 15 and 22, while avelumab was injected i.p. on days 8, 11, and 14 post-tumor instillation. (B) All mice were euthanized on day 29 and individual bladders were weighed to assess tumor burden. Solid black lines indicate the mean bladder weights in each group. *, $P < 0.05$; avelumab/saline vs. DPBS/BCG, **, $P < 0.01$; DPBS/saline vs. avelumab/BCG, **** $P < 0.0001$; DPBS/saline vs. avelumab/saline (Kruskal-Wallis test, Dunn's test). Data are from a representative experiment that was done three times with similar results. NS, not significant.

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^{luc} tumors were treated with DPBS/saline, DPBS/BCG (1.0 mg), avelumab (3×400 μg)/saline, or avelumab/BCG, as described in Figure 3. Mice were euthanized on day 29 and individual bladders from DPBS/saline (panels A, B), DPBS/BCG (panels D, E), avelumab/saline (panels G, H) and avelumab/BCG (panels J, K) were stained for CD4 (panels A, D, G, J) and CD8 (panels B, E, H, K) T cells as described in the Materials and Methods. Bar = 600 μm . Panels 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 the Materials and Methods. Data are from a representative experiment that was repeated with similar results. T, tumor.

Figure 5. Immunohistochemical detection of PD-L1 in the bladders of mice bearing tumors and treated with PBS (A), BCG (B), avelumab (C) or avelumab plus BCG (D)
Bar = 50 μ m, magnification - 100 $\times$. T, tumor.

Figure 6. Adaptive immunity mediates the antitumor effects of avelumab treatment of intravesical MB49^{luc} bladder tumors

(A) Groups of C57BL/6 mice were treated on days 1–4 and 6 with antibodies to deplete CD4, CD8, or on days 1 and 6 to deplete NK cells. Weekly antibody injections maintained the depleted state for each immune cell subset. Mice were instilled with MB49^{luc} tumor cells on day 4 and treated on days 8, 11, and 14 with 400 µg of either the control human Ig (circles) or avelumab alone (squares). avelumab was also administered to mice in which CD4 (triangles), CD8 (inverted triangles) or NK (diamonds) were depleted as described above. Mice were euthanized on day 29 and individual bladder weights were measured for tumor burden. Solid lines indicate the mean bladder weights for each group. ***, $P < 0.001$ (Kruskal-Wallis test, Dunn’s test) CD4-depleted groups vs. intact mice. NS, not significant.

(B) C57BL/6 mice (n = 38) bearing orthotopic tumors were treated with avelumab, which resulted in complete tumor eradication in 17 mice (based on imaging) by day 32. On day 36, those “immune” mice that were cured with avelumab immunotherapy (based on bioluminescence) were rechallenged orthotopically with the original dose of MB49 cells (inverted triangles). Challenge with wild-type MB49 cells allowed the differentiation on whether any tumor breakout was from the primary MB49^{luc} tumor cells (detected via

imaging) or from the wild-type MB49 challenge. Age-matched naïve mice (triangles) were challenged using the same conditions. All mice were euthanized on day 29 post tumor instillation and individual bladders were weighed to assess tumor burden. ****, $P < 0.0001$ (T-test, Mann-Whitney test); naïve vs. immune mice.

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