



*Microbial Science Archives (ISSN: 2583-1666)*

An International Research Journal

Journal homepage: [www.microbial.sciencearchives.org](http://www.microbial.sciencearchives.org)

<https://doi.org/10.47587/MSA.2023.3203>



Research Article

## Role of CD4 T-regulator cell and their subsets in bladder cancer

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Received: May 27, 2023/ Revised: June 17 / Accepted: June 21, 2023

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### Abstract

Tumor-infiltrating lymphocytes (TILs) are candidate as crucial biomarkers since certain cancers have high lymphocyte infiltration that obtain significant role in specific cancer types that can be contributed in tumor pathogenesis. In Bladder cancer found to be highly infiltrated with T-regulator cells, and it has been found to be associated with tumor progression and poor disease outcome as well. In this study, we assess the CD4 T- regulator cells and their subsets infiltration in tumor and autologous normal microenvironments of the urinary bladder and subsequently their significance in antitumor immunology with their clinicopathological influences of urinary bladder cancer.

**Keywords:** T-regulator, CD4, Subsets, Bladder, Cancer.

### Introduction

CD4+ regulatory T (Treg) cells are a CD4+ T cells subset that express forkhead box P3 (Foxp3) (Sakaguchi et al., 2008). Processes of self-tolerance and immune homeostasis are performed mainly by a great immunosuppressive cell which is the CD4+ regulatory T cell. These processes co-occur with a destructive effect on antitumor immune processes via a wide array of processes that inhibit effective antitumor immune responses together in a contact-dependent means and by secreting suppressive molecules; as a consequence, it will enhance tumor development and progression (Takeuchi et al., 2016). Generally, tumor microenvironment which shows high infiltration with Treg cells like urothelial malignancies believed to be proof of their role in tumor growth and metastasis (Mougiakakos et al., 2010). Also, poor prognosis and worse disease outcomes associated with high CD4+ Treg cells infiltration for many cancers (Sakaguchi et al., 2008). This added to a poor response to therapy for patients with bladder cancer who exhibit high infiltration of these immunosuppressive cells and for many other tumors (Baras et al., 2016). The degree of Foxp3+ T cells infiltration in cancer tissue for muscle-invasive bladder cancer, has been found to

be associated with patient prognosis (Parodi et al., 2016). Regulatory CD4+ T cells (Tregs) are essential to control T-effectors. This is achieved by two sets of regulatory T cell: those who are thymic (embryonic/neonatal) origin, and the other set generated in the periphery (peripheral Tregs) they have a critical influence in tumor growth and metastasis via the secretion of immunosuppressive cytokines like TGF $\beta$ , IL-10 (Loskog et al., 2007; Povoleri et al., 2013).

Bladder cancer cell line shows a reduction of CD25+ve regulatory CD4+ T cells induction when IL-10 and TGF $\beta$  receptors have been blocked (Liu et al., 2019). Cytotoxic T cells would be suppressed via these cytokines and so they inhibit immune defenses against cancer, they comprise a large percent of tumor-infiltrating lymphocytes in urinary bladder cancer (Murai et al., 2018). The survival rate in patients with NMIBC found to have a negative association with FoxP3 CD4+ Treg cells, also found a negative link between degree of tumor infiltrating FoxP3 T cell and recurrence-free survival (Murai et al., 2018). A shorter recurrence-free survival for NMIBC patients who showed a higher FoxP3 infiltration in the peri-tumor stroma after intravesical BCG treatment (Miyake et al., 2017). A great response to chemotherapy for

patients with MIBC with higher CD8/FoxP3 ratio for tumor infiltrating lymphocytes (Murai et al., 2018). The variation in the location of the assessed Tregs as if it is resided intra-tumoral or peritumoral the tumor result in a variable prognostic correlation for Tregs. On the other hand, other non-regulatory T lymphocytes can express FoxP3 or CD25 this may mask the outcomes if other markers have not been used to assess the T-regulator cells (Baras et al., 2016; Akkaya et al., 2019). CD4+ T cells can express T-bet and Foxp3 together although, the functions of T-bet and Foxp3 are contradictory in immune processes as well for antitumor immunity. The TGF- $\beta$  mediate the differentiation of CD4+ Treg cells to express both T-bet+Foxp3+ cells so take the phenotype of Th1-like Tregs. Then after converted from cells secreting IFN- $\gamma$  and act as antitumor T-bet+Th1 cells into T-bet and Foxp3-PD-1 CD4+ cells that inhibit antitumor immune responses. The dual expressing Tregs might be a critical extra-mechanism directed by TGF- $\beta$  for suppressing antitumoral immune responses (Kachler et al., 2018). The proinflammatory Tregs such as Th1-like Tregs and Th17-Tregs would be differentiated in such phenotype in the presence of IL-12 and IL-6 respectively (Arterbery et al., 2016). Since the Tregs effects of immune inhibition activity against tumors conquer efficient antitumor immune responses, Treg-targeting therapies if combined along with other treatment lines, like immune checkpoint inhibitors, radiotherapy, and chemotherapy, would result in to a harmonized antitumor activity. However, Immunotherapy against Tregs mostly act for clearance, their results would not be perfect. This may be due to effector T cells would be removed as a result of removing Tregs cells, leading to a profound inhibition in antitumoral immune responses. Since the clearance of Tregs cells is transient, so it will rise again to the same level before treatment. Thus, Tregs targeting immunotherapy have to be for controlling their number and inducing the differentiation of Th1-like Tregs phenotype (Liu et al., 2020).

## Materials and methods

### Patients and samples

Autologous tissues (normal and tumor) samples were taken from 17 patients undergoing tumor resection by Trans Urethral Resection of Bladder Tumor surgery (TURBT) for primary urinary bladder cancer at the Basra Teaching Hospital after written informed consent. Follow up period for assessment of disease recurrence was 1 years. The 17 autologous (tumor and normal) Fresh samples were directly transferred into Dulbecco's Minimal Essential Medium (DMEM) media EuroClone, S.P.A Italy, catalogue number ECM0728L, then tissue specimens placed on ice tissue samples were transferred immediately to Al Bayan group for advanced lab. Diagnostic, to assess the CD4 T-reg cells and their subsets infiltration in bladder cancer patients by the mean of flow cytometry.

### Inclusion Criteria

- Patients who diagnosed with urinary bladder cancer
- Primary tumor

### Exclusion Criteria

- Secondary tumors
- Recurrent disease
- Inflammatory disease like infection
- Patients on immune suppressive or modulating therapy.
- Patients known case of autoimmune disorders

### CD4 T- Cell subset assessment by flow cytometry

Single cell formation process for assessment by flowcytometry:

Preparing 2X TTDR To make 2X TTDR ((Tumor-Tissue Dissociation Reagent) from BD Bioscience USA), catalogue number (661563)

- Five mL of DMEM added to the amber vial containing TTDR.
- Gently agitated periodically for 15 minutes at room temperature to ensure complete reconstitution of the dried reagent.
- The reconstituted TTDR transferred to a labeled 50-mL conical centrifuge tube.
- Discarded the amber vial.
- Then Stored at 4°C until needed.

After weighed the tissue specimen, then to minced the tissue placed into a fresh, labeled 100 × 20-mm glass petri dish containing 5 mL of 37°C DMEM. Two scalpels were used to mince the tissue to minute pieces in the petri dish figure (3-1). The resulting tissue pieces was as small as possible Approximately 30 minutes before tissue mincing has been completed, placed the tube containing 2X TTDR (Tumor-Tissue Dissociation Reagent) from BD Biosciences (USA) in 37°C water bath not longer than 30 minutes prior to use. The contents of the petri dish (DMEM and tumor pieces) transferred into a conical tube containing 37 °C 2X TTDR. The final volume in the conical tube should be 10 mL (5 mL warm TTDR +5 mL minced tumor in DMEM).

### Digesting the minced tissue

The tubes containing the minced tissue and TTDR were incubated at 37°C for 30 minutes with mild but frequent agitation. After incubation, 25 mL of 1% BSA/DPBS/2 mM EDTA was added to the conical centrifuge tubes containing the dissociated tissue to bring the total volume to 35 mL. Passed the contents of each tube through a fresh 70- $\mu$ m cell

strainer into a fresh labeled conical tube. After washing the strainer with 10 ml DMEM media Then after the tubes were Centrifuged at 250g for 8 minutes at room temperature.

The supernatant removed then after resuspended the pellets in 2 mL of 1X BD Pharm Lyse™ solution from BD Bioscience, USA. Incubated for 15 minutes at room temperature. Then 40 mL 1% BSA/DPBS/2 mM EDTA added and centrifuged at 250g for 8 minutes at room temperature. Then removed the supernatant and resuspended pellets in 2 mL DPBS/2 mM EDTA.

**Cell stimulation for intracellular staining**

Leukocyte Activation Cocktail with Golgi Plug from BD Bioscience (USA), catalogue number (550583), used for T-cell activation to secrete cytokines for assessment by intracellular immunofluorescent staining.

- The cocktail was thawed at 37 °C in a water bath.
- Two µL of the cocktail added for every 1 mL of the dissociated samples and mixed thoroughly.
- Then after Placed in a 37°C humidified CO2 incubator for 6 hrs.
- After activation the cells washed with FACS Staining Buffer to be used in flow cytometric assessment.

**Fluorochrome-conjugated monoclonal antibodies staining**

- The cells washed with wash buffer
- Permeabilization Buffer added and then washed before the final wash step
- Resuspended the cells in a staining buffer,
- Then 50 µl of the suspension was added to each tube.
- Fluorochrome-conjugated monoclonal antibodies Table 1. added (5 µl)
- The mixture was incubated in the dark for 15-20 min at 4°C.
- The gating strategy planned by BD, Bioscience instructor.

**CD4<sup>+</sup>T reg cells (CD3<sup>+</sup>CD4<sup>+</sup> CD25<sup>+</sup> CD 127<sup>-</sup>)**

**Naïve T reg cell (CD3<sup>+</sup>CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>-</sup> CD45RA<sup>+</sup>)**

**Effector/Memory T reg (CD3<sup>+</sup>CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>-</sup> CD45RA<sup>-</sup>)**

**Statistical analysis**

IBN SPSS statistics version 22 program for statistical analysis has been used. The data is expressed as M ±SD. Wilcoxon Signed Ranks Test is used for paired samples, while Kruskal Wallis Test to assess multiple types of samples. Spearman's Correlations test was used to assess the presence of a correlation between the assessed parameters in autologous tumor and normal microenvironments of urinary bladder cancer patients. The statistically significant difference is considered at P<0.05.

**Results**

T-reg cells mean percentage of infiltration in tumor microenvironment of urinary bladder cancer was (1.10±0.93) significantly higher than that of T-reg cells infiltration in normal microenvironment (0.48±0.44), (p value ≤ 0.05) Table 1. There was no significant correlation between T-reg cells infiltration in tumor microenvironment and disease stage, tumor grade, LN involvement or tumor size (p value >0.05) Table 4.

The mean percentage of naïve T-reg cell infiltration in tumor microenvironment (62.2±41.3) higher than mean percentage of naïve T-reg cell infiltration in normal microenvironment (54.38±48.47), and this was not significant (p value > 0.05) Table 2. There is significant negative correlation between tumor size and naïve T-reg cell intra-tumor infiltration (p value ≤0.05), but there is no correlation found between T-reg cell infiltration and disease stage, tumor grade or LN involvement (p value>0.05) Table 4.

**Table 1. comparison of CD4 T-reg. cells and their subsets between normal and tumor bladder cancer tissue.**

<b>T-reg. normal</b>	0.48±0.44	0.0-1.35	0.046*
<b>T-reg. tumor</b>	1.10±0.93	0.13-2.9	

\*significant difference (p-value < 0.05).

**Table 2. Comparison of prevalence of effector/memory and naïve T-reg cells between normal and tumor microenvironment.**

CD4 T-reg. cell subset	Mean±SD.	Min.-Max.	Wilcoxon Signed Ranks Test
<b>Naïve Normal tissue.</b>	54.38±48.47	0-100	0.59
<b>Naïve Tumor tissue.</b>	62.2±41.3	0-100	
<b>Effector/memory Tumor tissue</b>	36.9±42.05	0-100	0.14
<b>Effector/memory Normal tissue</b>	17.3±37.3	0-100	

Significant difference if (p value ≤ 0.05).

**Table 3. Comparison between naïve and effector/memory T-reg subset prevalence in tumor tissue.**

T-reg cell subset in tumor tissue	Mean±SD.	P value
<b>Naïve Tumor</b>	<b>62.2±41.3</b>	<b>0.087</b>
<b>Effector/memory Tumor</b>	<b>36.9±42.05</b>	

Significant difference if (p value ≤ 0.05).

The mean percentage of Effector/memory T-reg cells infiltration in tumor microenvironment (36.92±42.1) was higher than their infiltration in normal urinary bladder tissue (17.3±37.3), but it was not significant (p value > 0.05) Table 2. We found higher mean percentage of naïve T-reg cell (62.2±41.3) than the mean percentage of effector/memory T-reg cell (36.92±42.1) in tumor tissue, but this was not significant (p value > 0.05), Table 3.

There is significant positive correlation exhibited between Effector/memory T-reg cells infiltration in tumor microenvironment and tumor size (p value ≤ 0.05), at the same time there is no correlation between them and disease stage, tumor grade or lymph node involvement (p value >0.05) Table 4. There is negative correlation between naïve T-reg and effector/memory T-reg cell infiltration in tumor microenvironment and this was not significantly negative correlation (p value ≤0.05) Table 5.

**Table 4. CD4 T-cell subset prevalence in tumor microenvironment in correlation to bladder cancer patient disease stage, tumor size, LN involvement.**

Cell		Stage	Tumor Grade	Tumor Size	L.N.
T-reg	R.	0.055	0.078	0.231	0.23
	P value	0.873	0.819	0.595	0.49
Naïve T-reg	R.	-0.252	0.271	-0.643	0.20
	P value	0.454	0.420	0.033*	0.53
Effector\memory	R.	0.210	-0.27	0.603	-0.28
	P value	0.535	0.409	0.049*	0.39

R. (Ratio),

\*Significance (p value ≤ 0.05).

**Table 5. CD4 T-reg cell subsets correlation of infiltration in tumor microenvironment.**

Spearman's correlation test	R.	Sig.
Naïve-Effector/memory T-reg.	-0.978*	0.00

Significant at the (\* 0.05, \*\*0.01) level

R. (Ratio), Sig.(significance).

### Discussion

The level of inflammation and lymphocyte tumor infiltration found to be correlated with patient response to therapy and overall survival (Pfanstiel et al., 2019). Studying Immune

evasion in bladder cancer can add further insight about the therapeutic strategy to improve patient response. Immune evasion is the key feature of malignant cells, which encourage proliferation, survival, and dissemination. Thus, recognizing how tumor evade immune responses could boost the efficiency of anticancer therapies by eluding immunosuppression caused by tumor and enhancing the anti-tumor immune responses (Dominguez-Gutierrez et al., 2019). Regulatory T cell known to induce immune tolerance and inhibit effective anti-tumor immune responses, so it enhances tumor initiation and progression (Takeuchi et al., 2016). Although Salama et al., have found a correlation between T-reg cells infiltrating tumor tissue in colorectal cancer and improved survival (Salama et al., 2009). Cancers that exhibited a high infiltration with CD4+ T-reg cell found to be associated with poor prognosis and disease outcome (Ward et al., 2015; Nagarsheth et al., 2017).

We found a significant higher infiltration of the T-reg in tumor tissue than their infiltration in normal tissue of urinary bladder cancer patients, these finding consistent with Chi et al. 2010 who found a higher percentage of circulating T-reg cells of urinary bladder cancer patients than the tumor tissue and higher than the percentage of circulating T-reg of healthy control (Chi et al., 2010). In pancreatic ductal adenocarcinoma T-reg cells found to be associated with later disease stages (Jin et al., 2014). Murai et al., found T-reg cell infiltration of tumor microenvironment in patients with non-muscle invasive urinary bladder cancer correlated with disease stage and grade (Murai et al., 2018). The present study found no correlation between T-reg cell infiltration in tumor tissue and disease stage, tumor size, grad, and LN involvement, this may be due to low sample size, all the above shows an important role of T-reg cells in tumor initiation and progression at the same time their possible role as a biological indicator of disease prognosis and possible therapeutic target for urinary bladder cancer patients. T-reg cell have been submitted to be differentiated in to naïve and effector/memory subtype (Kuczma et al., 2009). Since, there is limited studies about the impact of T-reg cell subsets in cancer generally and particularly in bladder cancer, this would make T-reg cell subset assessment a topic of interest. The effector/memory T-reg subset also found to be highly expressing tissue homing receptors like CCR3 and CCR5 which enhance cell recruitment into target tissue and subsequent tissue retention, also they found to infiltrate tumor microenvironment higher than the naïve T-reg cells as the disease progress and inhibit antitumor immune responses (Lin et al., 2009), in contrast to this study which found the prevalence of the naïve T-reg cell in tumor tissue higher than the prevalence of effector/memory T-reg cells, at the same time the prevalence of both effector/memory T-reg and naïve T-reg in tumor microenvironment was higher than their prevalence in normal tissue, our finding was consistent with Kotsakis et al. who found higher percentage of circulating naïve T-reg cells in patient with non-small lung cell lung carcinoma in comparison to healthy control, also they found no correlation between T-reg cell subsets and

clinicopathological features (Kotsakis et al., 2016), and this also was consistent with this study finding except in that it found a negative correlation of naïve T-reg cell prevalence in tumor tissue with tumor size and a positive correlation of effector/memory T-reg with tumor size, this reflect the effector/memory T-reg cells role as the possible cause of tumor progression, and their lower infiltration in tumor tissue in comparison to naïve T-reg cells might be explained by our finding of diminished tumor tissue infiltration with CD4 memory T-cells generally could affect effector/memory T-reg cell infiltration and so the percentage of effector T cell assessment might be more beneficial, at the same time we found a negative correlation between both T-reg subsets.

## Conclusion

The higher T-reg cell infiltration of tumor tissue than the normal tissue reflects a highly immune suppressive tumor microenvironment. T-reg cell subset (naïve and effector/memory) balance have a vital role in urinary bladder cancer development and progression. T-reg cell play a vital role in tumor immunology and further studies needed to establish T-reg subsets role in tumor immunology generally and in urinary bladder cancer particularly and other studies who found a correlation between T-reg cells and better disease outcome this may reflect the role of T-reg cell subset balance on disease prognosis, which can be used as prognostic indicator and as a target for immune therapy.

## Conflict of interest

The author hereby declares no conflict of interest.

## Consent for publication

The author declares that the work has consent for publication.

## Ethical Considerations

The study was approved by the institutional ethical committee.

## Funding support

The author declares that they have no funding support for this study.

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**How to cite this article**

Ali, H. O., Albadran, M. F. and Mezban, S. G. (2023). Role of CD4 T-regulator cell and their subsets in Bladder Cancer. *Microbial Science Archives*, Vol. 3(2), 70-75 <https://doi.org/10.47587/MSA.2023.3203>

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