Systemic and intravesical adoptive cell therapy of tumor-reactive T cells can decrease bladder tumor growth in vivo

Brittany L Bunch 1, Jennifer Morse,1 Sarah Asby,1 Jamie Blauvelt,1 Ahmet M Aydin,2 Patrick Innamarato2,1 Ali Hajiran,2 Matthew Beatty,1 Michael Poch,2,3 Shari Pilon-Thomas1,3,4

ABSTRACT

Background The therapeutic armamentarium of bladder cancer has been recently enriched with the introduction of new therapies including immune checkpoint inhibitors, receptor tyrosine kinase inhibitors and antibody drug conjugates, however treatment responses and duration of responses are still less than expected. Adoptive cellular therapy (ACT) using tumor-infiltrating lymphocytes (TILs) has potential to treat bladder cancer, as previously demonstrated by successful expansion of tumor reactive T cells from human bladder tumors.

Methods A model system using OT-I T cells and an ovalbumin expressing MB49 tumor cell line (MB49OVA) was developed to study ACT in bladder cancer. Systemic ACT-treated mice were given T cells intravenously after lymphodepleting chemotherapy and followed by interleukin (IL)-2 administration. Intravesical ACT treated mice were given T cells directly into the bladder, without chemotherapy or IL-2. TILs were isolated from MB49 orthotopic tumors and expanded ex vivo in IL-2. Immune cell infiltrates were analyzed by flow cytometry. T cell infiltration was studied using a CXCR3 blocking antibody.

Results Systemic ACT-treated mice had a decrease in tumor growth, increase in T cell infiltration and long-term immune protection compared with control-treated mice. OT-I T cells delivered intravesically were able to control tumor growth without lymphodepleting chemotherapy or IL-2 in MB49OVA orthotopic tumors. Intravesical delivery of TIL expanded from MB49 tumors was also able to decrease tumor growth in mice with MB49 orthotopic tumors. Blocking CXCR3 on OT-I T cells prior to intravesical delivery decreased T cell infiltration into the tumor and prevented the control of tumor growth.

Conclusions This study demonstrates how TIL therapy can be used in treating different stages of bladder cancer.

INTRODUCTION

In 2020, it is estimated that there will be 81,000 new cases of urinary bladder cancer and 18,000 deaths attributed to the disease in the USA.1 Approximately 70%–75% of all new bladder cancer cases present with superficial or non-muscle bladder cancer (NMIBC) in which the disease is confined to the mucosa or submucosa.2,3 Per clinical guidelines, intermediate-risk and high-risk NMIBC are initially treated locally with transurethral resection (TURBT) followed by therapeutic or adjuvant intravesical BCG.45 BCG was one of the first Food and Drug Administration (FDA)-approved immunotherapies to treat cancer in the 1970s, but over the past decade BCG supply has become increasingly limited due to manufacturing shortages.6 7 More recently, the FDA has approved systemic immune checkpoint inhibitors targeting programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) as treatment for patients with BCG unresponsive carcinoma in situ of the bladder and for patients with advanced bladder cancer who have previously received or are ineligible for cisplatin-based chemotherapy.8–13 However, the objective treatment response to immune checkpoint inhibitors in patients with bladder cancer remains lower than expected with an objective response rate ranging between 15% and 25% in all platinum pretreated advanced bladder cancer cases and about 25% and 30% in cases with high biomarker expression.8–12 Among the 20%–25% of patients who present with muscle-invasive bladder cancer (MIBC) at initial diagnosis, about 20%–40% eventually experience disease recurrence after radical cystectomy.15 The survival of patients with metastatic bladder cancer, approximately 5% of all cases at diagnosis, is <12 months after failing first-line cisplatin-based chemotherapy.16–18 The FDA has recently approved enfortumab vedotin, an antibody-drug conjugate targeting nectin-4, and erdafitinib, a fibroblast growth factor receptor tyrosine kinase inhibitor, in heavily pretreated patients with advanced disease. Enfortumab vedotin has an overall response rate of 44%; however, estimated overall survival is still <12 months.18 Likewise erdafitinib demonstrated a limited overall response rate (40%) in a particular subset of patients...
harboring genetic alterations of fibroblast growth factor receptor, with a limited duration of treatment response (an approximate progression-free and overall survival of 6 and 12 months, respectively). Therefore, there is a current pressing need to identify novel treatment strategies for patients with bladder cancer with high-risk localized, locally advanced and metastatic disease.

Adoptive cellular therapy (ACT) of tumor-infiltrating lymphocytes (TILs) is a personalized immunotherapy approach to treat solid tumors. Surgically resected tumor specimens are minced into small fragments (1–3 mm³) and cultured in high-dose interleukin (IL)-2 to promote expansion of T cells from within the tumor. Tumor-reactive TIL is selected, expanded to high numbers (10¹⁰), and then reinfused into the patient following non-myeloablative chemotherapy (NMAC). ACT of TIL has been successful in improving overall survival in patients with metastatic melanoma. Similar to melanoma, bladder cancer has been demonstrated to be an immunogenic tumor type, having one of the highest tumor mutation burdens among all cancer types, with resulting high levels of predicted neoantigen expression. Moreover, increased levels of CD8+ cytotoxic T cell infiltration within the tumor microenvironment has been associated with improved survival in patients with bladder cancer. Given these features, it is plausible that TIL therapy may be effective in targeting and treating both localized and metastatic bladder cancer tumors.

Our lab has previously shown the feasibility of expanding tumor-reactive T cells from patients with bladder cancer in vitro. In this study, we investigated the ability of tumor-reactive T cells to treat bladder cancer in vivo, using both systemic and intravesical TIL delivery methods. We found that intravesically delivered T cells are able to infiltrate bladder cancer tumors in part through CXCR3 signaling and are able to delay tumor growth. Results from this study provide rationale for delivering TIL either systemically or intravesically to treat bladder cancer.

**METHODS**

**Animals**

Female C57BL/6 mice were purchased from Charles River Laboratories (Indianapolis, Indiana, USA). OT-I transgenic mice (C57BL/6-Tg (TcraTcβb) 1100Mjb/J) were purchased from The Jackson Laboratory. Mice were housed and bred in the Comparative Medicine Facility at Moffitt Cancer Center where they were monitored daily. When tumors reached 400 mm² (subcutaneous) or 250–300 mm³ (orthotopic) mice were humanely euthanized with CO₂ followed by cervical dislocation.

**Cell lines and cell culture**

MB49 murine bladder cancer cells were a kind gift from Dr Jeffery Schlom (National Cancer Institute, Bethesda, Maryland, USA). An ovalbumin (OVA) expressing fluorescent MB49 cell line (MB49OVA) was generated by exposing cells to supernatants containing a lentiviral vector comprising a fluorescent ZsGreen (ZsG) protein and OVA. The viral supernatant was a kind gift from the Dr Brian Ruffell laboratory (H. Lee Moffitt Cancer Center, Tampa, Florida, USA). After transduction, ZsGreen tumor cells were sorted by FACS using BD FACSAria. MB49OVA tumor cells were passaged in vitro 4 times. OVA expression was confirmed by staining for H2-Kb bound to SIINFEKL peptide (25-D1.16, BioLegend). Cell lines were cultured in Complete Medium (CM) consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 mg/mL streptomycin, 100 U/mL penicillin, 50 mg/mL gentamycin, 0.5 mg/mL fungizone (all from Life Technologies, Rockville, Maryland, USA) and 0.05 mM 2-ME (Sigma-Aldrich, St. Louis, Missouri, USA). After thawing, cells were passaged no more than 10 times and routinely tested negative for mycoplasma.

**T cell isolation and culture**

Mice were inoculated with 5.0×10⁵ MB49 or MB49OVA cells subcutaneously at the right ventral abdomen or 1.0×10⁵ MB49 or MB49OVA cells orthotopically. T cells from MB49 or MB49OVA tumors were isolated using EasySep Mouse CD90.2 Positive Selection Kit II (Stem Cell Technologies) according to the manufacturer’s protocol. Isolated cells were plated in CM+IL-2 (100 IU, Prometheus).

**Systemic ACT treatment model**

MB49OVA cells (5.0×10⁵) were injected subcutaneously at the right ventral abdomen. Once tumors were palpable, mice received intraperitoneal injections of cyclophosphamide (200 µg) followed by fludarabine 24 hours later (100 µg). One day after fludarabine, OT-I T cells were isolated from splenocytes using EasySep Mouse CD8+ T Cell Isolation Kit (Stem Cell Technologies) according to the manufacturer’s protocol and delivered intravenously in 250 µL phosphate-buffered saline (PBS). IL-2 (2.5e5 IU) was given intraperitoneally every 12 hours for 3 days following T cell injections. Control mice did not receive T cells or IL-2 injections. Tumor size was measured every 2–3 days.

**Intravesical treatment model**

MB49 or MB49OVA cells were injected orthotopically. Briefly, mice were put under anesthesia and catheters were placed. Poly-L-lysine (50 µL at 1 µg/mL) was injected into the bladder for 10 min. Bladders were washed with PBS (50 µL) and tumor cells were injected for 30 min (1.0×10⁵ cells in 100 µL of PBS). The presence of tumor was confirmed by ultrasound-guided imaging using the Vevo 2100 imaging system (FUJIFILM VisualSonics). Approximately 7 days after injection when tumors were detected, OT-I T cells or MB49 TILs (isolated as described above) were infused directly into the bladder while mice were under anesthesia with catheters in place for 3 hours. Mice were not given lymphodepleting chemotherapy prior to...
T cell delivery or IL-2 post-T cell delivery. Tumor size was measured by ultrasound every 3–4 days.

**Murine tissue processing**

Tumors were processed into a single cell suspension by enzymatic digestion using Hanks’ Balanced Salt Solution (HBSS) (Life Technologies) containing 1 mg/mL collagenase, 0.1 mg/mL DNAse I and 2.5 U/mL of hyaluronidase (all from Sigma-Aldrich). Samples were incubated at 37°C for 45 min with constant agitation. Samples were passed through a 70 µm strainer and washed with CM. Red blood cells (RBCs) were lysed using RBC lysis buffer (Biolegend) for 5 min. Spleens were processed by physical dissociation through a 100 µm strainer followed by RBC lysis.

**Flow cytometry and tetramer staining**

Tumors and spleens were processed into single cell suspensions as described above. Cells (1×10⁶) were washed with 1 mL PBS. Zombie NIR viability dye (Biolegend, 1:1000) was added to each sample and incubated at room temperature for 30 min in the dark. Samples were washed with flow buffer (1.0 L PBS, 5% heat-inactivated fetal bovine serum, 1 mM EDTA, 0.1% sodium azide) and stained for OVA tetramer (MBL International, Woburn, Massachusetts, catalog# TB5001-1) at 4°C for 20 min in the dark followed by cell surface markers CD3 (catalog# 565643), CD8 (catalog# 558106), CD4 (catalog# 550954), CXCR3 (catalog# 562152) and CD45.1 (catalog# 560578, all from BD Biosciences, San Jose, California, USA) at 4°C for 20 min in the dark. Samples were run on a FACSCelesta flow cytometer (BD Biosciences) and analyzed using FlowJo V.10 software.

**Assessment of interferon-gamma by ELISA**

OT-I T cells were plated in a co-culture 10:1 with irradiated MB49 or MB49OVA tumor cells or OVA SIINFEKL peptide (1 µg/mL) as a positive control. Supernatants were collected after 48 hours. Interferon-gamma (IFN-γ) levels were analyzed using Mouse IFN-γ ELISA Kit II (RUO) (BD Biosciences) according to manufacturer’s protocol. Briefly, supernatants were diluted 1:10, 1:20 or 1:100 and plated along with the IFN-γ standard serial dilutions in wells containing 50 µL ELISA Diluent and incubated for 2 hours. Wells were washed 5 times. Working Detector (100 µL) was added to each well and incubated for 1 hour. Wells were washed 7 times. TMB One-step Substrate Reagent (100 µL) was added to each well and incubated for 30 min. Stop Solution (50 µL) was added to each well and absorbance was read at 450 and 570 nm.
Blocking CXCR3

OT-I T cells were isolated as described and incubated for 30 min on ice with either normal rat IgG isotype control or CXCR3 blocking antibody (500 µg, catalog# BE0249, BioXcell). In vitro, OT-I T cells were plated in normal CM in the upper well of a Corning Transwell insert with MB49OVA tumor digest conditioned media in the bottom. Cells were collected from the bottom chamber and counted 24 hours later. In vivo, mice were inoculated with orthotopic MB49OVA tumors as described above. Tumor-bearing mice were randomized and treated with antibody-coated OT-I T in 100 µL of PBS cells on day 7. Tumors were collected 3 and 24 hours after instillation, washed 3 times in PBS, digested and analyzed for CD45.1+ CD8+ T cells and OVA tetramer+ CD8+ T cells by flow cytometry. An additional cohort was monitored for tumor growth by ultrasound-guided imaging every 2–3 days.

Patient subjects

Two human bladder cancer TIL samples were screened for CXCR3 (catalog# 562558, BD Biosciences) expression by flow cytometry as described above. Informed consent was obtained prior to surgical resection under an IRB-approved study (MCC 18142). Both samples were from patients with muscle-invasive bladder cancer undergoing radical cystectomy. One patient had neoadjuvant cisplatin-based chemotherapy and one patient did not receive chemotherapy. TIL was expanded as previously described.26

Statistical analysis

For comparison of in vitro studies with two experimental groups, a Student's t-test was performed. For in vitro studies with more than two experimental groups, a one-way analysis of variance (ANOVA) was performed, followed by a Tukey’s post hoc comparison. For in vivo studies with two groups, the CGGC permutation test was used.27 For in vivo studies with three or more treatment groups, a two-way ANOVA (Tukey’s post hoc) was performed at each time point. GraphPad Prism was used to perform statistical analysis. Data with a p value <0.05 were deemed statistically significant.

RESULTS

MB49OVA transduced cells express OVA and are recognized by OT-I T cells

In order to study antigen-specific T cell response using ACT, we developed a model using a strong antigen and target T cell. MB49 cells were transduced to express OVA which is recognized by OT-I T cells. OT-I mice are transgenic mice containing TCRα-V2 and TCRβ-V5 specific for OVA peptide residues 257–264 in the context of H2Kb. Figure 1A,B show successful incorporation of ZsGOVA
and surface expression of H2Kb SIINFEKL in MB49OVA cells as determined by flow cytometry. To confirm OT-I T cells are specific for MB49OVA cells, a co-culture was performed with the parental MB49 cell line, MB49OVA cell line and OVA SIINFEKL peptide as a positive control. IFN-γ production was measured in the co-culture supernatants by an ELISA assay. OT-I T cells specifically recognize MB49OVA cells and not the parental MB49 cells that lack OVA (figure 1C). TILs were isolated from MB49OVA subcutaneous tumors in C57BL/6 mice and a co-culture assay was performed under the same conditions (figure 1D). TIL produced IFN-γ after co-culture with MB49OVA cells and to a lesser degree with the parental MB49 cells. These data validate our MB49OVA/OT-I model, which will be used in subsequent experiments.

**Systemic ACT using OT-I T cells decreases tumor growth in MB49OVA subcutaneous tumors**

To investigate the potential for systemic ACT to treat bladder cancer, MB49OVA cells (5.0×10^5) were injected subcutaneously into female C57BL/6 mice. Twenty days later when tumors were established, mice were randomized and treated with NMAC (cyclophosphamide/fludarabine) followed by intravenous infusion of OT-I CD8^+ T cells (5.0×10^6 cells/mouse) and intraperitoneal injection of IL-2. ACT-treated mice had a reduction in tumor growth compared with control-treated mice (figure 2A). On day 50, tumors and spleens were processed, and immune cell populations were examined by flow cytometry. CD45.1^+ T cells, a congenic marker used to distinguish the transferred OT-I T cells from the recipient mice T cells, were detected both in the spleen and tumors of ACT-treated mice and not detected in control-treated mice (figure 2B,C). In addition, there was an increase in CD3^+ , CD8^+ , CD4^+ and CD8^+ OVA tetramer^+ T cells in the tumors of ACT-treated mice (figure 2C). An increase in CD8^+ OVA tetramer^+ T cells was also measured in the spleens of treated mice (figure 2C), with no difference in total T cells (online supplemental figure 1A). ACT-treated mice that had complete tumor regression were subsequently challenged with the parental MB49 subcutaneous tumors 100 days later. All mice rejected MB49 tumors compared with naïve mice (online supplemental figure 2A). Splenocytes taken from re-challenged mice were able to produce IFN-γ in a co-culture with either MB49 or MB49OVA tumor cells (online supplemental figure 2B). Lastly, flow cytometric analysis of the splenocytes
confirmed the presence of both CD45.1+ CD8 T cells and OVA tetramer+ CD8 T cells over 100 days after adoptive transfer of OT-1 T cells. These data suggest that systemic ACT can decrease tumor burden in our model, and that adoptively transferred T cells can persist systemically. In addition, epitope spreading can occur as mice were able to reject rechallenge with the parental MB49 cell line lacking OVA.

**T cell migration into bladder tumors occurs within 3 hours after intravesical instillation**

To determine the potential to treat bladder tumors with intravesical TIL, we first determined how long it took T cells to migrate into the tumor. Mice were inoculated with MB49OVA cells (1.0×10⁵) orthotopically. The presence of tumor in the bladder was confirmed by ultrasound-guided imaging. One-week post-tumor inoculation, tumor-bearing mice were treated with OT-I CD8+ T cells (4.0×10⁶ cells/mouse) label with CellTrace Violet (CTV). Mice were put under anesthesia for 1–3 hours with catheters in place to prevent the cells from leaking out of the bladder. Bladder tumors were collected and washed with PBS. Tumors were digested and analyzed by flow cytometry for the presence of CTV-labeled T cells. After 3 hours, CTV+ cells were detectable within the tumor digest (**figure 3A**). These data suggest that 3 hours is sufficient for T cells delivered intravesically to migrate into the tumor.

**Intravesical delivery of OT-I T cells decreases tumor growth in MB49OVA orthotopic tumors**

Next, to test the efficacy of intravesical ACT, mice were inoculated with MB49OVA orthotopic tumors. One-week later, tumor-bearing mice were randomized into two groups. Mice were placed under anesthesia for 3 hours and treated with OT-I CD8+ T cells (4.0×10⁶ cells/mouse) or PBS. Tumor volume was monitored by ultrasound-guided imaging and quantified using Vevo Lab V.3.1.0 software. When mice reached end point, tumors and spleen were collected and processed. Flow cytometry analysis was performed to determine T cell infiltration as well as detect OVA tetramer+ and CD45.1+ adoptively transferred T cells. Intravesical ACT prevented tumor growth and improved survival compared with PBS-treated mice (**figure 3B,C**, representative ultrasound images **figure 3E**). Treated mice had an increase in CD3+, CD8+ and OVA tetramer+ T cells within the tumor (**figure 3D**). No differences were seen in bulk CD3+, CD4+, CD8+ or CD8+ OVA tetramer+ cell populations in the spleens, which was expected since this was a local therapy (online supplemental figure 1B-C). Results from this experiment confirmed intravesical T cell delivery can control tumor growth without lymphodepleting chemotherapy or IL-2.
TIL expansion from MB49 orthotopic tumors and intravesical ACT

While an OVA-based tumor model can be used to optimize treatment strategies, OVA is a highly immunogenic foreign antigen and does not represent the antigens found in patient tumors. To more accurately model ACT using TILs, orthotopic MB49 tumors (1.0×10^5) were established in mice. Tumors were collected after 2 weeks and TILs were isolated. TILs were plated in 100 IU IL-2. Over the span of 25 days, TILs increased in number from 1.5×10^6 (1 well) to 39.2×10^6 (24 wells) as seen in figure 4A. IFN-γ production was determined by ELISA after co-culturing TILs with MB49 tumor cells after 1 or 7 days in culture (figure 4B). The fold increase of IFN-γ was highest after 7 days in culture compared with the CM condition. TILs were predominately CD3⁺CD8⁺ T cells (90.2%, figure 4C). Next, TILs isolated from 20 MB49 orthotopic tumors and expanded in culture for 7 days was adoptively transferred intravesically into mice bearing orthotopic MB49 tumors on day 8. Tumor growth was abrogated in mice treated with TILs compared with PBS control-treated mice (figure 4D, online supplemental figure 3). Two mice per group were euthanized at 3 and 24 hours after transfer. Tumors were collected and screened for CD45.1⁺ T cells (figure 4D). Mice treated with TILs coated with an αCXCR3 antibody had a reduction in CD45.1⁺ T cells within the tumor at both time points. In a second cohort of mice, tumor growth was monitored by ultrasound after treatment. CXCR3 antibody-coated OT-I T cells were not able to delay tumor growth compared with control rat.

CXCR3 is responsible for T cell infiltration into bladder tumors post-ACT

Next, to determine a potential mechanism by which adoptively transferred T cells migrate from the intra-vesical space into bladder tumors, we first screened orthotopic MB49 and MB49OVA bladder tumors for the chemokines CXCL9, CXCL10, and CXCL11 by PCR after tumor digestion and RNA isolation (figure 5A). We then confirmed expression of T cell activation and migration marker CXCR3, which binds CXCL9/10/11, on OT-I T cells, MB49 TIL and two patient TIL samples by flow cytometry (figure 5B,C). Blocking CXCR3 decreased migration of OT-I T cells in vitro (online supplemental figure 4). Next, we adoptively transferred OT-I T cells (3.5×10^6) into the bladders of mice bearing orthotopic MB49OVA tumors. T cells were coated with a CXCR3 blocking antibody or control rat IgG (rIgG). Tumors were collected 3 and 24 hours after infusion and analyzed for CD45.1⁺ T cell infiltration by flow cytometry. (E) Tumor volume was measured by ultrasound. Precoating OT-I T cells with αCXCR3 decreased T cell infiltration into the tumor and abrogated the decrease in tumor growth seen with OT-I T cells coated with a control rIgG antibody. N=5 per group. Repeated 2 times. ns, not significant.

**Figure 5** CXCR3 mediates T cell infiltration into orthotopic ovalbumin expressing MB49 tumor cell line (MB49OVA) bladder tumor from intravesical space. (A) RNA was isolated from MB49 and MB49OVA tumor cells and analyzed for expression of chemokines CXCL9, CXCL10 and CXCL11 as determined by PCR. (B) OT-I T cells were isolated from spleen of transgenic mice and tumor-infiltrating lymphocytes (TILs) were isolated from MB49 orthotopic tumors. Expression of CXCR3 was determined on these cell populations by flow cytometry. (C) TIL samples expanded from patient bladder tumors were analyzed for CXCR3 expression by flow cytometry. In vivo, mice were treated with OT-I T cells coated with a CXCR3 blocking antibody or rat IgG (rIgG). (D) Tumors were collected 3 and 24 hours after infusion and analyzed for CD45.1⁺ T cell infiltration by flow cytometry. (E) Tumor volume was measured by ultrasound. Precoating OT-I T cells with αCXCR3 decreased T cell infiltration into the tumor and abrogated the decrease in tumor growth seen with OT-I T cells coated with a control rIgG antibody. N=5 per group. Repeated 2 times. ns, not significant.
IgG-coated OT-I T cells (figure 5E). These data suggest that CXCR3 is at least in part responsible for T cell infiltration into bladder tumors from the intravesical space.

**DISCUSSION**

In this study, we have demonstrated the ability of TIL delivered systemically or intravesically to delay tumor growth in a murine model. In traditional ACT, TIL is administered systemically in a single dose after NMAC and followed with systemic IL-2 therapy.20 21 Bladder cancer provides us with a unique opportunity to capitalize on the potential power of TIL therapy by using multiple modalities of tissue collection and different delivery routes to target multiple stages of the disease. First, tumor tissue obtained from either TURBT, radical cystectomy or biopsy of distant metastases could potentially be used as a source of TIL.26 Second, TIL therapy could be administered by different routes; systemic delivery could treat patients with metastatic bladder cancer or those who have undergone a cystectomy and intravesical delivery could treat patients with NMIBC as a bladder-sparing treatment option. Patients with MIBC could potentially receive TIL in a neoadjuvant or adjuvant setting. Systemic TIL therapy could also be combined with systemic immune checkpoint inhibitors, which has been shown to improve the efficacy of ACT in patients with metastatic melanoma.20 21 We anticipate intravesical TIL delivery would abrogate the need for NMAC and systemic IL-2. This would greatly reduce toxicity associated with TIL therapy. Additionally, intravesical TIL therapy has the potential to be delivered multiple times and could be given in combination with intravesical IL-2 without the toxic side effects associated with systemic IL-2.20

We have shown using a model with a strong antigen that either systemic or intravesical delivery of tumor-reactive T cells can elicit a profound antitumor response. After systemic OT-I T cell delivery, adoptively transferred T cells were detected within the mouse over 100 days after transfer, suggesting long-term systemic survival. Additionally, mice that were re-challenged with MB49 tumor cells that lack OVA completely rejected tumor growth. This suggests epitope spreading has occurred and that T cells were educated against other mutations and/or neoantigens in MB49 tumor cells. Unlike our OVA/OT-I experimental model, TILs expanded from patient tumor samples represents a heterogeneous T cell population with varying degrees of activation and tumor specificity. To model TIL therapy more accurately, we expanded TILs from orthotopic MB49 tumors ex vivo and tested for reactivity against MB49 tumor cells. TILs were then used to treat recipient mice with orthotopic MB49 tumors and was able to delay tumor growth. We were also able to identify an important role of CXCR3 in T cell migration from the intravesical space into the tumor. CXCR3 is expressed by human TIL samples and human bladder tumors have been shown to overexpress CXCL9, CXCL10 and CXCL11.31-35 We may be able to exploit this signaling pathway to increase the efficacy of TIL therapy. However, there may be other factors, such as cell-adhesion molecules and integrin signaling, which promote T cell attachment and infiltration into the tumor.

Moving forward, we will continue to investigate the optimal treatment approach for both systemic and intravesical TIL delivery, including combination treatments with anti-PD-1 or anti-PD-L1 immunotherapy. Future studies will investigate ways to improve T cell infiltration into tumors and decrease immunosuppressive cell populations, such as regulatory T cells and myeloid-derived suppressor cells, within the tumor microenvironment. Taken together, these studies suggest ACT of TIL has potential to treat patients with bladder cancer and could greatly improve the quality of life of patients.

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**Contributors** Conception and design: SP-T, BLB, MB. Development and methodology: BLB, JM, PI. Acquisition of data: BLB, JM, SA, JB, AMA. Analysis and interpretation of data: BLB, SP-T, MP. Writing, review and/or revision of the manuscript: BLB, AMA, AH, MP, SP-T. Administrative, technical or material support: BLB, JM, SA, JB. Study supervision: SP-T, BLB, MP.

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**Competing interests** Moffitt Cancer Center has licensed Intellectual Property (IP) related to the proliferation and expansion of tumor-infiltrating lymphocytes to lovance Biotherapeutics. Moffitt has also licensed IP to TuAura Biopharma. SP-T is an inventor on such IP. SP-T participates in sponsored research agreements with Proventus Biopharmaceuticals, lovance Biotherapeutics, Intella Therapeutics and Myst Therapeutics that are not related to this research. SP-T has received research support that is not related to this research from the following entities: State of Florida Bankhead-Coley Cancer Research Program (7BC08), NIH-NCI (U01 CA244101-00 and R01 CA239219-01A1) and V Foundation. Additionally, SP-T is a co-investigator on NIH-NCI (U54 CA193489-01A1 and R01 CA241559) research support, which is not related to this research.

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**Ethics approval** Animal studies have been approved by the Institutional Animal Care and Use Committee at University of South Florida. Patient consent was obtained prior to surgical resection of human bladder specimens; the study was approved and overseen by the Institutional Review Board (MCC18142).

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