

ORIGINAL RESEARCH



Expansion of tumor infiltrating lymphocytes (TIL) from bladder cancer

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ABSTRACT

Advanced bladder cancer patients have limited therapeutic options resulting in a median overall survival (OS) between 12 and 15 months. Adoptive cell therapy (ACT) using tumor infiltrating lymphocytes (TIL) has been used successfully in treating patients with metastatic melanoma, resulting in a median OS of 52 months. In this study, we investigated the feasibility of expanding TIL from the tumors of bladder cancer patients. Primary bladder tumors and lymph node (LN) metastases were collected. Tumor specimens were minced into fragments, placed in individual wells of a 24-well plate, and propagated in high dose IL-2 for four weeks. Expanded TIL were phenotyped by flow cytometry and anti-tumor reactivity was assessed after co-culture with autologous tumor digest and IFN-gamma ELISA. Of the 28 transitional cell bladder or LN tumors collected, 14/20 (70%) primary tumors and all of the LN metastases demonstrated TIL expansion. Expanded TIL were predominantly CD3⁺ (median 63%, range 10–87%) with a median of 30% CD8 + T cells (range 5–70%). TIL secreted IFN-gamma in response to autologous tumor. Addition of agonistic 4-1BB antibody improved TIL expansion from primary bladder tumors regardless of pre-treatment with chemotherapy. This study establishes the practical first step towards an autologous TIL therapy process for therapeutic testing in patients with bladder cancer.

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Introduction



Bladder Cancer is the fourth most common cancer in men and a leading cause of cancer death among men and women.¹ Despite radical cystectomy for local regional disease approximately 40% of patients will fail treatment for curative intent.² Those patients are subsequently treated with salvage chemotherapy and/or radiation. Most responses to chemotherapy are partial and of short duration with median overall survival for metastatic disease between 12 and 15 months.³ While recent advances in systemic therapy have demonstrated a slight improvement in overall survival, prognosis remains poor with a substantial need for clinical improvement.

Bladder cancer has long been recognized as a malignancy that is responsive to immune-based therapy. As early as the 1970s, patients with localized non-muscle invasive bladder cancer have been treated with intravesical immune therapy with Bacillus Calmette-Guerin (BCG) instillations.⁴ Induction intravesical BCG, considered standard of care for high risk non-muscle invasive disease, has multiple mechanisms of actions for anti-tumor activity. These mechanisms include direct infection of tumor cells and initiation of a Th1 mediated immune response with CD4 + T cells and CD8+ cytotoxic T lymphocytes. In addition, stimulated by BCG, Tumor Necrosis Factor related apoptosis ligand (TRAIL)


released by neutrophils have also been demonstrated to have anti-tumor effects in bladder cancer.⁵ In clinically advanced medications targeting the PD1/PDL1 pathway have demonstrated an improvement in overall survival of 4 – 8 months with some durable responses but with only a 20% response rate overall.⁶ Unfortunately, this still leaves median overall survival (OS) slightly over one year in patients with metastatic disease and even worse for non-responders.^{7,8}

Previous studies have identified and characterized tumor infiltrating lymphocytes (TIL) within bladder cancer specimens. The presence of CD8⁺ TIL is associated with improved survival in patients with muscle invasive urothelial carcinoma while the frequency of immunosuppressive regulatory T cells and macrophages correlates with reduced survival.⁹ Tumors characterized by high CD4⁺ T cells, low regulatory T cells, and low CD68⁺ or CD163⁺ macrophages are associated with prolonged recurrence free survival in patients that responded to BCG therapy.¹⁰ Thus, while the profile of anti-tumor and pro-tumor immune subsets in bladder cancer are predictive of clinical outcomes, the presence of TIL alone is not associated with the suppression of tumor growth.

One mechanism of harnessing the power of TIL is through adoptive cell transfer (ACT). ACT using tumor infiltrating lymphocytes (TIL) has shown promise for patients with

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metastatic melanoma, cervical cancer and ovarian cancer.^{11–15} We have previously demonstrated efficacy of TIL therapy for patients with metastatic melanoma.¹⁶ The goal of this study was to assess the feasibility of isolation and expansion, and assessment of *in vitro* efficacy of the tumor infiltrating lymphocytes from bladder tumor specimens obtained from chemotherapy-naïve and exposed cases for future use in adoptive cell therapy.

Results

Demographics of patient samples

Primary bladder tumors obtained from radical cystectomy specimens or LN metastases were collected from 28 bladder cancer patients. Eight primary tumors were contaminated upon initial culture. Thirteen samples were collected from patients (46%) who had received neoadjuvant chemotherapy (NAC). Two patients were complete responders to NAC. The feasibility of TIL expansion was evaluated in 20 primary bladder tumor samples and 7 lymph node (LN) metastatic lesions. Demographics are shown in Supplemental Table 1.

Expansion and phenotype of TIL from bladder tumor specimens

Resected primary bladder tumors or lymph node metastases were minced into small fragments and plated as one

fragment per well in media containing 6000 IU/mL of IL-2. At 4 weeks, the number of expanded TIL was measured. As shown in Table 1, TIL expansion from primary bladder tumors was demonstrated in 14/20 specimens. LN metastases were also collected from 7 patients and TIL expansion was measured in all samples including 3 whom primary tumors were contaminated.

When we evaluated the expansion of TIL from treatment naïve patients compared to patients treated with chemotherapy, better TIL expansion was measured from bladder tumors resected from patients that had not received chemotherapy (Figure 1). We also measured the phenotype of expanded TIL, including T cells and NK cells by flow cytometry. As shown in Figure 2, there was no difference in the expansion of CD3 + T cells, CD4 + T cells, or NK cells between patients treated with chemotherapy or treatment naïve. In contrast, CD8 + T cells were significantly increased in TIL expanded from bladder tumors that were chemotherapy naïve.

Phenotype of immune cells in digested tumor

To determine whether the differences in TIL expansion between untreated patients and patients treated with chemotherapy was due to differences in the infiltrates into bladder tumors, we measured subsets of immune infiltrates in the primary bladder tumors. After initial set-up of tumor fragments, the remaining tumor was digested to a single cell suspension. Flow cytometric analysis of

Table 1. Expansion of TIL from bladder tumor fragments.

Sample #	Source of Tumor	Number of Fragments Plated	Number of Expanded Fragments	Total TIL Number
1	primary	12	1	1.50E+06
2	primary	12	10	9.20E+07
3	primary	12	3	3.10E+07
4	primary	12	6	3.60E+07
5	primary		contaminated	
6	primary	5	4	8.00E+06
7	primary	12	2	<1e6
8	primary	7	1	<1e6
9	primary	5	0	0
10	primary	5	2	3.50E+06
11	primary	6	4	2.20E+07
12	primary	10	0	0
13	primary	10	6	1.20E+07
14	primary	8	8	9.70E+06
15	primary	10	4	1.23E+07
16	primary LN		contaminated	
17	primary	12	4	1.74E+07
18	primary		0	0
19	primary		contaminated	
20	primary	6	0	0
21	primary		contaminated	
22	primary LN		contaminated	
23	primary LN	12	9	7.84E+06
24	primary	12	contaminated	
25	primary LN	12	4	4.65E+06
26	primary LN	12	2	6.00E+06
27	primary LN	12	contaminated	
28	primary LN	12	11	3.40E+07
		12	4	1.89E+07
		12	3	2.69E+07
		12	4	9.84E+06
		12	12	9.95E+07
		12	7	3.95E+07
		5	5	6.97E+07

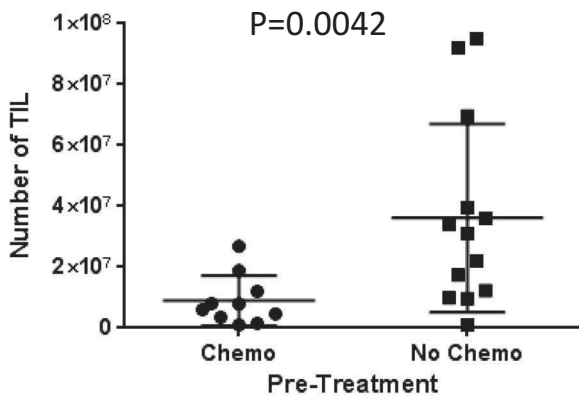


Figure 1. Expansion of TIL from bladder tumors. The total number of TIL expanded from bladder or LN tumor fragments was measured at 4 weeks after initiation of culture. Each point represents the total TIL generated from each fragment within an individual patient.

immune subsets was performed. We evaluated the percentage of CD3+, CD4+, and CD8 + T cells, CD56+ NK cells, CD19 + B cells, CD11b+ myeloid cells, and CD4+foxp3+

regulatory T cells (Tregs). As shown in [Figure 3](#), immune infiltrate patterns were similar in tumors resected from patients that were treatment naïve or treated with chemotherapy prior to surgery. These results suggest that pre-treatment with chemotherapy does not alter the immune cell composition within bladder tumors.

Reactivity of TIL to autologous tumor

We next measured the ability of expanded TIL to respond to autologous tumors. TIL expanded from each fragment was co-cultured with autologous tumor cells for 24 hours and supernatants were collected. IFN-gamma in supernatants was measured by ELISA. As shown in [Figure 4](#), in 6 representative patients, TIL from multiple fragments produced IFN-gamma in response to autologous tumor.

TIL growth after addition of anti-41bb antibody

We have previously demonstrated that addition of an agonistic anti-4-1BB antibody could improve the expansion of CD8 + T cells from melanoma tumor fragments.¹⁷ We evaluated

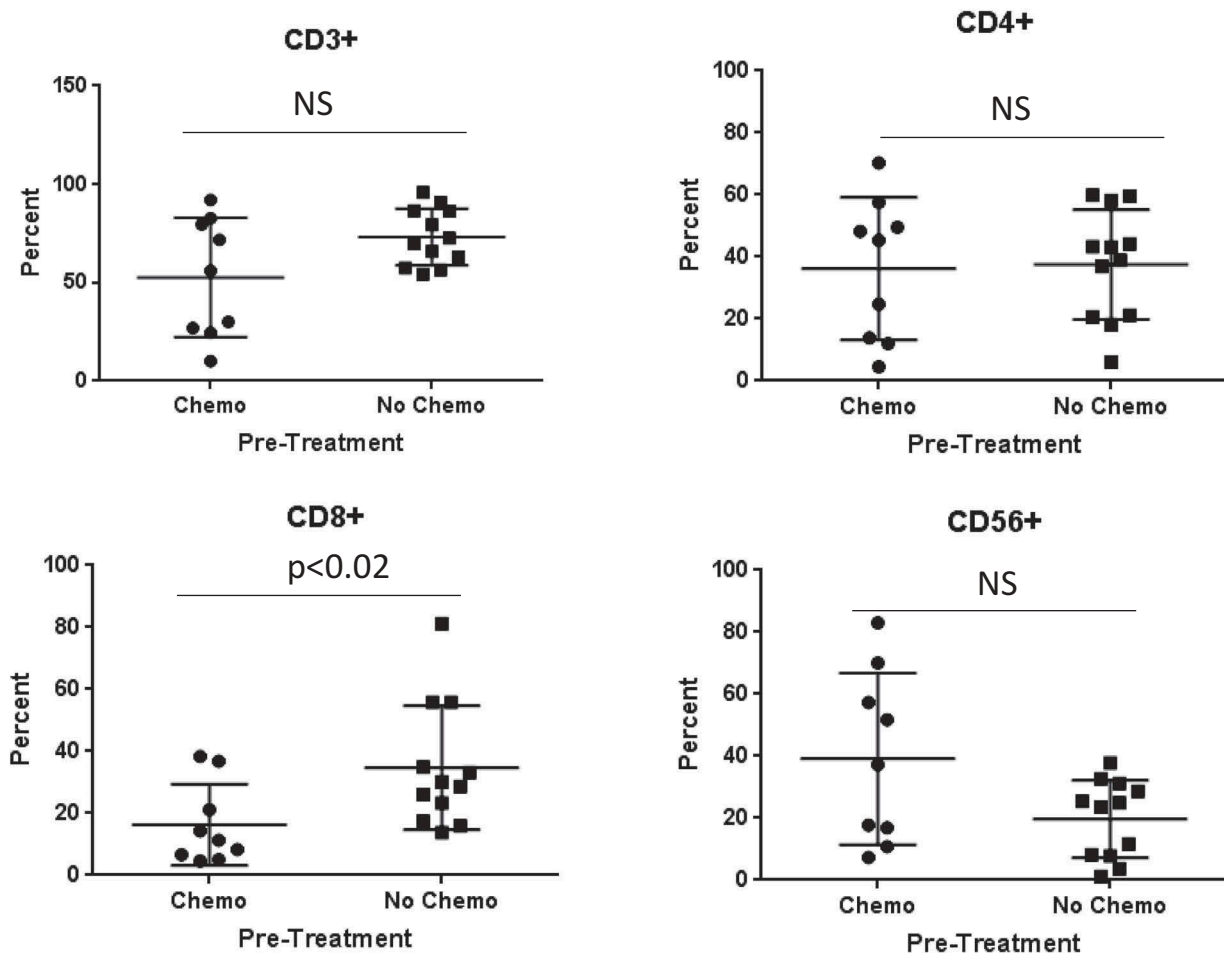


Figure 2. Phenotype of TIL expanded from primary bladder tumors. At four weeks after the initiation of TIL cultures, TIL were collected from each fragment and the percentage of CD3 + T cells, CD4 + T cells, CD8 + T cells, and CD3-CD56+ NK cells was measured by flow cytometry. Each point represents the mean percentage of cells generated from each fragment for an individual patient.

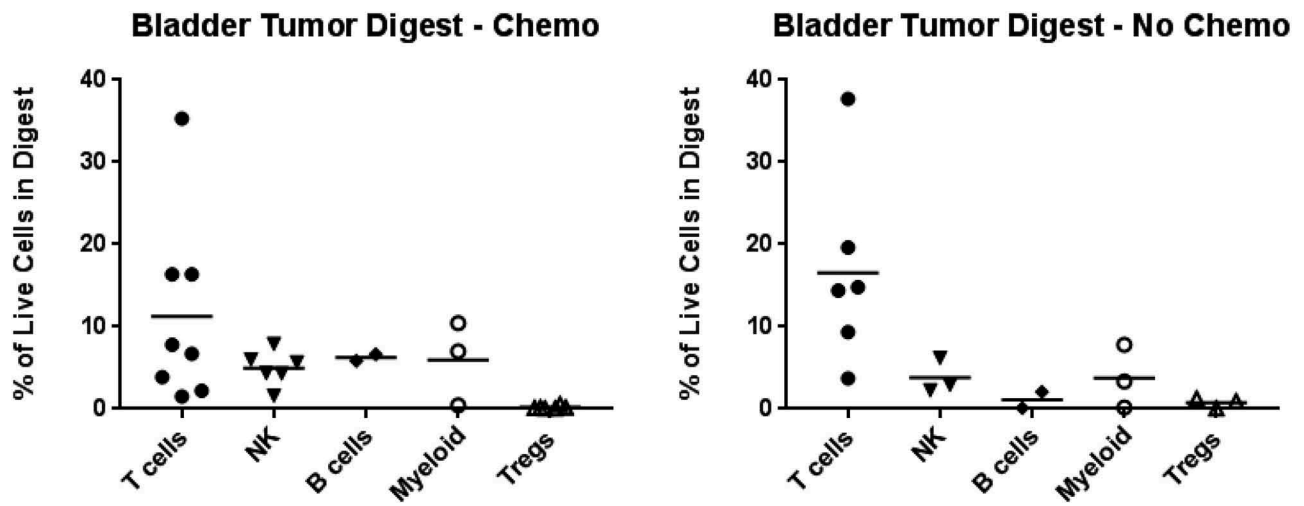


Figure 3. Immune infiltrates in primary bladder tumors. Primary bladder tumors were digested as described and the percentage of immune infiltrates were measured by flow cytometry.

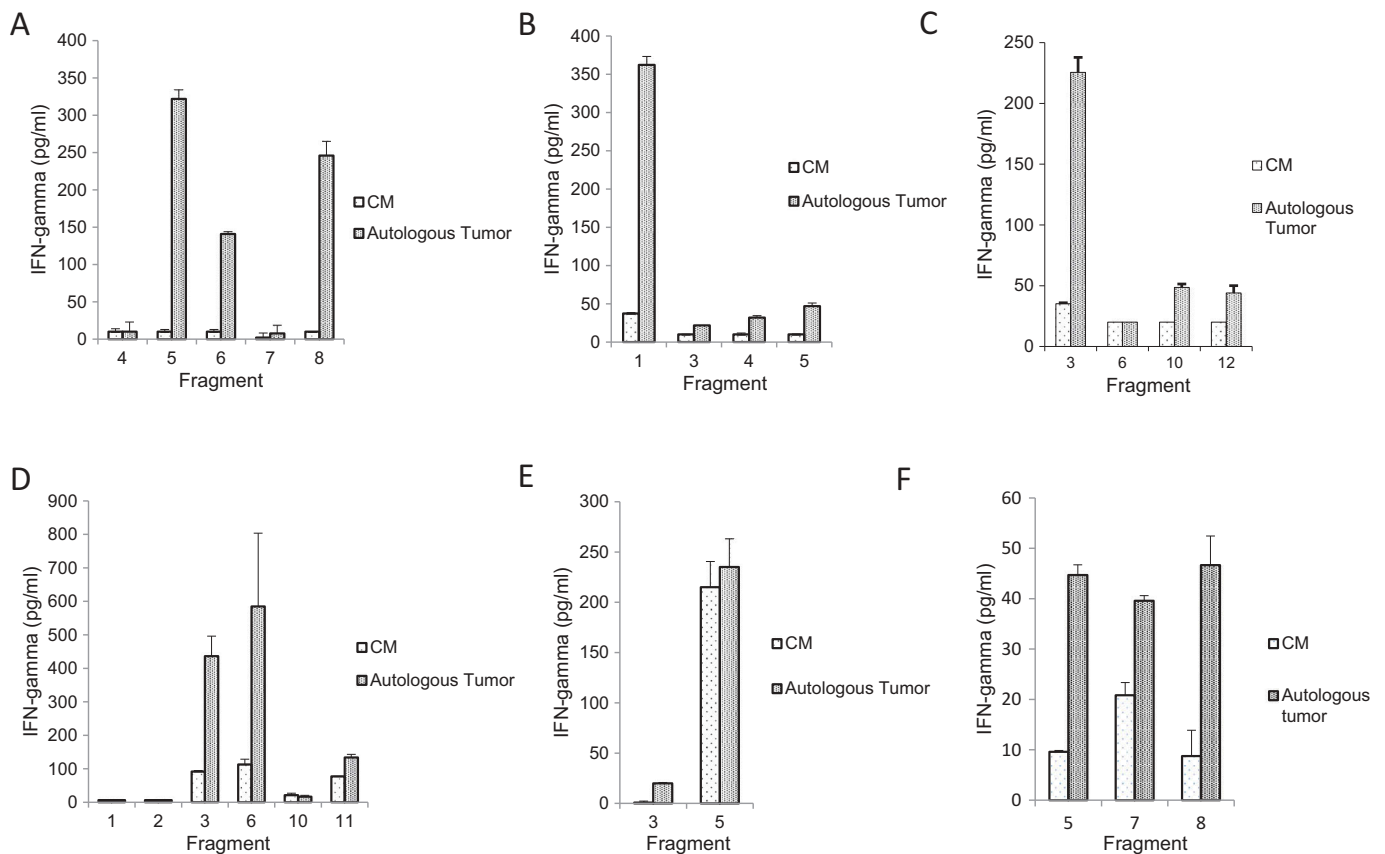


Figure 4. Reactivity of Bladder TIL. Expanded TIL was collected from individual fragments at 4 weeks after initiation of culture. TIL were co-cultured in complete media alone (CM), or at a 1:1 ratio with digested autologous tumor cells for 24 hours and supernatants were collected. IFN-gamma in supernatants was measured by ELISA. The results of 6 individual patients are shown.

whether 4-1BB agonism could also improve the expansion of T cells from primary bladder tumors. Addition of anti-4-1BB antibody to primary tumor fragments led to an increase in the number of fragments with TIL expansion and total TIL numbers (Table 2). Addition of 4-1BB agonistic antibody also improved the expansion of TIL from primary bladder tumors

resected from patients previously treated with chemotherapy. A significant increased expansion of CD8 + T cells within TIL was not measured after addition of anti-4-1BB antibody (Supplemental Figure 1). These results support the addition of agonistic 4-1BB antibody to expand overall TIL numbers from primary bladder tumor.

Table 2. Expansion of TIL after 4-1BB agonism.

Sample #	Number of Expanded Fragments		Total TIL Number		Neoadjuvant chemo
	IL-2	IL-2 + 4-1BB	IL-2	IL-2 + 4-1BB	
15	4	6	1.23E+07	2.65E+07	no
24	2	5	6.00E+06	2.40E+07	yes
26	4	5	1.89E+07	3.00E+07	yes
27	4	8	9.84E+06	1.10E+08	no
28	7	12	3.95E+07	1.32E+08	no
total	21	36	8.32 + 07	3.22E+08	

Rapid expansion protocol (REP) of bladder TIL

We also evaluated whether TIL expanded from primary bladder tumors could be expanded to clinically relevant numbers. Using a Rapid Expansion Protocol (REP), TIL were expanded over a one week period. Fold Expansion is shown in Supplemental Figure 2. In 3 of 4 samples, over 1000-fold expansion was measured, similar to our previous experience with melanoma. Together, these studies demonstrate the feasibility of expanding TIL for use in adoptive cell therapy strategies.

Discussion

Bladder cancer patients represent a promising population for the implementation of novel immunotherapeutic strategies. The purpose of the current study was to demonstrate feasibility of expansion of TIL from the primary tumors of patients with bladder cancer, including patients that had received NAC. In ovarian cancer patients, treatment with platinum or taxane-based chemotherapy led to an increase in CD8⁺ T cells at the tumor site but had no effect on pre-existing suppressive mechanisms.¹⁸ In esophageal cancer patients, treatment with NAC led to increased numbers of T cells infiltrating tumors.¹⁹ In this study, NAC did not significantly alter the types of immune cells infiltrating primary tumors of bladder cancer patients compared to patients that were chemotherapy naïve (Figure 3). For primary bladder specimens evaluated, 9 patients received NAC and 11 patients were chemo-naïve prior to surgery. Pre-treatment with NAC led to a slight decreased success rate of growing TIL from primary tumor (not including contaminated specimens), 66% of the samples yielded TIL while 73% of chemotherapy naïve primary tumors yielded TIL expansion. The time from NAC to surgery ranged from 36–175 days and did not have an effect on the ability to grow TIL from primary tumors. Interestingly, 1 patient who had a complete response to NAC had TIL growth, this is either secondary a microscopic focus of persistent tumor or lingering TIL at the site of the scar. However more importantly we were able to successfully harvest TIL from NAC and chemo naïve specimens.

Eight patients consented to this protocol demonstrated contamination within one week of culture and were discarded. Metastatic lymph node (LN) lesions were also collected from 7 patients and none of these samples demonstrated contamination. Contamination of primary samples was high in this study and may be due to the non-sterile conditions at the bladder site by presence of foley catheters.

As four of the lymph node samples were collected from patients in which the primary tumor was contaminated, breach of sterile technique at the time of tumor resection or at initiation of culture does not seem to be the reason for the contamination. While the phenotype of contaminants was not analyzed in these studies, it can be hypothesized that bacteria from the microbiome of the urinary tract may be involved.²⁰

The majority of TIL expanded from primary and LN tumors were CD3⁺ T cells. An increased percentage of CD8⁺ T cells expanded from the tumors of chemotherapy naïve compared to tumors from patients treated with NAC. We have previously shown that addition of an agonistic 4-1BB antibody can expand the number of reactive TIL from melanoma and pancreatic tumor fragments.^{17,21} Addition of agonistic 4-1BB antibody was also able to enhance the total number of TIL from primary tumors of bladder cancer patients that were chemotherapy-naïve or pretreated with NAC. In fact, in the two chemotherapy pretreated samples, agonistic 4-1BB was able to rescue expansion to an average of 2.7x10⁷ TIL. This number would fall into the 50th percentile of the chemotherapy-naïve group in Figure 1, and would be greater than any expansion seen in the NAC group.

In a review, McConkey *et al* compiled the results of four independent studies and identified four molecular subtypes of muscle invasive bladder cancer.²² One difference between the subtypes is the degree of cancer associated fibroblast (CAF) and immune cell infiltration. The infiltrated subtype (Cluster II) appears to be sensitive to immune checkpoint inhibitors. Our ability to grow TIL from tumor fragments may be associated with different molecular subtypes and requires further investigation.

In the early 1990s, Haas described the first isolation of TIL from urological tumors.²³ The majority of the lymphocytes were CD3⁺ T cells with variability in the CD4⁺/CD8⁺ T cell ratio after culture in high dose IL-2. Housseau *et al* subsequently characterized five TIL cultures from bladder cancer cultures and successfully evaluated *in vitro* cytotoxic effects against autologous tumor.²⁴ In this study, anti-tumor reactivity was measured in TIL expanded from primary and LN metastatic bladder tumors. While the specific antigens recognized by expanded TIL were not defined in this report, additional studies are ongoing to characterize neoantigens contained in bladder tumors. Evaluation of neoantigen-specific T cells as well as the diversity of T cell receptors within TIL of bladder tumors will be explored.

Traditional ACT strategies have focused on metastatic cancer patients in which one tumor lesion is resected for TIL growth and remaining lesions are untouched. Patients are then treated systemically with expanded TIL and regression of disease is measured. This study evaluated the feasibility of generating tumor-reactive TIL from primary bladder tumors and pelvic lymph node metastases in patients undergoing radical cystectomy. Of these patients, 50% are expected to relapse and would be potential candidates for TIL therapy at the time of disease progression. The translational approach would include TIL expansion from primary bladder tumors or lymph node metastases at the time of radical cystectomy. Tumor-reactive TIL would be cryopreserved. At progression, TIL numbers would be expanded in a REP and infused into the patient after treatment with a pre-conditioning regime such as cyclophosphamide

and fludarabine as previously described for the treatment of metastatic melanoma patients.¹⁶ Alternatively, to potentially avoid radical cystectomy, growth of TIL from transurethral surgical resection or resection of a lymph node metastatic lesion may allow for the expansion of TIL and subsequent neoadjuvant (or primary) administration followed by local delivery of TIL to primary bladder tumors.

Conclusions

In this report we establish that TIL in primary bladder tumors and lymph nodes can be harvested from patients, expanded and demonstrate anti-tumor activity in vitro. TILs can be grown from both chemotherapy-naïve and patients who have received NAC. This is the first step in bringing therapy known as Adoptive Cell Transfer to clinical trials for patients with bladder cancer.

Methods

Patient subjects

Patients older than 18 years of age with bladder tumor or bladder lesions suspicious for bladder tumor greater than 2 cm undergoing radical cystectomy were eligible for inclusion. Receipt of neoadjuvant systemic chemotherapy or prior intravesical BCG or chemotherapy were not contraindications to enrollment in the feasibility study. For patients that received neoadjuvant chemotherapy, the majority received Cisplatin-based chemotherapy. Those not eligible for Cisplatin received a Carboplatin-based regimen. Informed consent was obtained from all patients prior to tissue collection. The study was approved and overseen by the Institutional Review Board (MCC18142).

TIL cultures

Primary bladder tumors or lymph node metastases were minced into ~1–3 mm³ fragments and plated in media containing 6000 I.U./mL rhIL-2 (Prometheus). These cultures were expanded for up to four weeks in 24 well plates and confluent wells were split into additional wells. Some cultures were supplemented with 1 ug/ml anti-CD137 agonistic antibody (Urelumab, BMS-663513). TIL from each independent fragment was counted and flow cytometric analysis was performed.

Tumor digest

Remaining tumor material was mechanically and enzymatically digested using media containing 2% Collagenase Type IV and a GentleMACS Dissociator (Miltenyi, 130–093-235). Cells were counted by trypan blue exclusion and subjected to subsequent analysis or cryopreserved as functional assay targets.

Flow cytometry

Digested tumors were stained with fluorescent antibodies including CD3 (BD Biosciences, BDB563546), CD4 (BD Biosciences, BDB563875), CD8 (Biolegend, 300928), foxp3

(BD Biosciences, BDB560046), CD19 (BD Biosciences, BDB560728), and CD11b (BD Biosciences, BDB550019) to identify immune cell subsets. Expanded TIL was stained with fluorescent antibodies for CD3, CD4, CD8, and CD56 (BD Biosciences, BDB555516). All cells were stained with a Live/Dead Near-IR viability stain (Invitrogen, L10119) and fixed in 2% paraformaldehyde. Data were acquired on an LSR II flow cytometer and analyzed using FlowJo software (Treestar, Inc.).

Co-culture assay/elisa

TIL and tumor target cells were cultured at a 1:1 ratio (1x10⁵ cells each) overnight in round bottom 96-well plates. Autologous tumor cells from enzymatic digestion were used as targets. Supernatants were collected after 24 hours. IFN-gamma was measured using a Human IFNγ Quantikine ELISA Kit (R&D Systems, SIF50).

Rapid expansion of bladder TIL

Bladder TIL were stimulated with 30 ng/mL human anti-CD3 (OKT3, Ortho Pharmaceutical, CA-S 140608–64-6) in the presence of irradiated (5000 rad) allogenic PBMC feeder cells. TIL were cultured in REP Media I comprised of RPMI 1640, 2.05 mM L-glutamine (HyClone, Thermo Fisher Scientific, MT10040CM), 10% heat-inactivated human AB serum (Omega Scientific, HS-25), 55 μM 2-mercaptoethanol (Invitrogen, 21985023), and 10 mM HEPES Buffer (Mediatech, MT25060CI). On day 5, 70% of the media was replaced with REP Media II comprised of a 1:1 (v:v) mixture of REP Media I and AIM V (Invitrogen, 12055083). Media was supplemented with 6000 I.U./mL rhIL-2 on days 2 and 5. Cells were collected on day 8.

Disclosure of Potential Conflicts of Interest

This research work was supported by a Sponsored Research Agreement between Moffitt Cancer Center and Iovance Biotherapeutics. Moffitt Cancer Center has licensed Intellectual Property related to the proliferation and expansion of tumor infiltrating lymphocytes (TILs) to Iovance Biotherapeutics. SPT and AAS are inventors on such Intellectual Property.

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Notes on contribution

All the authors contributed and approved the final version. Design of the study: MP, MF, LK, JM, AS, and SP. Biological specimen procurement: MP, JZ, WS, JP, SG, and PS. Pathology: JD. Experimental work: MH, AJ, KK, MB, and PI. Data analysis and interpretation: MP, MH, AJ, KK, MB, PI, BB, LK, JM, AS, and SP. Final approval: MP, MH, AJ, KK, MB, PI, BB, MF, JZ, WS, JP, SG, PS, JD, LK, JM, AS, and SP.

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