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Programmed death-ligand 1 signaling pathway involves in bladder cancer growth and progression

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Abstract:

CONTEXT: Exploration of the biological property of programmed death-ligand 1 (PD-L1) signaling that may impact bladder tumor growth in humanized animals and cell culture.

AIMS: The aim of this study is to evaluate how PD-L1 signaling involves bladder cancer growth and progression.

SETTINGS AND DESIGN: This study design involves experimental *in vivo* and *in vivo* study.

SUBJECTS AND METHODS: A role of PD-L1 signaling pathway inhibition for bladder cancer growth was assessed in humanized immunodeficient animals carried main molecular subtypes of bladder carcinoma patient-derived xenografts and provided with selective anti-PD-L1 treatment; bladder cancer cells invasiveness was evaluated in mixed RT112/84 cells + $CD4^+$ cells culture incubated with PD-L1 blocker durvalumab. We used two-tailed Student's *t*-test to explore differences between main and control subgroups. Significance of intergroup comparison was measured with one-way ANOVA followed by the Tukey's or Newman–Keul's criterion. Survival curves were analyzed with Gehan's criterion with the Yate's correction. Differences were considered statistically significant at $P < 0.05$.

RESULTS: Anti-PD-L1 intervention increased survival of the animals carried both primary and relapsed luminal noninvasive, muscular invasive, and relapsed luminal bladder cancer xenografts. There was significant retardation of tumor volume duplication time in aforementioned subgroups correlated with PD-L1 expression. Durvalumab treatment in concentration-dependent manner inhibited tumor cells invasiveness of mixed RT112 + $CD4^+$ culture cells with its maximum at the highest studied concentration (10 μM).

CONCLUSIONS: Obtained data constituted the pivotal role of programmed cell death-1/PD-L1 signaling pathway in bladder cancer development and progression. The results will have major implications for further clinical investigations.

Keywords:

Anti-programmed death-ligand 1 treatment, invasiveness, metastasis, nonmuscular invasive bladder cancer, patient-derived xenograft

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Introduction

Recent investigations of bladder carcinoma have identified distinct molecular and genomic markers associated with the cancer progression, metastasis, and response to therapeutic manipulations. Several scientific teams have used whole-genome expression profiling and wide panels of molecular markers to classify bladder cancer into basal, luminal, and p53 subtypes.[1] The three intrinsic subtypes of bladder cancer have shown distinct clinical behaviors and responses to front-line chemotherapy. In the chemotherapy-naive setting, the nonmuscle invasive bladder cancer (NMIBC) of the basal subtype is more aggressive with shorter survival when compared to luminal subtype

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of NMIBC. On the other hand, basal bladder cancers have been more sensitive to particular chemotherapy, and patients with this form of the lesion have appeared to gain more benefits from front-line chemotherapy when compared to luminal subtypes.[2]

Programmed death-ligand 1 (PD-L1) is a member of the B7 family of co-stimulatory molecules; it is a cell surface glycoprotein that promotes apoptosis by binding to its surface receptor, programmed cell death-1 (PD-1), in T-cells and B-cells, thereby inhibiting host immune function. PD-L1 has also been implicated in tumor immune escape.[3-5]

Since the classification of bladder cancer into intrinsic molecular subtypes provided prognostic information and might help to identify a subgroup of patients with increased sensitivity to chemotherapy, we have figured out to perform experimental study in laboratory animals and cell lines to elucidate the role of PD-L1 signaling pathway for growth and progression of the main molecular subtypes of NMIBC.

The aim of this study was to evaluate how PD-L1 signaling pathway involved in bladder cancer growth and progression. The study secondary objectives were as follows: (1) to evaluate a role of PD-L1 signaling pathway inhibition for bladder cancer growth in humanized immunodeficient animals with main molecular subtypes of bladder carcinoma patient-derived xenografts (PDXs) provided with selective anti-PD-L1 drug treatment; (2) to assess NMIBC cells invasiveness in mixed RT112/84 cells + $CD4^+$ cells culture incubated with selective anti-PD-L1 antibody.

Subjects and Methods

Ethics

All the protocols for animal studies were reviewed and approved by the Ethics Committee of Sechenov University (Moscow, Russia) and Russian National Research Medical Center of Radiology (Moscow, Russia) at Joint meeting March 20, 2017, Number of Approval 14 (No. 14/03-2017).

Animals

Six- to eight-week-old immunodeficient NOG/SCID female mice were obtained from Pushchino specific-pathogen-free (SPF) Animals Breeding Center (Pushchino, Russia). Animals were raised in the facilities of Sechenov University and housed in separately ventilated cages. Mice were kept under SPF condition on natural daylight cycles. Autoclaved standard food and water were provided *ad libitum,* and room temperature (25°C ± 2°C) and humidity (60 ± 10%) were maintained.

Patient-derived xenografts establishment

Fresh tumor tissue samples were removed from patients through cystoscopy with biopsy or tumor resection at the National Research Medical Center of Radiology (Moscow). Informed consent to participate in the study was received from each patient. Fresh tumor tissues were divided into three pieces under sterile conditions as previously described.[6] One piece of each tissue specimen was immediately placed in particular medium without antibiotics and fetal bovine serum for storing at 4°C until engraftment. Another piece was cryopreserved for molecular biological examination, and the other piece was fixed in 10% formaldehyde for histological examination (HE). Before engraftment, we selected samples of luminal, p53, and basal subtypes of newly diagnosed NMIBC, relapsed luminal and basal tumor, and MIBC (1 sample of each kind) by HE, clinical cases reviewing, and GATA3, KRT5/6 expression analysis as well as PD-L1 expression level detection. Only PD-L1-expressing tumors were accepted for further inoculation. The piece of each subtype of the tumor assigned to engraftment was divided into small pieces and inoculated into the dorsal subcutis of a nude mouse. After the engrafted mass expanded to over quadruple its size, the xenograft tumor was harvested and directly re-transplanted for expansion in later serial generations using the same procedure. After the tumor tissue was passaged three times and HE confirmed the PDX to be a growing human tumor, the PDX line of each subtype of bladder cancer was considered as "established." The animals (n = 20 for each line), acceptors of PDXs, assigned to the referred subtypes of bladder cancer first underwent sub-lethal irradiation and then were subjected to simultaneous transplantation of human lymphocytes (5×10^7 cells/mouse) intraperitoneally and PDX pieces subcutaneously as mentioned above.[7,8]

Specific intervention and animals' surveillance – Pain control

When tumors were clearly palpable and reached a volume of 100–200 mm^3, animals carried each PDX line were randomly allocated into two subgroups (n = 10 in each subgroup). Animals of the first one received durvalumab (Imfinzi™, AstraZeneca) (118.0 mg/kg intravenously (IV), two times: first injection at the day of allocation and the other administration 4 weeks afterward the first one) while mice of the second subgroup received vehicle alone (control, n = 10; phosphate-buffered saline [PBS] at the same volume as that of the test group). Durvalumab murine dose was calculated on the base of acute toxicity data available, the drug efficacy and safety data obtained in clinical trials as an effective dose value for humans (10 mg/kg) multiplied on converting coefficient for mice (11.8).[9-11] Mice received injections through lateral tail vein with the assistance of Genie Touch™ Syringe Pump (Kent Scientific Corporation,

USA). Tumor growth was followed twice weekly from Day 1 after treatment cessation by serial caliper measurement. Tumor volume was calculated using well-known formula.[12] Tumor-doubling time in all the test and control groups was defined as the period required to double the initial tumor volume (200%). Animals' survival curves were plotted by mice death registering during the postinterventional period. Tumor growth was analyzed by maximal tumor inhibition (treated/control [T/C], calculated as [median tumor volume of treated mice/median tumor volume of control mice] ×100). We used facial scale model for pain control in the experimental groups.[13,14] All animals with moderate pain received ketoprofen (K1751, Sigma-Aldrich, Germany, substance purity >98%) intragastrically (100 mg/kg twice a day).[15] Mice with severe uncontrolled pain were euthanized. Tumors of both euthanized and perished animals were removed and measured. Lungs were harvested, and the number of visible surface metastases was counted.

Immunohistochemistry

To explore the expression level of luminal NMIBC marker GATA3 we used monoclonal antibody against human GATA3 (HG3-31 clone, dilution, 1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA). We used monoclonal antibody against human KRT5/6 (D5/16B4 clone, 1:50 dilution, Dako) as marker of basal subtype of bladder cancer. To detect the expression of PD-L1, 4-μm-thick histological sections were prepared and incubated with normal goat serum for 1 h at room temperature to block endogenous peroxidase activity. Sections were incubated at 4°C overnight with primary antibodies against PD-L1 (dilution, 1:200; catalog No. ab58810; Abcam, Cambridge, UK). Immunohistochemistry staining was performed using the VENTANA PD-L1 (SP263) Assay according to the manufacturer's protocols. Sections were counterstained with hematoxylin and then dehydrated, cleared, and mounted. To evaluate the specificity of the reaction, PBS was used for replacing the primary antibody as the negative control. Subsequent to being washed twice in PBS, the sections were incubated with biotinylated secondary antibody (dilution, 1:500; catalog No. E0466; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 20 min at room temperature, washed twice in PBS and then incubated with horseradish peroxidase-labeled streptavidin (dilution, 1:2,000; catalog No. K5007; Dako; Agilent Technologies, Inc., USA) for an additional 20 min. The results were visualized by fluorescence microscopy following chromogenic staining with diaminobenzidine (Wuhan Boster Biological Technology, Ltd.) and analyzed using particular software. The percentage of PD-L1-positive tumor cells among the total number of tumor cells was scored in five randomly selected high-power fields (HPFs) (×400) and percentages >25% were classified as PD-L1-positive.

Cell culture development

To evaluate how PD-L1 signaling pathway blocking impacts bladder cancer cells migration and invasion we carried out experiments in cell culture.[16] Grade 2 human bladder cancer RT112/84 cell line was obtained from and maintained as recommended by Experimental Oncology Institute of National Research Medical Oncology Center (Moscow, Russia). $CD4^+$ T-cells were isolated from human peripheral blood mononuclear cells and then cultured with RT112/84 cells at a 1:5 ratio for 48 h.

Invasion assay

Cell invasion assay was carried out with 24-well 8-μm-pore-size polycarbonate Nucleopore filters (Costar, USA).[17] Filters were stained with Diff-Quick (Fisher Scientific, USA), and cells were counted in 6 HPFs. RT112/84 cells (1×10^5 cells/100 μl) were added to the upper chamber of Matrigel-coated, 8-μm pore size polycarbonate inserts. Cells were allowed to invade matrix-coated inserts to the bottom chamber. Invasion assays were carried out for 24 h. Cells attached to the bottom surface of the inserts were stained with DiffQuick and counted. Results were represented as relative invasion to the appropriate control group.[18] Before loading into the chamber mixed RT112 cells with $CD4^+$ T-cells culture was incubated with 1, 5, and 10 μM PD-L1 inhibitor durvalumab (main groups) and a similar volume of PBS (control group).

Statistical data analysis

Data obtained were processed with the assistance of SPSS software (release 16.0 IBM, USA). We used two-tailed Student's *t*-test to explore differences between main and control subgroups. Statistical significance of intergroup comparison was measured with one-way ANOVA followed by the Tukey's or Newman–Keul's criterion. Survival curves were analyzed using Gehan's criterion with the Yate's correction. Differences were considered statistically significant at $P < 0.05$.

Results

Programmed death-ligand 1 expression and animals' survival

Six lines of main molecular subtypes of NMIBC heterotopic PDXs were successfully developed. All the lines of maternal tumors expressed PD-L1 [Figure 1] as did all tumors excised from animals of control subgroups. Specific anti-PD-L1 treatment sufficiently decreased the number of PD-L1-positive cells in PDXs of all durvalumab-treated mice. Survival of the animals, PDX carriers, was different in subgroups and depended on both the tumor molecular type and implemented intervention [Figure 1]. In control subgroup with luminal NMIBC animals began to perish from day 38

after the tumor engraftment until day 53 with average survival time (AST) of 46.2 ± 4.1 days. AST of mice carried basal NMIBC PDX was 43.4 ± 3.8 days, whereas AST of animals in the subgroup with p53 NMIBC was 41.5 ± 3.5 days. Survival of animals with relapsed lines of bladder cancer xenograft was the shortest among noninvasive tumors: 31.5 ± 2.4 days for GATA3-positive cancer (P = 0.003 when compared with primary tumor) and 27.3 ± 2.6 days for KRT5/6-expressed line (P = 0.005 when compared with primary tumor). In MIBC subgroup, AST of experimental mice was 37.8 ± 4.2 days. Anti-PD-L1 intervention prolonged animals' life expectancy to 83.0 ± 5.7 days (P = 0.001 when compared with appropriate control subgroup) in luminal primary NMIBC subgroup, to 74.5 ± 4.0 days (P = 0.001 when compared with noninterventional subgroup) in luminal relapsed bladder cancer subgroup, to 67.8 ± 3.5 days (P = 0.03 when compared with noninterventional subgroup) in basal primarily bladder cancer and to 77.8 ± 5.3 days (P = 0.002 when compared with noninterventional subgroup) in subgroup of animals carried MIBC PDX. There were no significant differences in AST among mice with basal relapsed and p53 NMIBC PDXs treated with durvalumab and animals of appropriate noninterventional subgroups.

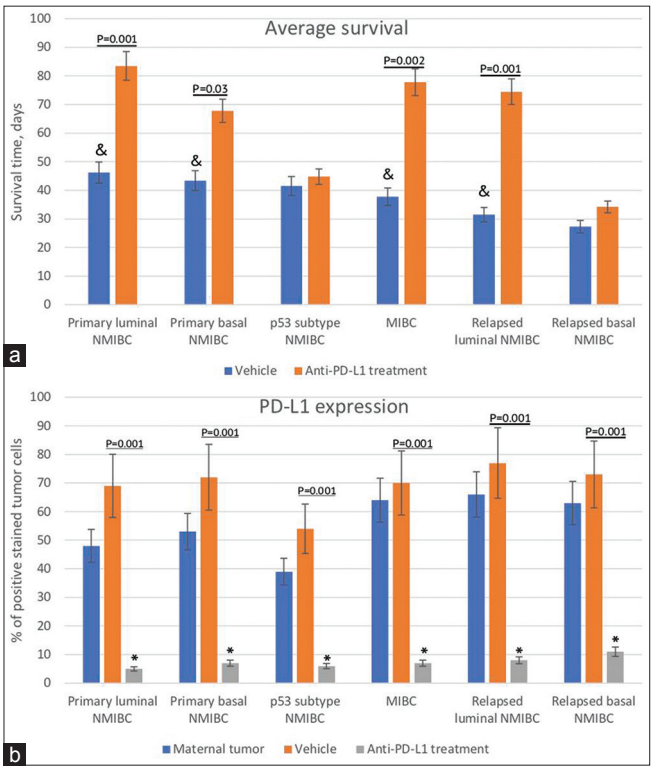

Figure 1: (a) Average survival of animals in experimental groups; Average survival (n = 10) presented in days of life (M ± MSE); $^{\&}P < 0.05$ when compared with control (Gehan's criterion with Yates's correction). (b) Programmed death-ligand 1 expression in bladder cancer patient-derived xenografts in dependence on anti-programmed death-ligand 1 treatment; programmed death-ligand 1 expression depicted in average % of positively stained tumor cells (M ± MSE) in maternal tumor used for engraftment, in patient-derived xenograft of control subgroup mice and animals utilized specific therapy (n = 20 in maternal tumor group; n = 10 in each subgroup); $^{*}P < 0.05$ (one-way ANOVA, Newman–Keuls's criterion)

Tumor growth

Anti-PD-L1 treatment (durvalumab 118.0 mg/kg IV two times) inhibited tumor growth in animals of experimental subgroups [Table 1] except p53 subtype NMIBC and relapsed basal NMIBC subgroups where T/C index was under 50 value at all check-points, and there was no difference in tumor-doubling time in comparison with relative vehicle control. T/C index measured on days 7, 14, and 21 after the second anti-PD-L1 antibody IV injection was >75 in primary luminal NMIBC subgroup at all check-points, in MIBC and relapsed luminal NMIBC subgroups on Day 7 of measurement. 21.2 ± 2.8 days after anti-PD-L1 treatment, cessation PDX volume duplication was registered in primary luminal bladder cancer subgroup versus 9.4 ± 0.3 days

Table 1: Anti-programmed death-ligand 1 treatment inhibits bladder cancer patient-derived xenografts growth and lung metastasis (initially n=10 in each subgroup)

Tumor subtype	T/C index			Subgroup of animals	M±MSE	
	Day 7	Day 14	Day 21		Tumor-doubling time, days	Number of metastasis
Primary luminal NMIBC	89	80	76	V	9.4±0.3	24.6±3.9
				D	21.2±2.8†	0±0†
Primary basal NMIBC	71	65	63	V	10.8±1.6	16.5±2.4
				D	17.3±1.7†	4.3±1.7†
p53 NMIBC	32	17	4	V	8.3±0.9	44.7±4.5‡
				D	12.7±2.6	16.2±5.8†
MIBC	76	62	50	V	9.6±0.7	56.1±6.4‡
				D	15.2±1.4†	13.5±4.3†
Relapsed luminal NMIBC	77	58	53	V	10.0±0.5	47.8±6.1‡
				D	18.3±1.9†	7.2±3.5†
Relapsed basal NMIBC	47	29	17	V	10.1±0.7	63.4±7.5‡
				D	13.4±2.1	22.8±5.4†

†P<0.05 when compared with relative control subgroup (Student's t-test); ‡P<0.05 when compared with primary both luminal and basal NMIBC subgroups (one-way ANOVA, Tukey's criterion). V: Vehicle (control subgroup), D: Durvalumab-treated mice, MIBC: Muscle invasive bladder cancer, NMIBC: Non-MIBC, T/C: Treated/control

in control subgroup (P = 0.001). 17.3 ± 1.7 days after completion of experimental treatment, we registered tumor volume doubling in primary basal NMIBC subgroup versus 10.8 ± 1.6 in control mice (P = 0.01). In subgroup of animals carried MIBC PDX tumor volume doubled after 15.2 ± 1.4 days of treatment cessation (P = 0.05 when compared with vehicle). Duplication of relapsed luminal NMIBC tumor volume was observed 18.3 ± 1.9 days (P = 0.03 when compared with vehicle) after completion of pharmacological intervention.

Anti-metastatic property

All PD-L1-expressed bladder cancer PDXs gave lung metastasis, but there were significant differences in their number [Table 1]. Durvalumab 118.0 mg/kg IV prevented metastatic action of primary luminal NMIBC PDXs and significantly decreased number of visible superficial lung metastasis given by: primarily basal bladder cancer PDXs to 4.3 ± 1.7 (16.5 ± 2.4 in control, P = 0.03), p53 subtype PDXs to 16.2 ± 5.8 (44.7 ± 4.5 in control, P = 0.01), MIBC PDXs to 13.5 ± 4.3 (56.1 ± 6.4 in control, P = 0.001). In relapsed tumor subgroups, the anti-metastatic property of specific anti-PD-L1 treatment remained. Thus, number of superficial lung metastasis of mice carried relapsed luminal bladder cancer PDX and utilized durvalumab was 7.2 ± 3.5 versus 47.8 ± 6.1 in subgroup of animals received vehicle alone (P = 0.001), and similarly, IV introduction of anti-PD-L1 antibody lowered metastasis number in animals with relapsed basal bladder cancer to 22.8 ± 5.4 versus 63.4 ± 7.5 in control group (P = 0.03).

Assessment of invasiveness

In 24 h after mixed RT112 + CD4$^+$ culture, cells were allowed to invade Matrigel-coated chamber inserts we counted Diff-Quick-stained tumor cells. Durvalumab in concentration-dependent manner inhibited invasive property of cells [Figure 2] with its maximum at the highest concentration studied (10 μM).

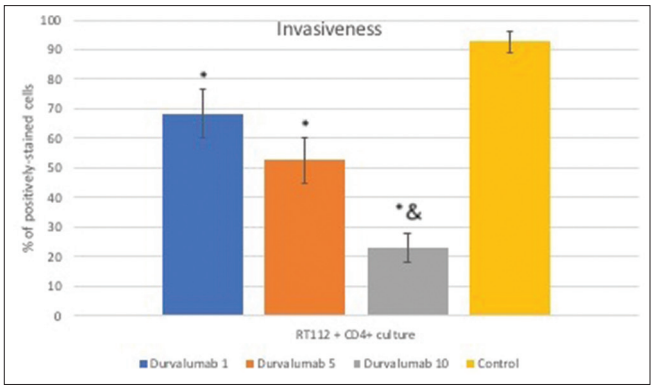

Figure 2: Invasiveness of mixed RT112 + CD4$^+$ culture cells depends on anti-programmed death-ligand 1 antibody concentration (μM, n = 6 for each concentration): Results represented percentage of Diff-Quick-stained tumor cells; *P < 0.05 in comparison with control, $^{\&}P$ < 0.05 intergroup comparison (one-way ANOVA, Tukey's criterion)

Discussion

The important role of PD-1/PD-L1 signaling axis in tumor growth and progression has been shown for many malignancies such as melanoma, orofacial carcinomas, malignant neoplasia of the kidney, lung, bladder, etc.[19-21] Thus, Vandeveer *et al*. showed promising antitumor activity of avelumab, an antibody to PD-L1 in MB49luc *in vitro* model of nonmetastatic noninvasive bladder carcinoma.[22] High predictive potency of PD-L1 mRNA expression in NMIBC was identified by Breyer *et al*.[23] Identification of luminal, basal, and p53 subtypes of bladder cancer highlighted molecular patterns in the tumors' progression, prediction, and their different response to Bacillus Calmette-Guerin (BCG) immunotherapy.[1] At the same time, involvement of PD-L1 signaling in different subtype of NMIBC growth and progression remained unclear. To explore the issue, six PD-L1 expressed heterotopic PDXs were developed in humanized NOG/SCID mice. Among them, four animal models represented primary bladder tumors such as GATA3-expressed/luminal, KRT5/6-expressed/basal, p53 subtype of NMIBC, and muscular-invasive bladder cancer. Two groups of mice were used to establish relapsed luminal and basal forms of NMIBC. We used durvalumab, an IgG1T monoclonal antibody to PD-L1 approved by the FDA for previously treated patient with advanced bladder cancer immunotherapy in 2017, to block PD1/PD-L1 signaling in each animal PDX model.

Having comparatively analyzed animals' survival curves and tumor volume it turned out that PD-L1 blockade inhibited tumor growth in not all subtypes of bladder carcinoma. In particular, relapsed basal and p53 NMIBC PDXs tolerated anti-PD-L1 treatment despite significant depression of PD-L1 expression in comparison with appropriate control subgroups. Conversely, statistically significant stagnation of metastatic activity of all subtypes of established PDXs underlined involvement of PD-L1 signaling in the mentioned process. Possible explanation of the results acquired may be found in the heterogeneity of T-cells response to specific therapy in cohorts of patient with different disease history, molecular profiles, and previously endured interventions.[24-27]

Tumor cells of human Grade 2 nonmuscular invasive bladder cancer RT112 culture mixed with CD4$^+$ cells and incubated with 1, 5, and 10 μM durvalumab displayed different invasiveness depended on anti-PD-L1 antibody concentration. This is consistent with previously obtained data of concentration-dependent inhibition of T-cells PD-L1 activity by durvalumab in mixed lymphocyte activation assay.[1,28] However from the point of our

study concept, it would be more profitable to assess PD-L1-associated invasiveness on models represented all molecular subtypes of bladder carcinoma. That shall be a subject for further exploration.

In summary, PD-1/PD-L1 signaling blockade depressed metastatic activity of all main molecular subtypes of human primary NMIBC relapsed NMIBC, MIBC on animal PDX model of tumors expressed PD-L1. Growth of both relapsed basal bladder cancer and p53 NMIBC was not inhibited by anti-PD-L1 treatment despite significant depression of PD-L1 expression. PD-L1 inhibition depressed tumor cells invasiveness of mixed RT112, human grade 2 bladder cancer, cells + $CD4^+$ cells culture.

Conclusions

1. Specific anti-PD-L1 treatment sufficiently decreased the number of PD-L1-positive cells in PDXs of all durvalumab-treated mice. Survival of the animals, PDX carriers, was different in subgroups and depended on both the tumor molecular type and intervention implemented. Survival of animals with relapsed lines of bladder cancer was the shortest among noninvasive tumors
2. Anti-PD-L1 intervention prolonged animals' life expectancy and depressed tumor growth in the majority of subgroups assigned to treatment except for ones with relapsed basal type of bladder cancer and p53 subtype of NMIBC
3. Durvalumab inhibited metastatic activity in all subgroups of animals, PD-L1-expressed bladder carcinoma PDX carriers
4. Monoclonal anti-PD-L1 antibody in concentration-dependent manner inhibited tumor cells invasiveness of mixed RT112 + $CD4^+$ culture cells with its maximum at the highest studied concentration (10 μM).

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Conflicts of interest

There are no conflicts of interest.

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