Bladder Cancer Immunomodulatory Effects of Intravesical Nitazoxanide, Rapamycin, Thalidomide and Bacillus Calmette–Guérin (BCG)

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Running Head: Intravesical Nitazoxanide, Rapamycin, Thalidomide and Bacillus Calmette–Guérin (BCG)

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Abstract

Purpose: To understand the effect of Nitazoxanide (NTZ), Rapamycin, Thalidomide, alone and in combination with BCG on bladder cancer (BC) histopathology and programmed death-ligand 1 (PD-L1) and anti-cytotoxic T-lymphocyte antigen 4 (CTLA4) expression.

Methods: Female Fisher-344 rats underwent intravesical N-methyl-N-nitrosourea (MNU) followed by weekly intravesical treatment with saline (controls, n = 10), BCG (n = 10), NTZ (n = 8), BCG plus NTZ (n = 8), Rapamycin (n = 10) BCG plus Rapamycin (n = 10), Thalidomide (n = 10), and BCG plus Thalidomide (n = 10), and euthanized after eight weeks and their bladders were investigated for BC and PD-L1 and CTLA4 expression.

Results: Rapamicyn alone and in combination with BCG had the lowest number of bladder neoplasias in the histopathology exam (1/10). Neoplastic lesions were found in 4/10 BCG recipients, 5/10 Thalidomide recipients, 4/10 Thalidomide plus BCG recipients, 5/8 NTZ and 3/8 NTZ plus BCG recipients. Adding NTZ to BCG increased the expression of PD-L1 and adding Rapamycin or Thalidomide decreased PD-L1 and CTLA4 expression compared to BCG alone. Rapamycin alone significantly increased CTLA4 and slightly increased PD-L1 expression but its combination with BCG significantly decreased both markers. Thalidomide had a similar effect; however, it was only slightly different from the control and BCG alone groups.

Conclusion: Intravesical BCG combination treatment seems to effectively prevent BC development in an immunecompetent clinically relevant animal model, introducing Thalidomide, Nitazoxanide, and specially Rapamycin as candidates in the intravesical immunotherapy advancement. Our study contributes in understanding the mechanism of cancer immunotherapy.

Key-words: immunecheckpoint, bladder cancer, nitazoxanide, rapamycin, thalidomide, BCG

Introduction

Around 3% of all global cancer arise from the bladder [1]. In the United States, bladder cancer (BC) is the 6th most common cancer and in Brazil, around 11,370 new cases are estimated for the year 2022, and about 70% are non-muscle invasive BC [NMIBC] [2].

Currently, the mainstay of treatment for NMIBC is resection of the tumor followed by weekly intravesical injection of Bacillus Calmette–Guérin (BCG) [3]. BCG is an imperfect treatment and about 40% of its recipients' experience recurrence. Recently, a new immunotherapy called immune checkpoint inhibition (ICI) has been approved for advanced BC and BCG resistant NMIBC. In this treatment modality, certain cell surface molecules are targeted that are known to help the cancer cells escape the immune system [4]. Programmed death-ligand 1 (PD-L1) and anti-Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) are the two main targets of this treatment modality.

Nitazoxanide (2-(acetyloxy)-N-(5-nitro-2-thiazolyl) benzamide) (NTZ) is an antiprotozoal drug that is previously shown by our group to have a synergistic effect with BCG on BC [5]. Thalidomide (N-alpha-phthalimido-glutamine) is a drug that has recently been re-purposed as a potential cancer treatment due to its immunomodulatory and pro-apoptotic effects. Thalidomide has also been shown by our group to enhance the effect of BCG on BC [6]. Rapamycin blocks the mammalian target of Rapamycin (mTOR) pathway and is generally considered to have anti-proliferative and immunosuppressive effects [7-8].

In this study, we report our findings with NTZ, Rapamycin, and Thalidomide. Our main aim was to study the immunomodulatory effect of each compound alone and in combination with BCG on immunecompetent rat orthotopic BC and possible alterations in PD-L1 and CTLA4 expression and their correlation with histopathological findings.

Methods

The immunecompetent rat orthotopic BC model, including doses and administration mode, was previously described in articles published by our group [5,6; 9,10]. In short, after ethics committee approval (4540-1/2017; 3163-1; and 3164-1), 80 isogenic female Fisher-344 rats, 7 weeks old, underwent four intravesical instillations of N-methyl-N-nitrosourea (MNU) (1.5 mg/kg; Sigma-Aldrich) dissolved in 0.2 ml of saline via a 22-gauge angiocatheter every 15 days (week 0, 2, 4, 6).

At week eight, rats were randomly allocated to the eight groups (n=10) of weekly intravesical treatment: Control group with 0.2 ml saline solution; BCG alone group (2×10^6 CFU Connaught), NTZ alone (300 mg/kg Nitazoxanide), BCG plus NTZ (2×10^6 CFU BCG plus 300 mg/kg Nitazoxanide), Rapamycin alone (3 µg), BCG plus Rapamycin (2×10^6 CFU BCG plus 3 µg Rapamycin), Thalidomide alone (20 mg/ kg), BCG plus Thalidomide (2×10^6 CFU BCG plus 20 mg/ kg Thalidomide). All drugs were dissolved in a 0.2 ml saline solution and applied weekly for six weeks. Four rats expired during the MNU-inducing process; therefore, 2 were deducted from the NTZ group and 2 from the NTZ + BCG group, finishing with 76 animals at the end of the process.

On week 15 all surviving animals were euthanized, the bladder was extracted, formaldehyde fixated, and 6 µm slides were prepped for histopathology hematoxylin-eosin staining) and immunohistochemistry (IHC).

Histopathological analysis

The urinary bladder samples were classified according to the World Health Organization/International Society of Urological Pathology consensus [11]. The uropathologist who interpreted the hematoxylin and eosin stains was blinded to treatment groups. For clinical significance, lesions were clustered into two groups: Normal or non-neoplastic = no lesion, hyperplasia, papillary hyperplasia or dysplasia. Neoplastic = non-invasive papillary carcinoma (pTa-low or high grade); carcinoma in situ (pTis); or invasive papillary carcinoma (pT1).

Immunohistochemistry

IHC was performed on all extracted bladders regardless of presence or absence of neoplastic lesions. CTLA-4 (CD152) was stained using rabbit anti-rat, polyclonal (Invitrogen, PA5-79090) and PD-L1 using rabbit anti-rat, polyclonal (Invitrogen, PA5-20343) according to standard overnight protocol. First, the tissue section was deparaffinized and then rehydrated before incubating with the primary antibody overnight (PDL1-1 or CTLA-4). Enzyme-conjugated secondary antibodies were then applied and visualized by adding the enzyme-specific substrate [12].

Image Acquisition

Images were captured using Zeiss Imager Z1 upright microscope (Zeiss, Germany) equipped with an AxioCam MRc5 camera (Zeiss, Germany), interfaced with a Microsoft computer. Light and camera settings were controlled using the AxioVision V4.6 (Zeiss, Germany) software, resulting in average background values of 63±13 milliseconds (mean ± standard deviation) for the red, green, and blue channels. Images were captured at 40X objective lenses. All IHC samples were photographed in 8 random slides (except in the Rapamycin and

Rapamycin+BCG groups, we were only able to photograph 4 slides each because tissue was diminished).

Image Analysis

The cytoplasmic area staining intensity of carcinoma-positive individuals was obtained using ImageJ 1.50b (National Institute of Health, USA) using IHC Profiler, an open-source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. The images were analyzed according to the degree of expression of the markers PD-L1 and CTLA-4 in each sample, which were described in percentage staining of each slide in four different ways: %high-positive, %low-positive, %positive, and %negative; additionally, they were described using a "score" for each slide ranging 0 to 3 (0 = negative, 1= low positive, 2= positive and 3= high positive) [13].

Statistical analysis

For markers, each slide was treated as a separate observation and slides from the same group were clustered because the animals were isogenic. The staining percentages were reported as continuous variables between zero and 100 and since they were not normally distributed, we used the Kruskal-Wallis test for comparison which is a non-parametric version of ANOVA test. The null hypothesis (H_0) for this test was "a random observation from each group is equally likely to be above or below a random observation from another group".

The markers' scores were treated as categorical variables with four levels 0, 1, 2, and 3 (**Supplementary Figures 1 and 2**). In the statistical analysis we tested the H_0 of independence, i.e. the frequency of each score was not dependent on the treatment. Pairwise comparison was

performed using Fisher's exact test and a multidimensional test of all groups was performed using Pearson's chi-squared test.

In pairwise comparisons, each significant *p*-value (<0.05) was adjusted for multiple testing using the Holm method. H_0 was rejected if adjusted *p*-value was <0.05. Only significant *p*-values (after adjustment) are reported in the figures. All analyses were performed using R version 4.1.2 on RStudio platform 2022.07.1 with the packages tidyverse and ggstatsplot [14].

Results

Histopathology

The histopathology distribution is shown in **Supplementary Table 1**. Eight (80%) control rats developed bladder neoplasia, followed by 5 (62.5%) in the NTZ and 5 (50%) in the Thalidomide recipients. The groups treated with BCG, and Thalidomide + BCG both presented 4 (40%) animals with cancer; Nitazoxanide + BCG had 3 (37.5%) rats with neoplasia and both Rapamycin and Rapamycin + BCG presented the fewest number of neoplasia with 1 (10%) animal each.

Fisher's exact tests comparing the number of bladders with neoplasia to bladders with no lesions or non-neoplastic lesions (hyperplasia, dysplasia, etc.), were statistically significant in both Rapamycin and Rapamycin plus BCG (*p*-Value = 0.0055) when compared to controls. When compared to BCG there was a tendency towards improvement that was not statistically significant

Immunohistochemistry

A total of 560 biopsy slides were prepped, out of which, 80 were controls (only exposed to MNU and treated with saline). The remaining slides were taken from bladders that received the following intravesical treatments: 80 BCG, 80 NTZ, 80 NTZ + BCG, 40 Rapamycin, 40 Rapamycin + BCG, 80 Thalidomide, and 80 Thalidomide + BCG. **Supplememtary Figures 1** and **2** show representative images of 0 = negative, 1= low positive, 2= positive and 3= high positive of PDL-1 staining and CTLA4 staining, respectively.

- *PD-L1 staining percentage and intensity* (**Supplementary Figure 3**)

Compared to controls, combination treatment with BCG plus NTZ had the most dramatic effect on PD-L1 expression; (positive percentage: median 21.8% NTZ+BCG vs 3.8% in controls, p=0.001; high-positive percentage: 2.5% in NTZ+BCG vs. 0.1% in controls, $p < 0.0001$).

On the other hand, Rapamycin plus BCG significantly reduced the percentage of PD-L1 positive and high positive cells (median 0.7% positive and 0.0% high-positive, $p < 0.0001$ for both compared to BCG alone and control) and significantly increased the percentage of negative cells (median 79.7% vs 56.4% control and 39.1% BCG alone, $p < 0.0001$ for all).

- CTLA4 staining percentage and intensity (**Supplementary Figure 4)**

BCG alone increased overall expression of CTLA4 (positive percentage: median 18.8% stained positive vs 2.0% of controls, $p < 0.0001$; high-positive percentage: median 2.8% vs <0.1%, $p < 0.0001$). Both Thalidomide and Rapamycin caused increased percentage of high positive and positive. However, adding BCG significantly annulled this effect of both drugs; the slides rarely stained positive for CTLA4 and became predominantly negative in combination therapy ($p < 0.0001$ for all comparisons).

- PD-L1 and CTLA4 score comparison (**Figure 1)**

BCG alone did not significantly change PD-L1 score compared to controls after Holm correction of p-value acquired from two-sided Fisher's exact test (FIGURE 3). However, CTLA4 expression score was significantly changed by BCG treatment alone: while the percentage of score 3 was zero in controls, 14% of BCG treated slides scored 3 in the CTLA4 expression and the percentage of score 2 changed from 6% in controls to 30% in BCGs ($p < 0.0001$).

NTZ alone significantly changed the PD-L1 expression score compared to controls ($p = 0.011$); score 2 became less common (1% NTZs vs 17% controls) and the slides predominantly scored 0 or 1. The combination of BCG + NTZ, on the contrary, increased high scores in PD-L1 expression and created the highest percentage of score 3 (25%) compared to other treatment groups; adding BCG to NTZ significantly increased both scores 3 and 2 compared to each treatment alone or to controls ($p < 0.0001$ for all three comparisons). In contrast, CTLA4 score was not changed by NTZ alone ($p = 0.51$), and its combination with BCG had an effect similar to BCG alone ($p = 0.069$ comparing BCG vs BCG + NTZ in CTLA4 score.

Rapamycin alone did not significantly change the PD-L1 score ($p = 0.08$) but increased the CLTA4 score ($p = 0.0009$). Interestingly, combining Rapamycin with BCG completely reverted the effect of each drug alone on both CLTA4 and PD-L1 scores. Both drugs increased CTLA4 high scores when administered alone; however, their combination significantly decreased high CTLA4 scores. The slides that were treated with Rapamycin plus BCG combination had no score 2 (compared to 17% of controls, 10% of Rapamycin alone, and 30% of BCG alone recipients), and were mostly score 0 (74%). A similar effect was seen with PD-L1 expression, however, the

difference between Rapamycin alone and Rapamycin + BCG combination was not statistically significant in PD-L1 score ($p = 0.08$).

Thalidomide alone significantly changed CTLA4 expression ($p = 0.0012$) while it had no significant effect on PD-L1 (p=0.16). Its combination with BCG significantly decreased score for both markers. While Thalidomide alone had a 27% score 2 or 3, its combination with BCG had no scores 2 or 3 in CTLA4 expression. Thalidomide alone had a 24% score 2 or 3 in PD-L1 expression while its combination with BCG had no score 3 and only 1% score 2.

Discussion

The Food and Drug Administration (FDA) approved the first PD-L1 inhibitor in 2016 for cancer treatment and for BCG resistant NMIBC in 2021 [15]. Fewer studies are performed on CTLA4 inhibition in urothelial cancer, however, trials on combination therapy with PD-L1 and CLTA4 inhibition are underway [16-18]. In this study, we compared three potential anti-neoplastic immunomodulator drugs alone and in combination with BCG on neoplasm formation and expression of PD-L1 and CLTA4 in a clinically relevant immunecompetent BC animal model.

The current study expands the previous data by comparing each drug and their combination with BCG, in addition, we analyzed distinct molecular targets, the immune checkpoints expression of PD-L1 and CLTA4, improving our understanding of the immunosuppressive properties of these molecules, and their anti-cancer immune activity, as promising future treatment options.

The literature has some options of dose and administration mode of Rapamycin in experimental cancer models. Kinkade et. al in 2008 suggested that Rapamycin should dissolved in 100% ethanol to make a working stock of 25 mg/ml and then diluted to 1.25 mg/ml in a solution

of 5.2% Tween-80, 5.2% PEG400 in sterile water and delivered intraperitoneal at 10 mg/kg, for a systemic delivery [7]. We chose to do a local delivery (intravesical), using 15 mg/ml rapamycin diluted in 0.2 ml saline solution, as presented in our previous study in 2015 [10].

Rapamycin's maintenance therapy has been explored to prevent relapse after NMIBC resection in a clinical trial, due to its antiproliferative effect, and potential relapse prevention [19]. Combination therapy with intravesical Rapamycin plus BCG significantly changed the effect of both BCG alone and Rapamycin alone on CTLA4 and PD-L1's expression. Both markers were significantly less expressed when combination treatment was used compared to BCG alone or Rapamycin alone. This effect was more prominent on CTLA4 expression: while CTLA4 was highly expressed with BCG alone treatment, it was almost absent when Rapamycin was added. Histopathology of those bladders that received Rapamycin with or without BCG revealed a better outcome compared to both BCG alone or controls. While neoplasia was observed in only 1/10 (10%) of both Rapamycin recipients and Rapamycin plus BCG recipients, 40% of the BCG alone recipients and 80% of controls had neoplasia in their bladders.

Thalidomide and BCG combination has been studied *in vitro* and *in vivo*, most of which show reduced BCG-induced inflammation after Thalidomide administration [20-21]. One phase II clinical trial tested a combination of oral Lenalidomide plus BCG therapy on BC patients. The trial was stopped early with a very limited number of participants (15 combination treatments vs 2 controls) due to adverse events in the combination arm [22]. In this and in our previous *in vivo* study [6] we proposed intravesical administration to minimize adverse events.

Thalidomide alone increased the expression of both CTLA4 and PD-L1 while its combination with BCG negated such effect. Overall, Thalidomide + BCG did not markedly change the two immune checkpoint markers compared to controls or BCG alone, indicating that their

histopathology advantages may be independent of expression of the markers. In our previous study we performed western blotting on the rat bladders for molecules involved in angiogenesis (VEGF: Vascular endothelial growth factor, and HIF: Hypoxia-inducible factor) as well as molecules involved in mTOR-related proliferation (4E-BP1: Elongation-initiation factor 4E-binding protein-1 and p70S6 kinase-1). HIF, VEGF, and p70S6K1 were down-regulated, while 4E-BP1 was up-regulated in Thalidomide and Thalidomide + BCG groups. Cell turnover was lower in the treatment groups. Overall, we can conclude that Thalidomide + BCG has potential anti-neoplastic activity by impairing angiogenesis and cellular proliferation.

NTZ is shown to stimulate autophagy and inhibit the mTOR pathway [23]. In 2021, Sun et al. showed that in urothelial cancer, NTZ causes mitochondrial damage while impairing the mitophagy flux (lysosomal degradation of damaged mitochondria); aggravating this effect with an autophagy inhibitor (Chloroquine) promoted apoptosis in the cells [24]. The effect of NTZ on the immune system is mostly hypothetical; one study showed NTZ reduced T cell proliferation and cytokine production [25].

NTZ alone slightly decreased PD-L1 expression compared to controls and BCG alone. However, combination of NTZ + BCG increased number of PD-L1 high-positive cells while low-positives became less common and overall expression of the molecule did not change significantly compared to controls or BCG alone (%negative). This indicates that the cells expressed PD-L1 more strongly after combination therapy. CTLA4 expression, on the contrary, did not differ between BCG alone and NTZ + BCG combination treatment while NTZ alone- was almost identical to controls. This indicates that NTZ had no effect on CTLA4 expression.

Strength of our study is presence of controls (placebo and gold standard treatment BCG) in a clinically relevant immunecompetent animal model [9], as well as cell-based analysis of

results. In this study we compared histopathology slides based on the treatment they were exposed to, instead of grouping slides of one animal together and comparing animals. The strength of this method is that the cells became the focus of the study instead of the animals. These animals are inbred from the same clone (isogenic), this gave us the advantage of increasing our observations while limiting the number of sacrificed animals. Our study contributes in understanding the complicated mechanism of cancer immunotherapy. Further studies to better understand the multidrug approach can increase our knowledge and enable the development of safe and effective treatments for this very challenging disease.

The limitation of our study is the absence of dose titration, relying on previous studies for the best dose of every treatment, and though the model was extensively validated, neoplasia growth was not confirmed before treatment initiation using imaging methods [5]. Furthermore, some of the used drugs may have had interferences when used in combinations, however, the separate injection would make it not clinically feasible. Current results might support future studies with less instilations and with sequential use of combined immunotherapies, which may improve the outcome by allowing BCG to increase anti-cancer immune activity without interference with the immunosuppressive properties of NTZ, Rapamycin, or Thalidomide.

Conclusion

Intravesical BCG combination treatment seems to effectively prevent bladder cancer development in a clinically relevant immunecompetent animal model, showing Thalidomide, Nitazoxanide, and specially Rapamycin as candidates in the intravesical immunotherapy advancement, suggesting that combination therapies could be well tolerated and potentially more

promising than single agents. Furthermore, adding Rapamycin or Thalidomide decreases both markers, while adding NTZ to BCG increases the expression of PD-L1, compared to BCG alone.

Author Contributions:

DLA, MJ, ACCS: data collection, analysis, statistics, and manuscript writing

LOR: supervision, data analysis, manuscript editing.

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Declaration of Interest:

The authors report no conflicts of interest.

Financial Disclosure:

The authors declare that they have no relevant financial interests.

Data Availability Statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with Ethical Standards:

This study was approved at University of Campinas Ethics Commitee on Animal Experimentation under numbers: 4540-1/2017; 3163-1; and 3164-1.

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TABLES

SUPP. Table 1: Description and frequency of bladder lesions observed following treatment.

	Control (n=10)	BCG (n=10)	Thalidomide (n=10)	Thalidomide + BCG (n=10)	Rapamycin (n =10)	Rapamycin + BCG (n =10)	Nitazoxanide (n =8)	Nitazoxanide + BCG (n =8)
Normal or Non-Neoplastic	2 (20%)	6 (60%)	5 (50%)	6 (60%)	9 (90%)	9 (90%)	3 (37.5%)	5 (62.5%)
Neoplastic	8 (80%)	4 (40%)	5 (50%)	4 (40%)	1 (10%)	1 (10%)	5 (62.5%)	3 (37.5%)
Low grade	2 (20%)	2 (20%)	5 (50%)	2 (20%)	1 (10%)	1 (10%)	3 (37.5%)	2 (25%)
pTis	3 (30%)	2 (20%)	0	0	0	0	0	0
pTa	3 (30%)	0	0	2 (20%)	0	0	2 (25%)	1 (12.5%)
pT1	0	0	0	0	0	0	0	0
p-Value*		0.17	0.35	0.17	0.0055	0.0055	0.60	0.15

*p-Values obtained using Fisher's exact test comparing each treatment group to the control based on the number of bladders with neoplasia to bladders with no lesions or non-neoplastic lesions (hyperplasia, dysplasia, etc.)

Figures

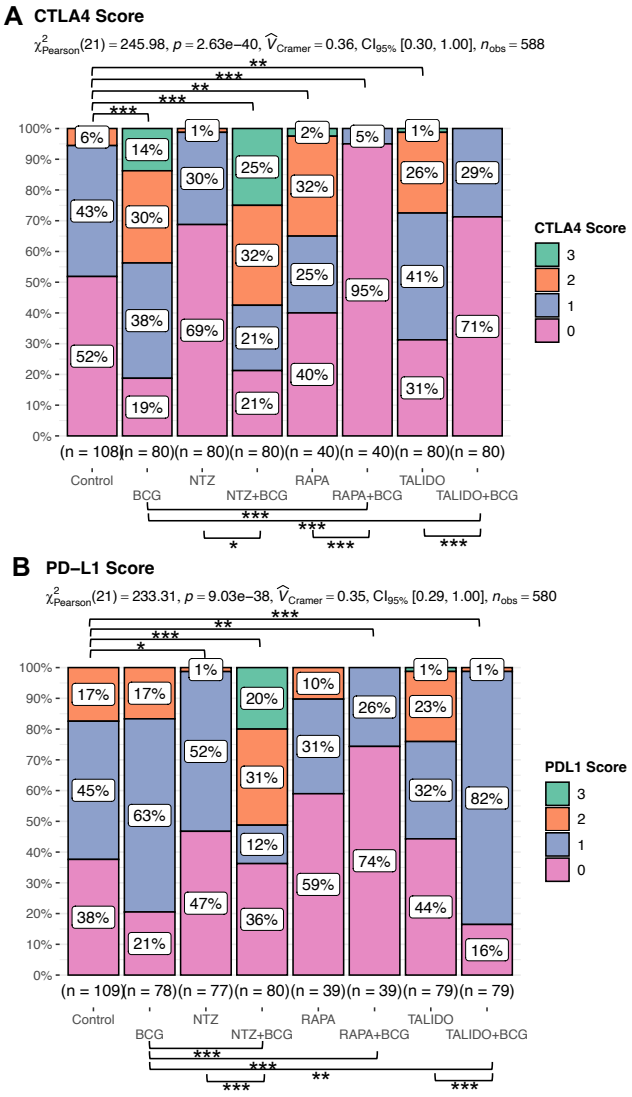
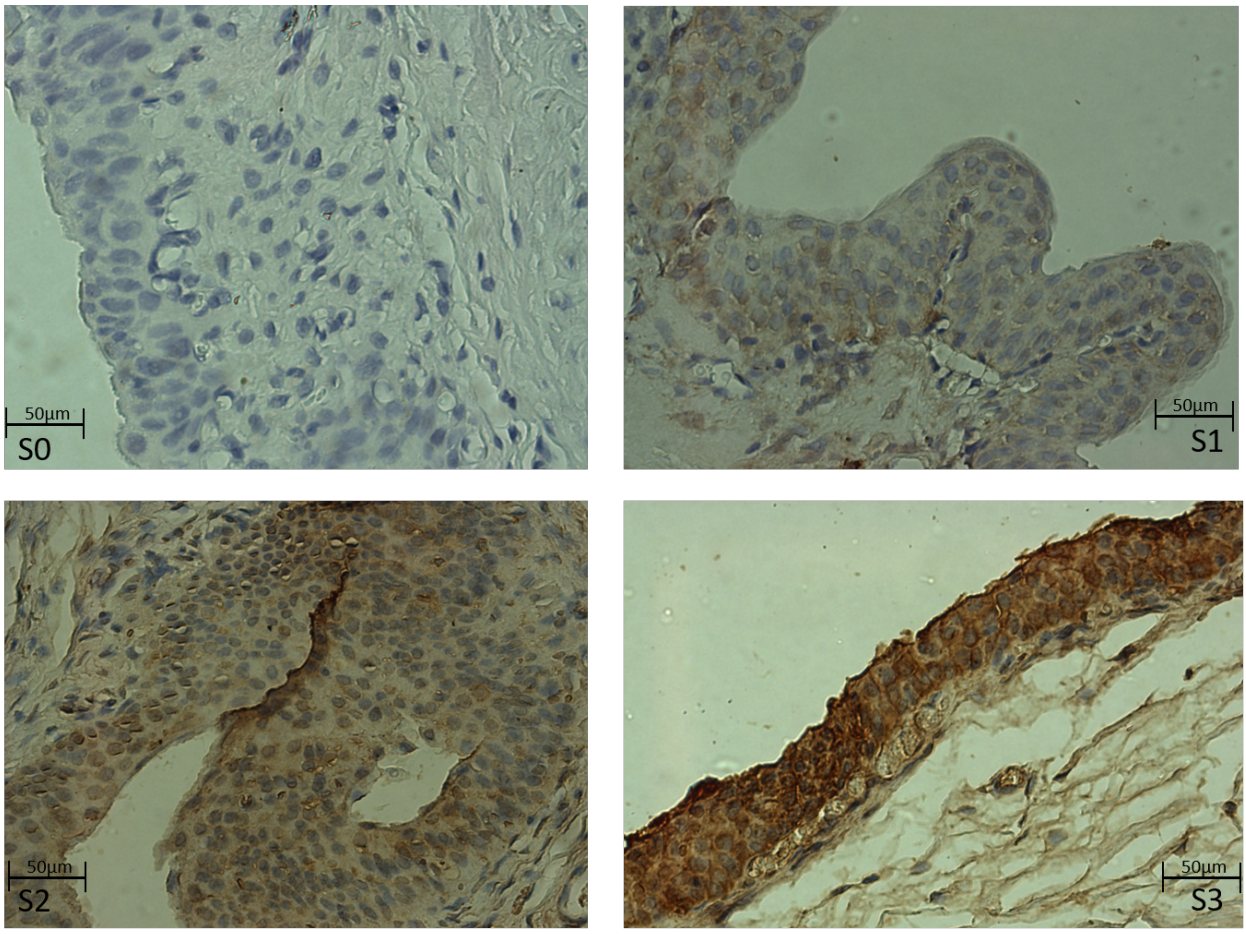
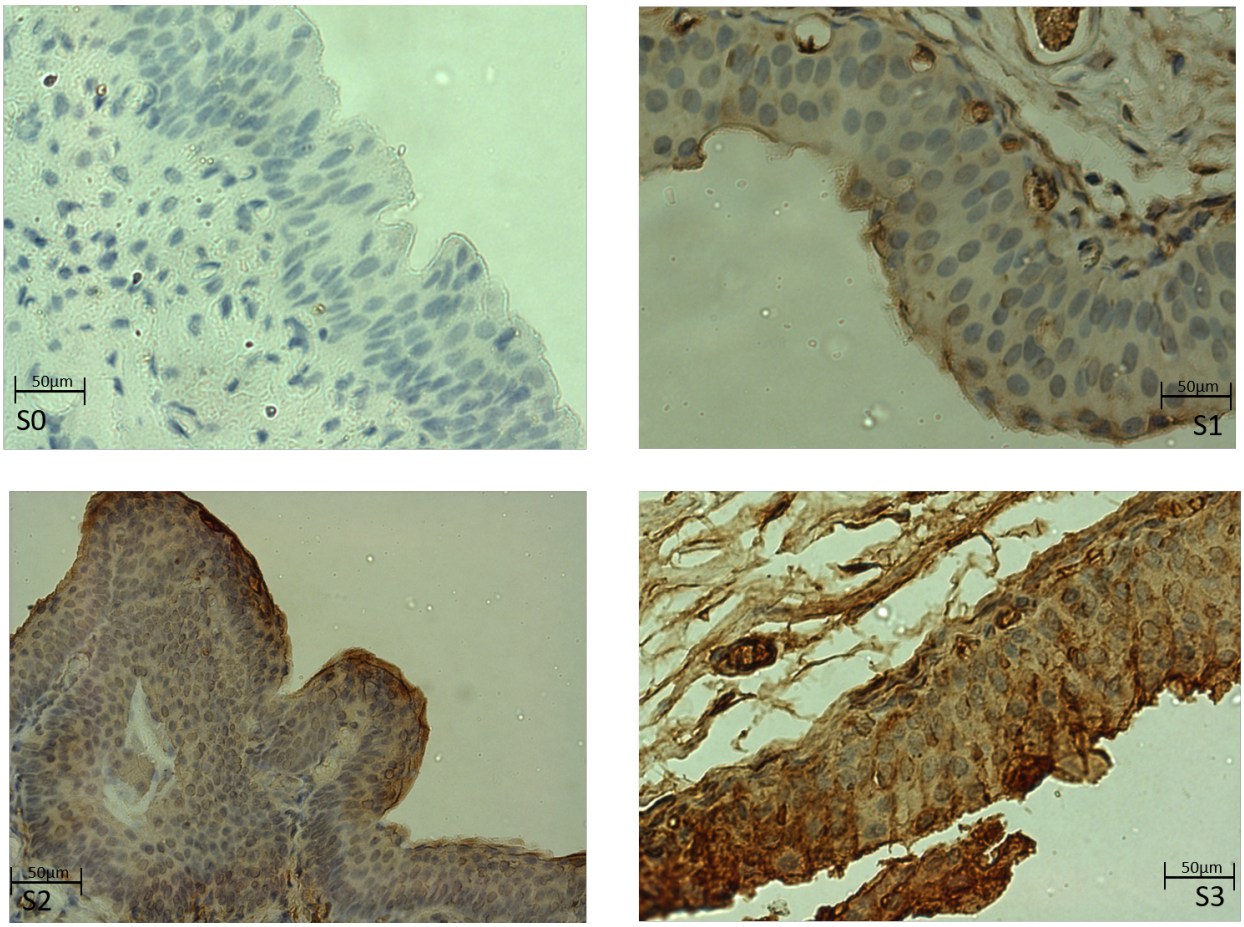


Figure 1: The markers CTLA4 (A) and PD-1(B) expression level for each treatment group were described using a "score" ranging 0 to 3 (0 = negative, 1= low positive, 2= positive and 3= high positive) for each slide; Multidimensional test of all treatment groups was performed using

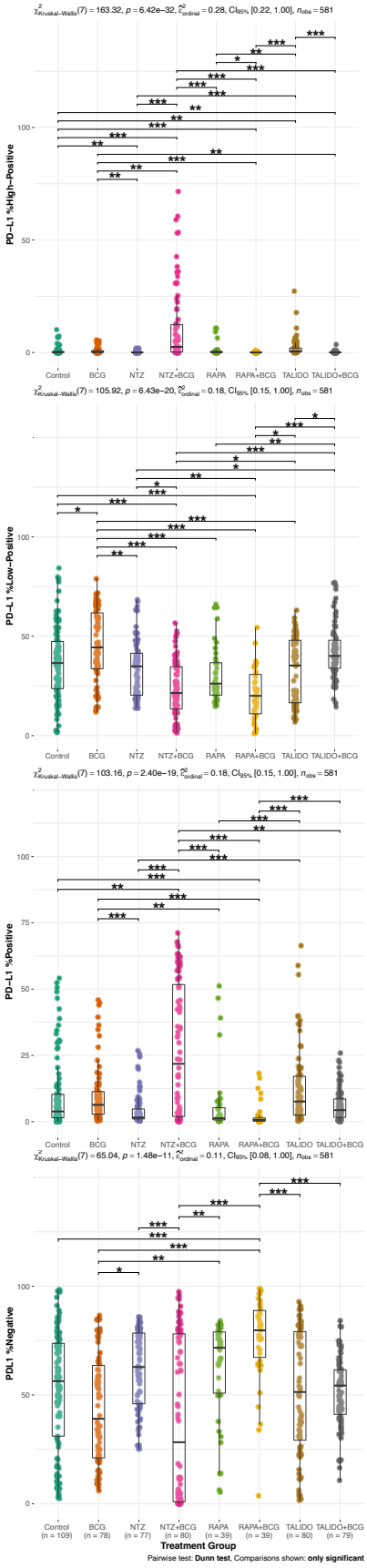
Pearson's chi-squared test with *, *p*-value between 0.005 and 0.0005 shown with ** and *p*-value<0.0005 shown with ***.



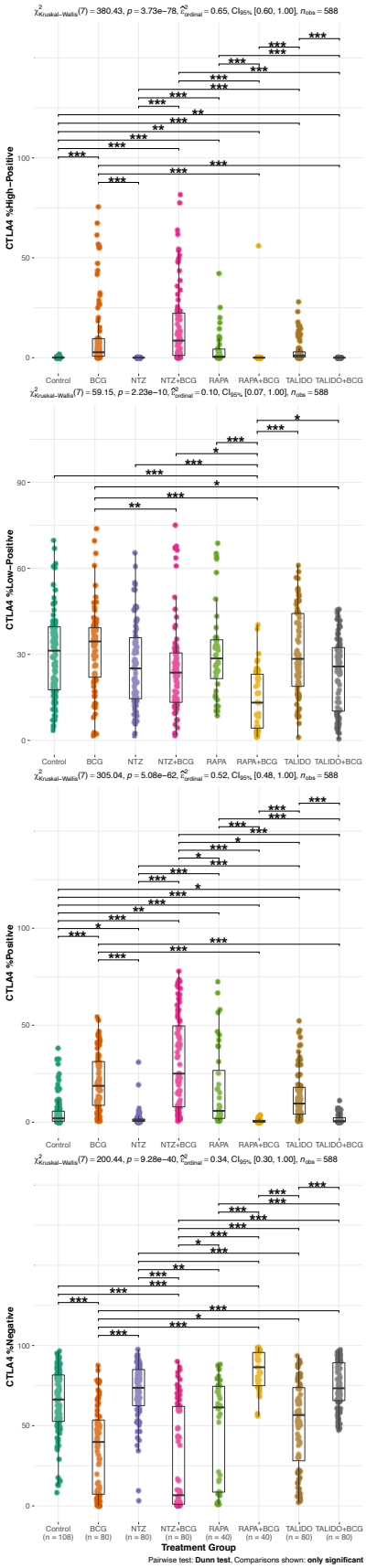
Supplementary Figure 1. Immunohistochemical staining for PDL1. The markers results were described using a "score" for each slide from 0 to 3: **S0** = negative, **S1**= low positive, **S2**= positive and **S3**= high positive.



Supplementary Figure 2. Immunohistochemical staining for CTLA-4. The markers results were described using a "score" for each slide from 0 to 3: **S0** = negative, **S1**= low positive, **S2**= positive and **S3**= high positive.


$\chi^2_{Kruskal-Wallis}(7) = 163.32$, $p = 6.42e{-}32$, $\hat{\epsilon}^2_{ordinal} = 0.28$, $CI_{95\%}$ [0.22, 1.00], $n_{obs} = 581$
PD−L1 %High−Positive
$\chi^2_{Kruskal-Wallis}(7) = 105.92$, $p = 6.43e{-}20$, $\hat{\epsilon}^2_{ordinal} = 0.18$, $CI_{95\%}$ [0.15, 1.00], $n_{obs} = 581$
PD−L1 %Low−Positive
$\chi^2_{Kruskal-Wallis}(7) = 103.16$, $p = 2.40e{-}19$, $\hat{\epsilon}^2_{ordinal} = 0.18$, $CI_{95\%}$ [0.15, 1.00], $n_{obs} = 581$
PD−L1 %Positive
$\chi^2_{Kruskal-Wallis}(7) = 65.04$, $p = 1.48e{-}11$, $\hat{\epsilon}^2_{ordinal} = 0.11$, $CI_{95\%}$ [0.08, 1.00], $n_{obs} = 581$
PDL1 %Negative
Control (n = 109)
BCG (n = 78)
NTZ (n = 77)
NTZ+BCG (n = 80)
RAPA (n = 39)
RAPA+BCG (n = 39)
TALIDO (n = 80)
TALIDO+BCG (n = 79)
Treatment Group
Pairwise test: Dunn test, Comparisons shown: only significant

Supplementary Figure 3: PD-L1 expression level of slides taken from bladder of rats treated with different drug combinations; the level is described as %high-positive, %low-positive, % positive, and %negative. Kruskal-Wallis test was performaded and adjusted for multiple testing using the Holm method. Only significant *p*-values (after adjustment) are shown with *p*-value between 0.05 and 0.005 shown with *, *p*-value between 0.005 and 0.0005 shown with ** and *p*-value<0.0005 shown with ***.


$\chi^2_{Kruskal-Wallis}(7) = 380.43$, $p = 3.73e{-}78$, $\hat{\epsilon}^2_{ordinal} = 0.65$, $CI_{95\%}$ [0.60, 1.00], $n_{obs} = 588$
CTLA4 %High−Positive
$\chi^2_{Kruskal-Wallis}(7) = 59.15$, $p = 2.23e{-}10$, $\hat{\epsilon}^2_{ordinal} = 0.10$, $CI_{95\%}$ [0.07, 1.00], $n_{obs} = 588$
CTLA4 %Low−Positive
$\chi^2_{Kruskal-Wallis}(7) = 305.04$, $p = 5.08e{-}62$, $\hat{\epsilon}^2_{ordinal} = 0.52$, $CI_{95\%}$ [0.48, 1.00], $n_{obs} = 588$
CTLA4 %Positive
$\chi^2_{Kruskal-Wallis}(7) = 200.44$, $p = 9.28e{-}40$, $\hat{\epsilon}^2_{ordinal} = 0.34$, $CI_{95\%}$ [0.30, 1.00], $n_{obs} = 588$
CTLA4 %Negative
Control (n = 108)
BCG (n = 80)
NTZ (n = 80)
NTZ+BCG (n = 80)
RAPA (n = 40)
RAPA+BCG (n = 40)
TALIDO (n = 80)
TALIDO+BCG (n = 80)
Treatment Group
Pairwise test: Dunn test, Comparisons shown: only significant

Supplementary Figure 4: CTLA4 expression level of slides taken from bladder of rats treated with different drug combinations; the level is described as %high-positive, %low-positive, % positive, and %negative. Kruskal-Wallis test was performaded and adjusted for multiple testing using the Holm method. Only significant *p*-values (after adjustment) are shown with *p*-value between 0.05 and 0.005 shown with *, *p*-value between 0.005 and 0.0005 shown with ** and *p*-value<0.0005 shown with ***.