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Intravesical Anti-PD-1 Immune Checkpoint Inhibition Treats Urothelial Bladder Cancer in a Mouse Model

Austin N. Kirschner,*,† Jian Wang, Anne Rajkumar-Calkins, Kevin E. Neuzil and Sam S. Chang‡

From the Department of Radiation Oncology (ANK, JW, AR-C), Vanderbilt University Medical Center, Nashville, Tennessee, Vanderbilt University School of Medicine (KEN), Nashville, Tennessee, and Department of Urology (SSC), Vanderbilt University Medical Center, Nashville, Tennessee

Abbreviations and Acronyms

BCG = bacillus Calmette-Guérin

IHC = immunohistochemistry

NMIBC = nonmuscle-invasive bladder cancer

PD-1 = programmed cell death protein 1

PD-L1 = programmed death-ligand 1

Purpose: Nonmuscle-invasive bladder cancer is treated by resection within the bladder and bladder instillment with bacillus Calmette-Guérin or chemotherapy. For bacillus Calmette-Guérin-refractory disease, systemic anti-PD-1 (programmed cell death protein 1) immune checkpoint inhibition is a treatment. Our aim is to test whether intravesical instillment with anti-PD-1 inhibitor treats localized bladder cancer as effectively as systemic administration.

Materials and Methods: We investigated an orthotopic mouse model of urothelial bladder cancer using MBT2 cells instilled into the bladders of syngeneic, wild-type C3H mice. Groups of 10 mice received each treatment for comparison of intravesical anti-PD-1, intraperitoneal anti-PD1, and intravesical chemotherapy. The primary outcome was overall survival and secondary outcomes included long-term immunity and toxicity.

Results: Anti-PD-1 administered by bladder instillment (intravesical route) successfully treats localized bladder cancer and has similar overall survival to anti-PD-1 by systemic route. Anti-PD-1 by either route provides a significant survival advantage over control antibody. Anti-PD-1 increases CD8+ cell infiltration in tumors, particularly when administered intravesically. Antibody treatment avoids toxicity observed for intravesical chemotherapy. Mice who cleared their tumors after initial treatment were rechallenged with tumor engraftment 3–9 months later without any additional treatment. Initial anti-PD-1-treated mice did not grow tumors when rechallenged, which suggests long-term immunity exists, but initial mitomycin-treated mice readily grew tumors indicating no immunity occurred by chemotherapy treatment.

Conclusions: Intravesical administration of anti-PD-1 is a promising treatment route for localized bladder cancer, with comparable overall survival to systemic

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Declarations: Ethics approval and consent to participate. All research involving vertebrate animals was performed in strict accordance with protocols M/14/182 and M1700134 approved by Vanderbilt's Institutional Animal Care and Use Committee (IACUC). All procedures were conducted according to applicable national guidelines, including appropriate analgesics and anesthesia to ameliorate and minimize animal suffering.

All data generated or analyzed during this study are included in this published article.

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* Correspondence: Department of Radiation Oncology, Vanderbilt University Medical Center, 2220 Pierce Avenue, B1003 PRB, Nashville, Tennessee 37232 (telephone: 615-322-2555; FAX: 615-936-3363; email: austin.kirschner@vumc.org).

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anti-PD-1 in this mouse model. Intravesical anti-PD-1 increases CD8+ T cells in treated tumors and long-term immunity was seen to tumor rechallenge.

Key Words immunotherapy; programmed cell death 1 receptor; disease models, animal; urinary bladder neoplasms; tumor microenvironment

INTRAVESICAL instillation of bacillus Calmette-Guérin, an immunotherapy agent, has been used to treat nonmuscle-invasive bladder cancer for more than 40 years.[1–3] The putative mechanism for immune activation is internalization of BCG by urothelial and dendritic cells causing release of cytokines that recruit lymphocytes (especially T-cells), natural killer cells, macrophages, and neutrophils, which cause tumor cell death from direct cytotoxicity and apoptotic factors.[4] Clinical practice guidelines for NMIBC recommend intravesical BCG given as a 2-hour instillation once weekly for 6 weeks for induction, followed by maintenance at intervals (eg 3 weekly instillations at 3 months, 6 months, and every 6 months for up to 3 years).[5]

However, intermediate and high risk patients, especially those with high grade disease (T1G3), tend to do poorly with up to 50%–70% failing BCG treatment, up to 20%–30% progressing to invasive disease by 5 years, and up to 15% cancer-specific mortality at 5 years.[6–8] Intravesical chemotherapy may be used as salvage therapy for BCG-refractory NMIBC, but these typically have lower efficacy, except for mitomycin-C.[9] Both intravesical BCG and intravesical chemotherapy treatments have significant rates of local and systemic adverse events, including bladder irritation and granulomatous cystitis in 27%–84% of patients, gross hematuria in 21%–72%, and fever in 27%–44%.[9] Therefore, new therapeutic approaches are needed to improve the management for NMIBC.[1]

Immune checkpoint inhibitors, such as anti-PD-1 or anti-PD-L1 antibodies, activate T cells to fight a tumor by blocking the suppressive signal caused by interaction of the receptor/ligand pair between immune cells and tumor cells. These agents are U.S. Food and Drug Administration (FDA)-approved or being studied for platinum-ineligible/refractory metastatic urothelial carcinoma, including nivolumab,[10] pembrolizumab,[11] atezolizumab,[12] durvalumab,[13] and avelumab.[14] Pembrolizumab (anti-PD-1) was recently FDA-approved for BCG-refractory NMIBC based on 96 patients in the KEYNOTE-057 clinical trial with a 41% complete response rate and 16.2 months median duration of response.[15] However, systemic administration of immune checkpoint inhbitors often results in side effects, such as fatigue (16%–33% of patients) and up to 10% of patients experience high grade toxicities of the cutaneous, endocrine, gastrointestinal, pulmonary, renal, hematologic, rheumatic, and neurological systems.[16]

We hypothesized that anti-PD-1 antibody given by intravesical route will effectively treat bladder tumors while having a very good safety profile with limited systemic effects. Therefore, as a proof-of-principle project for future translation study, we investigated the intravesical route for immune checkpoint inhibition, comparing efficacy and toxicity to the systemic route or intravesical chemotherapy.

MATERIALS AND METHODS

Study Population

Female C3HeB/FeJ mice were purchased from Jackson Laboratory (Ellsworth, Maine). All research involving vertebrate animals was performed in strict accordance with protocol M1700134 approved by Vanderbilt's Institutional Animal Care and Use Committee (IACUC). All procedures were conducted according to applicable national guidelines, including appropriate analgesics and anesthesia to ameliorate and minimize animal suffering. MBT2 cells were purchased from Sekisui Xenotech LLC (Kansas City, Kansas), supplied from the Japanese Collection of Research Bioresources Cell Bank. Cells were authenticated by short tandem repeat analysis and confirmed free of *Mycoplasma* (CellCheck Mouse Plus, IDEXX BioAnalytics, Columbia, Missouri), and grown in cell culture in Dulbecco's Modified Eagle Medium (DMEM, Corning, Durham, North Carolina) supplemented with 10% fetal bovine serum (Corning) and 1% penicillin-streptomycin (Gibco, Gaithersburg, Maryland).

Syngeneic, Orthotopic Mouse Model of Urothelial Carcinoma in the Bladder

To establish bladder tumors, 0.3 million MBT2 tumor cells in 0.1 mL phosphate-buffered saline were instilled for 30–60 minutes into the bladders of C3H mice via 24-gauge angiocath (BD Biosciences, San Jose, California) as the intravesical route method.[17,18] Starting 1 week after tumor cell engraftment, mice were treated once weekly for 6 weeks and then observed thereafter. After bladders were manually emptied of urine by gentle pressure, mice were given 0.1 mL of pH 7 antibody dilution buffer (BioXcell, Lebanon, New Hampshire) containing 0.2 mg anti-murine PD-1 antibody (clone RMP1-14, BioXcell) given by intravesical route[19] or by intraperitoneal injection (systemic route), 0.1 mg mitomycin-C chemotherapy by intravesical route, or 0.2 mg isotype control antibody (clone 2A3, BioXcell). Mouse bladder tumors were confirmed as localized to the bladder in a subset of mice 2 weeks after instillment with MBT2 cells. Magnetic resonance imaging was obtained using a 7.0T Varian single channel magnetic resonance imaging scanner with T2-weighted imaging. Survival and body weights were recorded. The most common terminal endpoint was large tumor burden causing death. To ensure reproducibility of the data, at least 3

independent biological replicate cohorts were tested with each containing 10 mice per treatment condition.

Tumor Rechallenge Mouse Model

Mice completely clearing their bladder tumors were used for long-term rechallenge studies. Two million MBT2 cells in 50%–70% Matrigel in phosphate-buffered saline were engrafted subcutaneously in the flank 49–238 days following last treatment administration. No treatment was given after engraftment. Survival was recorded and tumor graft volumes were measured at least once weekly by digital calipers using the modified ellipsoidal formula: 0.5 × length × width.[2]

Immunohistochemistry

To study the immune cell infiltrate, bladder tumors were uniformly harvested 2 weeks after starting treatment. They were fixed in 10% zinc-formalin (Fisher Scientific, Suwanee, Georgia) at room temperature overnight, then transferred to 70% ethanol for paraffin embedding. Immunohistochemical staining for murine CD8 was performed on adjacent sections with staining by hematoxylin & eosin for identification of tumor tissue. Slides were placed on the Leica Bond Max IHC stainer. All steps besides dehydration, clearing, and coverslipping were performed on the Bond Max. Slides were deparaffinized. Heat-induced antigen retrieval was performed on the Bond Max using their Epitope Retrieval 2 solution for 20 minutes. Slides were incubated with anti-CD8 (cat#14-0808-80, eBioscience Inc, San Diego, California) for 1 hour at a 1:1,000 dilution and then incubated with rabbit anti-rat secondary (BA-4001, Vector Laboratories, Inc., Burlingame, California) for 15 minutes at a 1:2,000 dilution. The Bond Polymer Refine detection system was used for visualization. Slides were then dehydrated, cleared, and coverslipped. Whole slide imaging of immunostaining was performed in the Digital Histology Shared Resource at VUMC (www.mc.vanderbilt.edu/dhsr). Immunostained tissue slides were imaged on a Leica SCN400 Slide Scanner (Leica Biosystems, Lincolnshire, Illinois) at 20× magnification to a resolution of 0.5 μm/pixel. Quantification was performed using QuPath software[20] for positive cell detection, using sigma level 1.5 and mean cell intensity single threshold 0.2 of the CD8 stain.

Statistical Analysis

For a power of at least 0.7 with an alpha of 0.05, we calculated a sample size of 10 per condition assuming a tumor establishment rate of 70% and an estimated quadrupling of median overall survival with treated compared to control animals. Overall survival was compared using log-rank (Mantel-Cox) test (GraphPad Prism). Rechallenge graft volumes were compared using a 2-way ANOVA with multiple comparisons to the control group for each timepoint (GraphPad Prism). Immunohistochemical staining was analyzed by 2-sided Fisher's exact test and chi-square test (GraphPad Prism). Body weights were compared by unpaired 2-tailed T-test with Welch's correction (GraphPad Prism).

RESULTS

Intravesical anti-PD-1 provides effective treatment for bladder tumors. We used a syngeneic orthotopic murine model of urothelial bladder carcinoma using the MBT2 urothelial cell line. This model mimics the origin of most human bladder cancers, as it was developed by carcinogen exposure. This model allows reliable formation of bladder tumors to test tumor growth and responses to treatment without modifying the tumor microenvironment or adjacent normal tissues.

We optimized the intravesical tumor cell inoculation dose and timing before starting treatment. Depending on tumor inoculate and engraftment incubation time, early cohorts showed rapid development of metastatic disease as early as 2 weeks after tumor engraftment (data not shown). Disease remained within the bladder when starting treatment 1 week after intravesical MBT2 tumor cell engraftment. For comparison of survival outcomes, 10 mice per condition were tested with at least 3 independent experimental replicates. Mimicking the schedule for patient treatments for NMIBC, we administered treatments once weekly for 6 weeks and compared results from systemic versus intravesical routes for anti-PD-1 antibody, as well as intravesical isotype control antibody and intravesical mitomycin-C chemotherapy. Remarkably, anti-PD-1 antibody treatment provided comparable excellent survival for both the systemic and intravesical routes, which were both superior to control antibody and mitomycin-C treatment. A representative experimental cohort of 40 mice is shown (fig. 1, *A*). Similarly, detectable palpable disease was less common in the mice treated with anti-PD-1 antibody (by either route) and mitomycin-C compared to control antibody treatment (fig. 1, *B*). Three of the mice treated with control antibody never developed palpable bladder tumors, consistent with prior studies showing successful engraftment of MBT2 cells of 70% after bladder instillment.[19] Although none of the mice treated with mitomycin-C developed a tumor, there was significant toxicity that caused decreased survival (fig. 1, *A*). Mice who died from tumor burden had disease spread to localized/regional and metastatic sites, typically bladder or periurethral masses (fig. 1, *C*), kidney metastases, peritoneal metastases, and/or lymph node involvement. The improved survival of mice treated by intravesical application of anti-PD-1 antibody suggests it is a useful approach for the treatment of bladder cancer.

Intravesical anti-PD-1 treatment provides durable immunity to tumor rechallenge. Mice with complete bladder tumor regression and long-term survival were rechallenged with subcutaneous engraftment of MBT2 cells. The subcutaneous grafts were measured twice weekly for 49 days after engraftment; no treatment was administered to any mice during this time. Based on the results in early cohorts (not shown) and figure 1, *A*, 49 days was chosen as a surveillance timepoint to give ample time for tumor growth, as this was at least 3 weeks longer than the

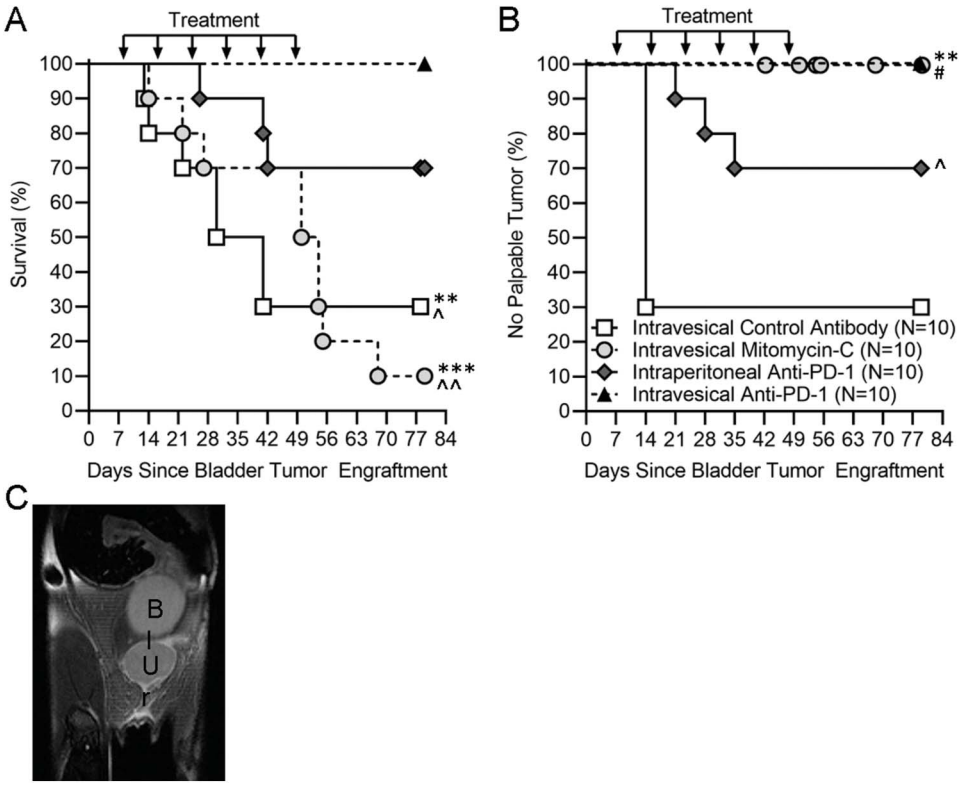

Figure 1. C3H mice with orthotopic intravesical engraftment of MBT2 bladder tumors treated with anti-PD-1 immune checkpoint inhibitor. *A*, survival for 4 treatment groups (10 per group) is shown, with significantly superior survival for anti-PD-1 antibody treatment given by intravesical route compared to intravesical isotype control antibody (double asterisks indicate p=0.0013) and intravesical mitomycin-C (triple asterisks indicate p <0.001). Intraperitoneal route (systemic) anti-PD-1 antibody was also superior to intravesical isotype control antibody (caret indicates p=0.048) and intravesical mitomycin-C (double carets indicate p=0.0208). Anti-PD-1 antibody by intravesical versus intraperitoneal route (systemic) had trend toward improved survival (p=0.067). p Values are by log-rank test. *B*, compared to intravesical isotype control antibody, detectable tumor by palpation was significantly less prevalent for anti-PD-1 antibody treatment given by intravesical route (double asterisks indicate p=0.0014), intraperitoneal route (caret indicates p=0.038), and for intravesical mitomycin-C (number symbol indicates p=0.014). *C*, example of mouse with regional disease in bladder (Bl) and urethra (Ur), with mass seen on T2-weighted magnetic resonance imaging coronal view.

tumors required for implantation and significant growth in the bladder. The mice that were previously treated with anti-PD-1 antibody (both intraperitoneal and intravesical route) for their initial bladder grafts demonstrated an inability to grow grafts, suggesting an immunity to the tumor cell line (fig. 2). Conversely, the mice that were previously treated with either control antibody or mitomycin for their bladder tumor were able to support the growth of subcutaneous grafts when rechallenged with MBT2 tumor cells, suggesting that they did not have immunity.

Intravesical anti-PD-1 increases CD8+ cells in the bladder tumor. The tumor-immune microenvironment was investigated by quantifying murine CD8+ cells by immunohistochemical staining of the grafts harvested 2 weeks after final treatment with control or anti-PD-1 antibody (fig. 3, *A*). Despite lack of palpability (fig. 1, *B*), mice treated with intravesical anti-PD-1 antibody still had tumor deposits seen on IHC. To account for varying quantities of tumor examined, we normalized CD8+ cell infiltration to total tumor cells by computing ratios of CD8+ cells to tumor cells. Compared to control treatment, mice treated with anti-PD-1 antibody by intravesical and intraperitoneal routes had significantly greater CD8+ cells within the tumor (fig. 3, *B* and *C*). This is consistent with the known effects of anti-PD-1 antibody causing increased activation of CD8+ cells and tumor infiltration.[21,22]

Intravesical anti-PD-1 causes less toxicity compared to intravesical chemotherapy. Toxicity between the treatment groups was compared by measuring body weight and physical appearance for each animal. All antibody treatments by intravesical and intraperitoneal (systemic) routes did not cause any detectable toxicities. However, intravesical mitomycin-C given once weekly for 6 weeks caused significant toxicity, which was quantified by body weight loss (fig. 4, *A*). Starting around week 3 of treatment, visible scarring of cutaneous tissues in the pelvis and periurethral region were noted, suggesting local irritation from the chemotherapy agent instilled in the bladder (fig. 4, *B*). Most significantly, death without disease occurred, associated with loss of body weight (fig. 1, *A*).

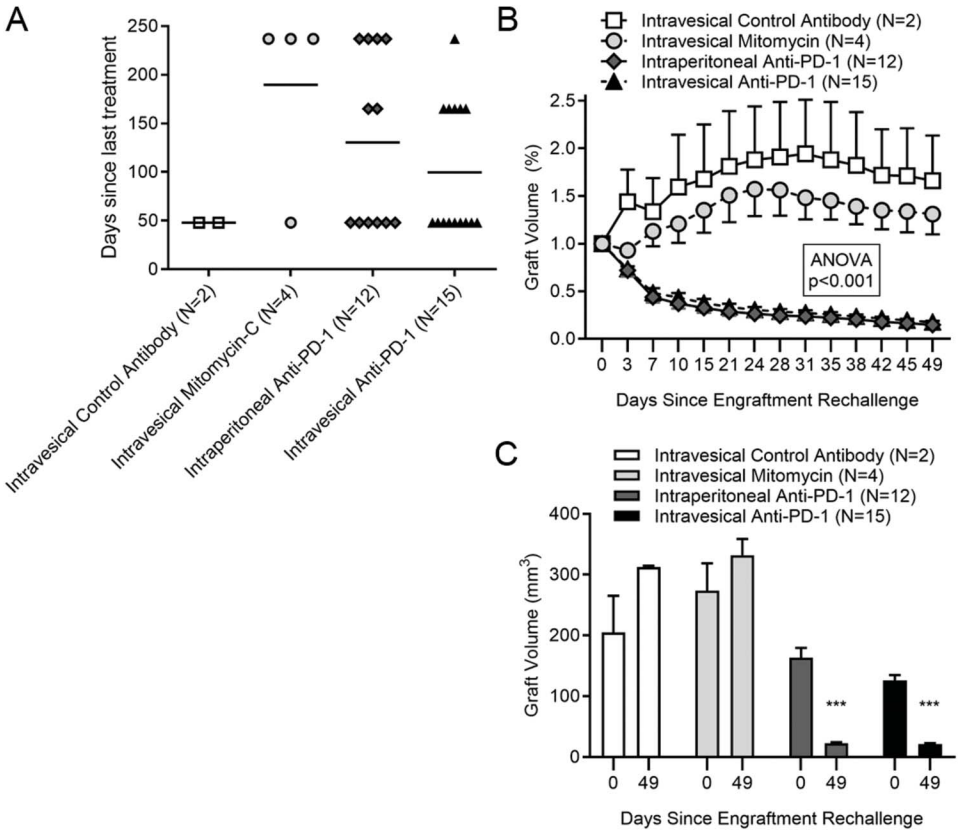

Figure 2. Long-term immunity to tumor rechallenge in mice that cleared their orthotopic bladder tumors. Mice from separate experiments were combined by original treatment for rechallenging. *A*, subcutaneous MBT2 grafts were implanted in mice that cleared their initial bladder tumors (range 49–238 days after finishing initial treatment), which was not different between treatment groups ($p=0.1345$, ANOVA). *B*, inoculate of 2 million MBT2 cells in matrigel placed subcutaneously was cleared by mice that previously received anti-PD-1 antibody treatment (by either intravesical or intraperitoneal routes) for their initial bladder tumors, but tumor grafts grew for mice previously treated by mitomycin-C and control antibody ($p<0.001$, ANOVA). *C*, graft volumes on rechallenge day 0 compared to day 49 show a significant decrease in mice previously treated with anti-PD-1 antibody (triple asterisks indicate $p<0.0001$, unpaired 2-sided T-test). This suggests anti-PD-1 treatment leads to immune surveillance that is active against tumor cell line, but mitomycin-C and control antibody do not enhance immune response. Error bars represent±SEM.

DISCUSSION

The preclinical model is representative of human disease. The syngeneic mouse model was selected to allow treatment effects to be studied in the presence of an intact immune system, ie a wild-type mouse. The tumor cell line MBT2 used in this project was generated in 1977 from C3H/He mice by carcinogen exposure, making it a good model for human bladder cancer. We use C3HeB/FeJ mice, a substrain that was more readily available. However, it does not seem MBT2 cells are allogeneic to C3HeB/FeJ mice, as the engrafted cells established local and metastatic disease at significant rates indicating they are syngeneic to the immunocompetent host mice. The MBT2 bladder engraftment model is aggressive. Using our instillment approach, MBT2 bladder tumors inadequately treated were found to progress rapidly to muscle-invasion and metastatic disease 2–3 weeks after bladder implantation.

Intravesical anti-PD-1 treats localized bladder tumors to improve survival and confers long-term immunity. The data presented herein demonstrates anti-PD-1 immune checkpoint inhibitor treatment using an intravesical route provides robust control of localized disease in a highly aggressive urothelial orthotopic bladder cancer preclinical model. Although clinical data indicate urothelial bladder cancer may respond to systemic anti-PD-1 or anti-PD-L1 therapy, data are not yet available for efficacy or safety when the drug is given by intravesical route for localized disease. To assess treatment response for disease localized to the bladder, treatments were begun 1 week after engraftment. Outcome data show that local therapy in the bladder provided effective local control, which speaks to the significant efficacy of this intravesical treatment approach. Furthermore, disease control was similar between intraperitoneal (a commonly used approach for systemic antibody therapy in mice) and intravesical routes for anti-PD-1 antibody, which suggest the immune response is activated efficiently even with intravesical delivery.

Remarkably, long-term immunity was conferred by intravesical application of anti-PD-1 antibody, as

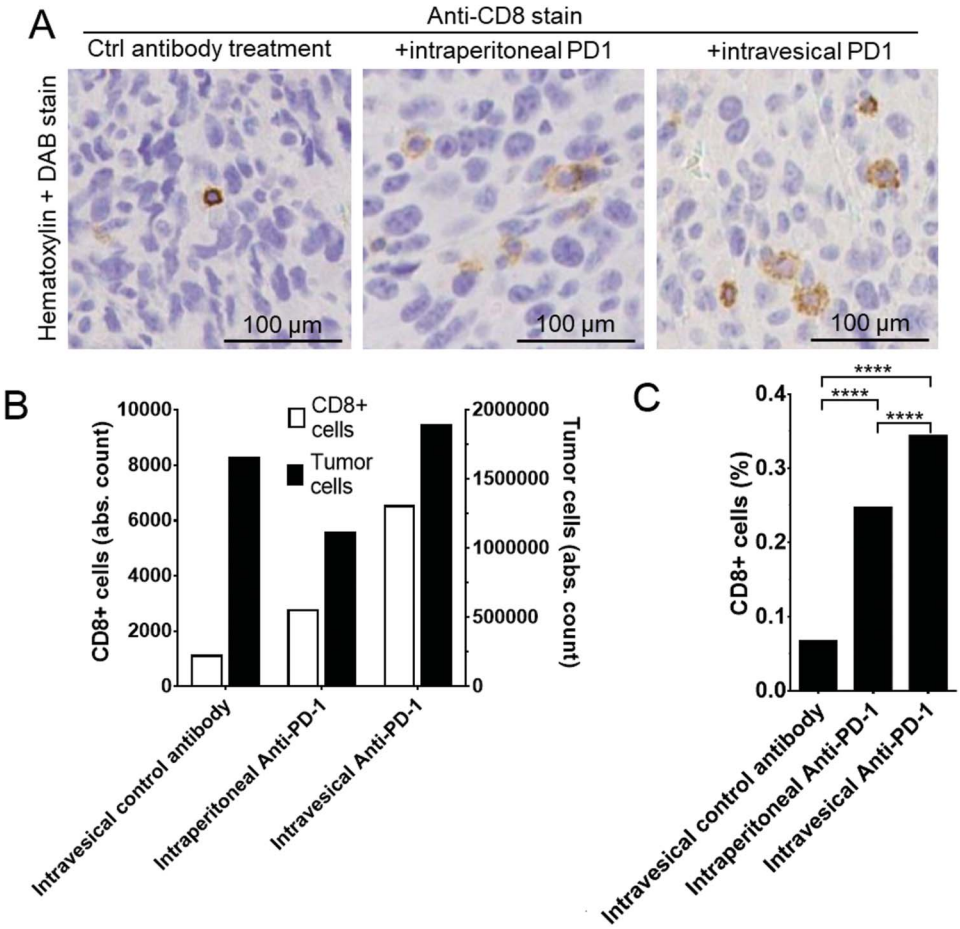

Figure 3. Immune infiltrate in orthotopic bladder tumor microenvironment from at least 5 independent bladder tumors per treatment condition with summed cell count data. Treatment with anti-PD-1 antibody by intraperitoneal or bladder (intravesical) route shows increased CD8 stained cells by immunohistochemical staining compared to treatment with intravesical control (*Ctrl*) antibody (*A*). Absolute (*abs.*) count of tumor cells identified on hematoxylin & eosin (*right y axis*) (*B*) and CD8+ cells identified in IHC (*left y axis*) and (*C*) ratio of CD8+ cells to total tumor cells. Triple asterisks indicate p <0.0001, chi-square test.

demonstrated by the lack of tumor engraftment upon rechallenge using a subcutaneous location. This suggests the potential for greater durability of anti-tumor response mediated by the immune system, as compared to intravesical chemotherapy or BCG treatment in patients who require repeated treatments and maintenance therapy in common clinical practice.

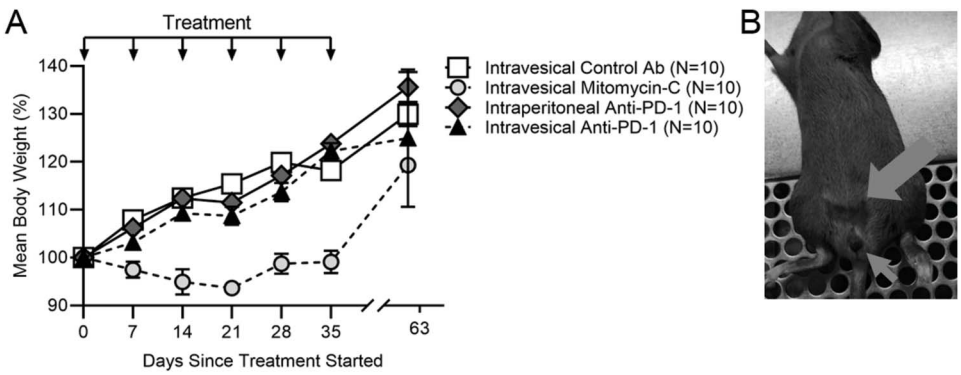

Figure 4. Toxicity from treatment. *A*, mean body weight for each treatment group (10 per group) indicates significant weight loss due to intravesical mitomycin-C compared to intraperitoneal anti-PD-1 (p=0.021), intravesical anti-PD-1 (p=0.037), and intravesical control antibody (p=0.0120, unpaired 2-tailed T-test with Welch's correction). Error bars represent±SEM. *B*, gross image of mouse treated with intravesical mitomycin-C that developed pelvic cutaneous scarring and hair loss (*large arrow*) superior to the urethral meatus (*small arrow*).

Intravesical anti-PD-1 enhances tumor-related CD8+ T-cell populations. To further understand the mechanism for reduced tumor growth following immune checkpoint treatment, immunohistochemistry was used to characterize the tumor immune microenvironment. As shown in figure 3, a higher absolute count and increased percentage of CD8+ cells, consistent with cytotoxic T cells, was found infiltrating the tumors treated with anti-PD-1 antibody. This finding is consistent with one known mechanism for anti-PD-1 treatment: causing the activation and increased tumor-infiltration of CD8+ cells.[21,22] Both systemic and intravesical anti-PD-1 has an increase in tumor-related CD8+ cells compared to control antibody treatment, indicating that the mechanism is likely similar for both routes. In fact, significantly greater CD8+ cells were found with intravesical anti-PD-1 antibody treatment compared to intraperitoneal route, which suggests that the local application within the bladder may provide a higher drug concentration at the tumor site.

Toxicity in the preclinical model. Our study has various inherent weaknesses. Despite a higher local concentration of anti-PD-1 antibody, we saw no bladder toxicity in comparison to the mitomycin-C administration, which caused frank scarring perivesically and weight loss in mice. Mitomycin-C prevents bladder tumor growth but contributes to death in mice over time, which is consistent with published mouse models.[23] Future studies comparing efficacy may be undertaken with lower doses of mitomycin-C to minimize the risk of excessive treatment-induced toxicity.

One of our untested hypotheses is that immunotherapy administration via bladder instillment instead of systemic route would spare toxicity in humans. Immunotherapy has significant toxicity in humans, which is not well reflected in a majority of mouse models.[24] Therefore, we would not anticipate seeing differences in side effects from anti-PD-1 in this preclinical model, given that immunotherapy related adverse events generally have low prevalence in mice. We assert that our model shows preclinical equivalence between bladder instillment vs systemic administration, providing the rationale for further exploration in clinical trials.

Our study evaluates immunotherapy in the treatment of bladder tumors but omits use of intravesical BCG, the first neoplastic-directed immunotherapy. As mentioned previously, the clinical response to BCG in humans is already known—most patients achieve a good but temporary response, with frequent relapse. While pre-administration with BCG could theoretically "supercharge" anti-PD-1 antibody therapy and is undergoing clinical investigation with intravesical BCG followed by intravenous pembrolizumab, we wished to fundamentally establish whether intravesical administration of immunotherapy alone is a route worth pursuing.

CONCLUSIONS

As clinical trials develop to test immune checkpoint inhibitors for the treatment of localized bladder cancer, it is important to recognize that an immune checkpoint treatment given within the bladder may provide excellent efficacy while limiting systemic exposure that could lead to broad side effects. Indeed, of more than 2 dozen trials that include use of anti-PD-1 or anti-PD-L1 antibodies that are enrolling patients with bladder tumors, only 2 utilize intravesical administration of anti-PD-1 antibody (NCT02808143 and NCT03167151). The preclinical model presented herein provides a framework for further investigating the approach for giving immune checkpoint inhibitor by intravesical route, which should be optimized and more widely utilized in future clinical trials.

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