Clinical implications of T-Cells CX3CR1^{+}, Toll-like Receptor 4 signaling pathway, and immune checkpoints in Non-Muscle Invasive Bladder Cancer

Implicações clínicas das células T CX3CR1^{+}, via de sinalização do receptor Toll-like 4 e checkpoints imunológicos no Câncer de Bexiga Não-Músculo Invasivo

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ABSTRACT
Background: This study characterized and compared the molecular profiles of CX3C chemokine receptor 1 (CX3CR1, a marker of T-cell differentiation), Toll-like receptor 4 (TLR4)-mediated interferon signaling pathway, and immune checkpoints in the different histological stages of non-muscle invasive bladder cancer (NMIBC), aiming the investigation of these biomarkers as a criterion of clinical response to immunotherapy. Methods: Seventy-five formalin-fixed paraffin-embedded samples of bladder were obtained from 34 to 96-year-old patients (mean 65 years) with NMIBC diagnosis in University of Campinas (UNICAMP) and Paulinia Municipal Hospital/ Brazil. Subsequently, the samples were divided into 3 groups (n= 25 samples per group): pTis group, high-grade pTa group, and pT1 group; and submitted to immunohistochemistry analysis: TLR4-mediated IFN-γ production signaling pathway (TRIF, TBK1, IRF-3, IFN-γ), CX3CR1+CD8+ T-cells, immune checkpoints (PD-1/PD-L1 and CTLA-4), and regulatory T (Treg) cells (FOXP3). The retrospective anonymous study was approved by the local ethics committee (Clinical Trial: RBR-6swqd2). Results: pTis group showed the lowest activation of TLR4-mediated IFN-γ signaling pathway when compared ($p<0.01$) to high-grade pTa and pT1 groups. Both the immunoreaction intensity and positive cells percentage were lower ($p<0.01$) for TLR4, TRIF, IRF-3, and IFN-γ in the pTis group with respect to other groups. No statistical difference was found between high-grade pTa and pT1 groups for these biomarkers. Likewise, CX3CR1 immunoreactivities were remarkably lower ($p<0.01$) in the pTis group in comparison with high-grade pTa and pT1 groups, which did not show statistical differences between them. Furthermore, immune checkpoints (PD-1/PD-L1 and CTLA4) and $FOXP3^+$ Treg cells immunoreactivities were significantly higher ($p<0.01$) in the high-grade pTa and pT1 compared to the pTis group. Conclusions: Our data demonstrated that pTis stage was characterized by an immunosuppressive microenvironment in comparison with pTa and pT1 stages, showing decreased TLR4-mediated interferon signaling pathway and low activation of $CX3CR1^+CD8^+$ T-cells; which implies in low sensitivity to immunotherapy. The larger number of $FOXP3^+$ Treg cells in pTa and pT1 was correlated with intensified immune checkpoints immunoreactivities, indicating higher sensitivity to immunotherapy. Finally, these biomarkers may be useful in the clinical management of patients with NMIBC.

Keywords: Bladder Cancer, Toll-like receptor 4, CX3CR1.

RESUMO
Objetivos: Este estudo caracterizou e comparou os perfis moleculares do receptor 1 de quimiocina CX3C (CX3CR1, um marcador de diferenciação de células T), da via de sinalização do interferon mediada pelo receptor *Toll-like* 4 (TLR4) e dos *checkpoints* imunológicos nos diferentes estágios histológicos do câncer de bexiga não-músculo invasivo (CBNMI), visando a investigação desses biomarcadores como critério de resposta clínica à imunoterapia. Métodos:

Setenta e cinco amostras de bexiga fixadas em formalina e embebidas em parafina foram obtidas de pacientes com idade entre 34 e 96 anos (média de 65 anos) com diagnóstico de CBNMI na Universidade de Campinas (UNICAMP) e Hospital Municipal de Paulínia/Brasil. Posteriormente, as amostras foram divididas em 3 grupos (n= 25 amostras por grupo): grupo pTis, grupo pTa de alto grau e grupo pT1; e submetidas às análises imunoistoquímicas: via de sinalização para produção de IFN-γ mediada por TLR4 (TRIF, TBK1, IRF-3, IFN-γ), células T $CX3CR1^{+}CD8^{+}$, *checkpoints* imunológicos (PD-1/PD-L1 e CTLA-4) e células T reguladoras (Treg) (FOXP3). O estudo retrospectivo anônimo foi aprovado pelo comitê de ética local (Ensaio Clínico: RBR-6swqd2). Resultados: O grupo pTis apresentou a menor ativação da via de sinalização do IFN-γ mediada por TLR4 quando comparado ($p<0,01$) aos grupos pTa e pT1 de alto grau. Tanto a intensidade da imunorreação quanto a porcentagem de células positivas foram menores ($p<0,01$) para TLR4, TRIF, IRF-3 e IFN-γ no grupo pTis em relação aos outros grupos. Não foram encontradas diferenças estatísticas entre os grupos pTa e pT1 de alto grau para esses biomarcadores. Da mesma forma, as imunorreatividades de CX3CR1 foram notavelmente menores ($p<0,01$) no grupo pTis em relação aos grupos pTa e pT1 de alto grau, os quais não mostraram diferença estatística entre eles. Além disso, as imunorreatividades dos *checkpoints* imunológicos (PD-1/PD-L1 e CTLA4) e das células Treg $FOXP3^{+}$ foram significativamente maiores ($p<0,01$) nos grupos pTa e pT1 de alto grau em relação ao grupo pTis. Conclusões: Nossos dados demonstraram que o estágio pTis foi caracterizado por um microambiente imunossupressor em relação aos estágios pTa e pT1, mostrando diminuição da via de sinalização do interferon mediada por TLR4 e baixa ativação de células T $CX3CR1^{+}CD8^{+}$ e, portanto, resultando em possível baixa sensibilidade à imunoterapia. O maior número de células Treg $FOXP3^{+}$ nos estadios pTa e pT1 foi correlacionado com as maiores imunorreatividades dos *checkpoints* imunológicos, indicando maior sensibilidade à imunoterapia. Finalmente, esses biomarcadores podem ser úteis no manejo clínico de pacientes com CBNMI.

Palavras-chave: Câncer de Bexiga, receptor Toll-like 4, CX3CR1.

1 INTRODUCTION

Bladder cancer is one of the most prevalent tumors in the world, with non-muscle invasive bladder cancer (NMIBC) being the most common in the urinary tract[1,2]. There is a pressing medical need to develop new diagnostic and therapeutic approaches for NMIBC, as its overall prognosis has not changed over the past 30 years.

The relationship between malignant tumors and the immune system is very close, as cancer is immunogenic, that is, capable of triggering an inflammatory response. In the NMIBC microenvironment, several immune cells can be found, such as: natural killer (NK) cells, tumor-associated macrophages (TAMs), dendritic cells (DCs), neutrophils, eosinophils and lymphocytes[3]. These cells are associated with the initiation of innate and adaptive immunity responses and create a complex inflammatory microenvironment, which can inhibit or stimulate tumor growth [3, 4]. In the innate immune response, the protagonists of this interaction with the tumor are NK cells and macrophages[4]. NK cells are able to destroy several types of malignant

cells that express a small amount of major histocompatibility complex (MHC) class I or NK cell activating ligands presented by antigen presenting cells (APCs) such as dendritic cells and macrophages[4].

Therapeutic strategies that enhance and modulate the migration of T cells to tumor microenvironment (TME) are fundamental in the control of several types of cancer, including NMIBC[5]. One of these strategies is the modulation of Toll-like receptors (TLRs). Activation of TLRs is the initial step in a cascade of events that lead to the stimulation of innate immunity, characterized by pro-inflammatory cytokines secretion and adaptive immune responses[5, 6]. TLR signaling can be classified in two ways: canonical pathway (depending on MyD88) for inflammatory cytokines: TNF-α, interleukins (IL-6, IL-1β, and IL-10) and; non-canonical (TRIF-dependent) pathway that activates interferon regulatory factors (IRF-3, IRF-5 and IRF-7) for the production of interferons (IFNs)[7, 8]. Activation of TLRs affects the recruitment of immune cells into the TME, and increases the activity of antitumor effector cells, such as cytotoxic T cells and NK cells; while at the same time blocking the activity of regulatory T cells (Treg) and myeloid cells, which have immunosuppressive function[5, 6].

The expression of TLRs has been reported in a variety of tumors, including hepatocellular, colorectal, prostate, and bladder carcinoma[9], and is often associated with disease prognosis[10]. However, the accurate outcome of TLR signaling in cancer remains a challenge[11]. For example, the antitumor role of TLRs is associated with their stimulation by PAMPs (pathogen-associated molecular patterns)/DAMPs (damage-associated molecular patterns) on APCs, resulting in a cancer-specific cytotoxic response[12, 13]. As different cell populations express TLRs, which are differentially stimulated by PAMPs/DAMPs, the role of TLR signaling in TME needs to be better understood.

The immune cycle in NMIBC is a multi-step process that can be targeted by treatments at different levels. Antigens released from NMIBC cells are captured by APCs, processed, and displayed on major histocompatibility complex (MHC)-I and -II molecules for presentation to T cells. Effector T cells are initiated and activated in response to antigen presentation tumor. Activated T cells kill NMIBC cells after binding to the NMIBC tumor antigen on MHC-I through the T cell receptor (TCR). However, the immunosuppressive TME can hinder the immune control in NIMBC[2, 4, 14, 15]. Recently, CX3C chemokine receptor 1 (CX3CR1) was discovered to be a marker of T cell differentiation[16]. CX3CR1 is stably expressed on $CD8^+$ T cells through unidirectional differentiation of $CX3CR1^-CD8^+$ T cells during the effector phase[16], which provides an advantage as a biomarker compared to transiently expressed molecules on T cells.

Immune checkpoints are immune system regulators that control immune effector function by maintaining a balance between inhibitory and costimulatory signals. Its role is to protect tissues from damage due to excessive immune response, but also to prevent autoimmunity[17, 18, 19]. However, it has been shown that tumor cells take advantage of this system to evade immune surveillance, upregulating the expression of immune checkpoints, and activating these negative regulators in tumor-specific immune cells[18, 20, 21]. The main immune checkpoints that have been successfully targeted in cancer treatments are PD-1 (programmed death-1), its ligand PD-L1 (programmed death ligand-1), and cytotoxic T lymphocyte antigen-4 (CTLA-4). Inhibition of these T-cell negative regulators with monoclonal antibodies enhances the immune response in many cancers (e.g., melanoma and non-small cell lung cancer)[22, 23, 24, 25]. Immunotherapies that block immune inhibitors, such as CTLA-4 and PD-1/PD-L1, allow the patient's immune response to act to eliminate the tumor, which leads to increased overall survival even in cases of advanced cancers[14].

Due to the inherent complexity of the immune response, patient selection and the development of biomarkers to guide the identification of patients who will derive the greatest benefit from a given immunotherapy remain critical. Identifying consistent changes in cellular function that occur in neoplastic tissue could revolutionize the way cancer is treated. Thus, this study characterized and compared the molecular profiles of CX3CR1, TLR4-mediated interferon signaling pathway, and immune checkpoints in the different histological stages of NMIBC, aiming the investigation of these biomarkers as a criterion of clinical response to immunotherapy.

2 MATERIALS AND METHODS

2.1 HUMAN SAMPLES

Seventy-five formalin-fixed paraffin-embedded samples of bladder were collected from patients, the median age was 65 years (34 to 96-year-old), with a ratio of 2 men to each woman, with the diagnosis of urothelial lesion, obtained at the University of Campinas (UNICAMP) and Paulinia Municipal Hospital/ Brazil. Positive history for tobacco occurred in 80% (60/75). Only the cases in which the previous transurethral resection of bladder Tumor (TURBT) had the same grade tumor than the sample analyzed in the study were included in the analyzes. The classifications of neoplastic bladder lesions were based on the consensus proposed by the International Society of Urological Pathology/World Health Organization[26], after review by a senior uropathologist. After, bladder tumor samples were divided into 3 groups (n= 25 samples per group): pTis group, high-grade pTa group, and pT1 group.

The retrospective anonymous study was approved by the local ethics committee (Clinical Trial: RBR-6swqd2).

2.2 IMMUNOHISTOCHEMICAL ANALYSIS

All bladder tumor samples were submitted to immunohistochemistry analysis. Bladder tumor samples were sectioned at 5 µm thickness using a Slee CUT5062 RM 2165 rotary microtome (Slee Mainz, Mainz, Germany) and collected on silanized slides. Antigenic retrieval was performed by incubating the sections in citrate buffer (pH 6.0) at 100°C in a microwave oven. Endogenous peroxidase was blocked using Peroxidase Blocker (EasyPath EP12-20523, Sao Paulo, Brazil) with subsequent incubation in 5% Goat Serum blocking solution (EasyPath EP12-20523, Sao Paulo, Brazil) for 10 minutes at room temperature. Subsequently, the TLR4, TRIF, TBK1, IRF-3, IFN-γ, CX3CR1, PD-1/ PD-L1 and CTLA4 antigens were located using specific primary antibodies: anti-TLR4 (RRID:AB_2205016), anti-TRIF (RRID:AB_2201578), anti-TBK1 (RRID:AB_783995), anti-IRF3 (RRID:AB_2890146), anti-IFN-γ (RRID:AB_315493), anti-CX3CR1 (RRID:AB_2890638), anti-FOXP3 (RRID:AB_783444), anti-PD-1/PD-L1 (sc-293425, mouse monoclonal, Santa Cruz Biotechnology) and anti-CTLA4 (RRID:AB_10988256); diluted in goat serum 1% (EasyPath EP12-20523, Sao Paulo, Brazil) 1% and stored overnight at 4°C. EasyLink One (EasyPath EP-12-20504, Sao Paulo, Brazil) was used for antigen detection according to the manufacturer's instructions. After washing with TBS-T buffer, the sections were incubated with HRP-polymer (EP-12-20503) from the EasyLink One kit (EasyPath, Sao Paulo, Brazil) for 25 min and, subsequently revealed with diaminobenzidine (DAB), counterstained with Harris Hematoxylin and evaluated on the DM2500 photomicroscope (Leica, Munich, Germany).

To evaluate the intensity of antigen immunoreactions in the urothelial cells of the bladder tumors, ten fields were selected with 400x magnification for each patient and each antibody. The immunostaining results were analyzed using the Image J software (https://imagej.nih.gov/ij/) in Macro Profile Analysis from the selection of the urothelium and quantification of positive urothelial cells (adapted from previous protocol)[27]. Total immunoreactivity was obtained as the result of the percentage of urothelial cells negative for a given antibody subtracted from 100%, that is, the values represent the total number of urothelial cells in the field that showed immunoreaction for the evaluated antibody.

2.3 STATISTICAL ANALYSIS

The immunohistochemical analyses were represented as mean ± standard deviation. Total immunoreactivity average was calculated for each group and for each antibody and the data were submitted to the Analysis of variance (ANOVA), complemented by Tukey's test or the Kruskal-Wallis non-parametric analysis of variance followed by the Dunn post hoc in cases of lack of data normality (evaluated previously by the Shapiro-Wilk normality test). The software used for analysis was GraphPad Prism, version 7.00 (GraphPad Software Inc., San Diego, California, USA) and BioEstat 5.0 (Sociedade Civil Mamirauá/CNPq, Belém, PA, Brazil). Statistical significance was 5% ($p<0.05$) for all applied tests.

3 RESULTS AND DISCUSSION

3.1 IMMUNOREACTIVITY OF THE TLR4-MEDIATED IFN-Γ SIGNALING PATHWAY

The non-canonical TLR4 signaling pathway is responsible for inducing the production of interferons (IFNs), which have antitumor effects by inducing TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand), a potent inducer of cell death in malignant neoplastic cells[28]. The tumor suppressor action of the immune system depends on the actions of IFN-γ, which stimulates several antiproliferative and tumoricidal pathways in macrophages and tumor cell lines[29], in addition to playing an early role in protecting against metastases[30, 31]. Furthermore, IFNs are involved in the activation mechanisms of the innate immune system for tumor detection and modulation of T cell responses[32]. Activation of $TCD8^+$ lymphocytes against tumors was impaired in mice lacking STING (Stimulator of interferon genes). Woo et al. [32] demonstrated, in vitro, that through STING and IRF-3, the production of IFNs and the activation of dendritic cells (DCs) were triggered by DNA from neoplastic cells, indicating the involvement of a cytosolic DNA detection mechanism. In vivo models, DNA from malignant neoplastic cells was detected inside host antigen-presenting cells (APC), and this result correlated with the production of IFNs and activation of STING[32].

TLR4 is decreased in tumor tissues compared to surrounding tumor tissues or normal tissues, which is also positively correlated with overall survival rate (hazard ratio [HR] = 0.38) and cancer-specific survival rate (HR = 0.15) of patients with bladder cancer[33]. Our study detailed the TLR4 signaling pathway immunoreactivities in the different histological stages of NMIBC. The pTis group showed the lowest activation of TLR4-mediated IFN-γ signaling pathway when compared ($p<0.01$) to high-grade pTa and pT1 groups (Table 1). Both the immunoreaction intensity and positive cells percentage were lower ($p<0.01$) for TLR4, TRIF, IRF-3, and IFN-γ in the pTis group with respect to other groups (Figures 1A – 1O). No statistical

differences were found between high-grade pTa and pT1 groups for these biomarkers (Table 1).

3.2 IMMUNOREACTIVITIES OF THE CX3CR1, FOXP3, AND IMMUNE CHECKPOINTS

The urinary bladder has a unique immune microenvironment. On the one hand, the bladder employs several non-specific defense strategies to remain uninfected [34]. These strategies include a thick mucin barrier, urination, and secretion of antibacterial agents such as β-defensins and cathelicidin[34]. On the other hand, bladder has an immunosuppressive environment to prevent undesirable immune responses[34, 35]. In addition, the bladder tumor microenvironment is immunosuppressive. Bladder tumors can cause inefficiency of the tumor infiltrating lymphocytes (TILs) in eradicating tumor cells[36] as well as have been associated with increased levels of regulatory $FOXP3^+$ T cells (Tregs) and Th1 response inhibitory cytokines [e.g., transforming growth factor-β (TGF-β) and IL-10]. Therefore, the strategies that bladder tumors employ for immune evasion are key to the development of immunotherapies against bladder cancer[37].

Our study demonstrated that CX3CR1 immunoreactivities were remarkably lower ($p<0.01$) in the pTis group in comparison with high-grade pTa and pT1 groups, which did not show statistical differences between them (Table 1; Figures 2A, 2B, 2C).

The immune checkpoints (PD-1/PD-L1 and CTLA4) and $FOXP3^+$ Treg cells immunoreactivities were significantly higher ($p<0.01$) in the high-grade pTa and pT1 when compared to pTis group (Table 1; Figures 2D – 2L).

Table 1: Total Immunoreactivity (%) in the groups for the different antigens.

	Groups (n=10 fields/ group)		
Antigens	pTis	High-grade pTa	pT1
TLR4 *	66.20 ± 2.6 a	79.64 ± 5.6 b	71.15 ± 5.1 b
TRIF *	60.17 ± 3.2 a	76.96 ± 3.4 b	75.33 ± 4.0 b
TBK1	65.93 ± 4.1 a	73.96 ± 4.7 b	76.82 ± 13.2 b
IRF3 *	66.56 ± 5.2 a	82.88 ± 6.7 b	76.25 ± 5.3 b
IFN-γ	61.17 ± 15.6 a	77.74 ± 10.7 b	76.64 ± 11.7 b
CX3CR1	45.59 ± 15.3 a	74.25 ± 1.9 b	78.30 ± 7.7 b
FOXP3 *	71.79 ± 3.6 a	84.03 ± 2.5 b	80.80 ± 4.5 b
PD-1/PD-L1	51.84 ± 10.5 a	77.55 ± 3.4 b	72.78 ± 10.6 b
CTLA4	49.98 ± 8.4 a	79.80 ± 3.5 b	78.51 ± 7.4 b

Values expressed as mean ± standard deviation. ANOVA Kruskal-Wallis, Dunn's Test. *ANOVA, Tukey test. On the same line, values followed by different letters indicate a significant difference between groups ($p<0.01$).

Figure 1: Immunostaining of the antigens in the urinary bladders: TLR4 (A - C); TRIF (D - F); TBK1 (G - I); IRF-3 (J - L); IFN-γ (M - O). pTis group (A, D, G, J, M); High-grade pTa group (B, E, H, K, N); pT1 group (C, F, I, L, O). Bars = 50 µm.

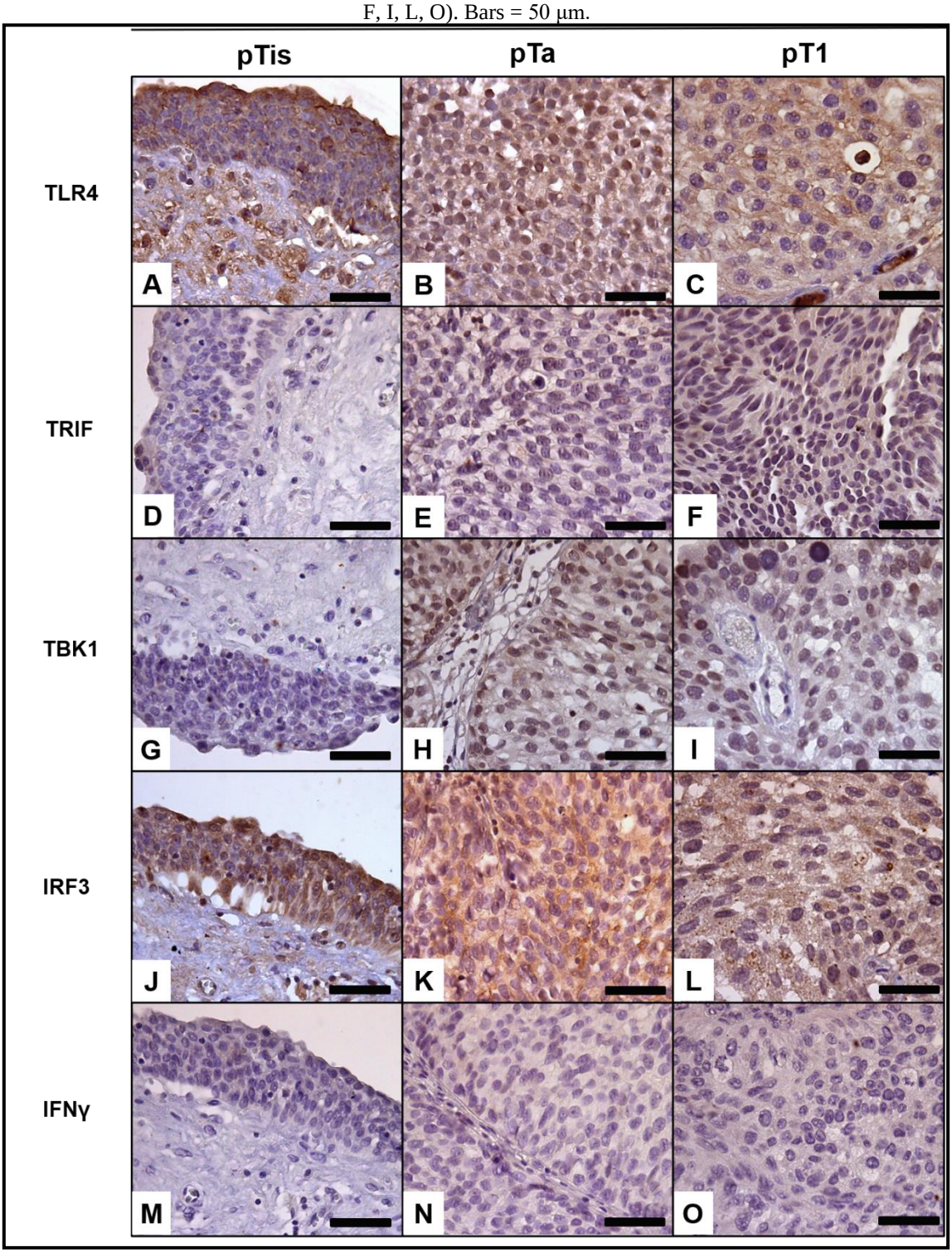
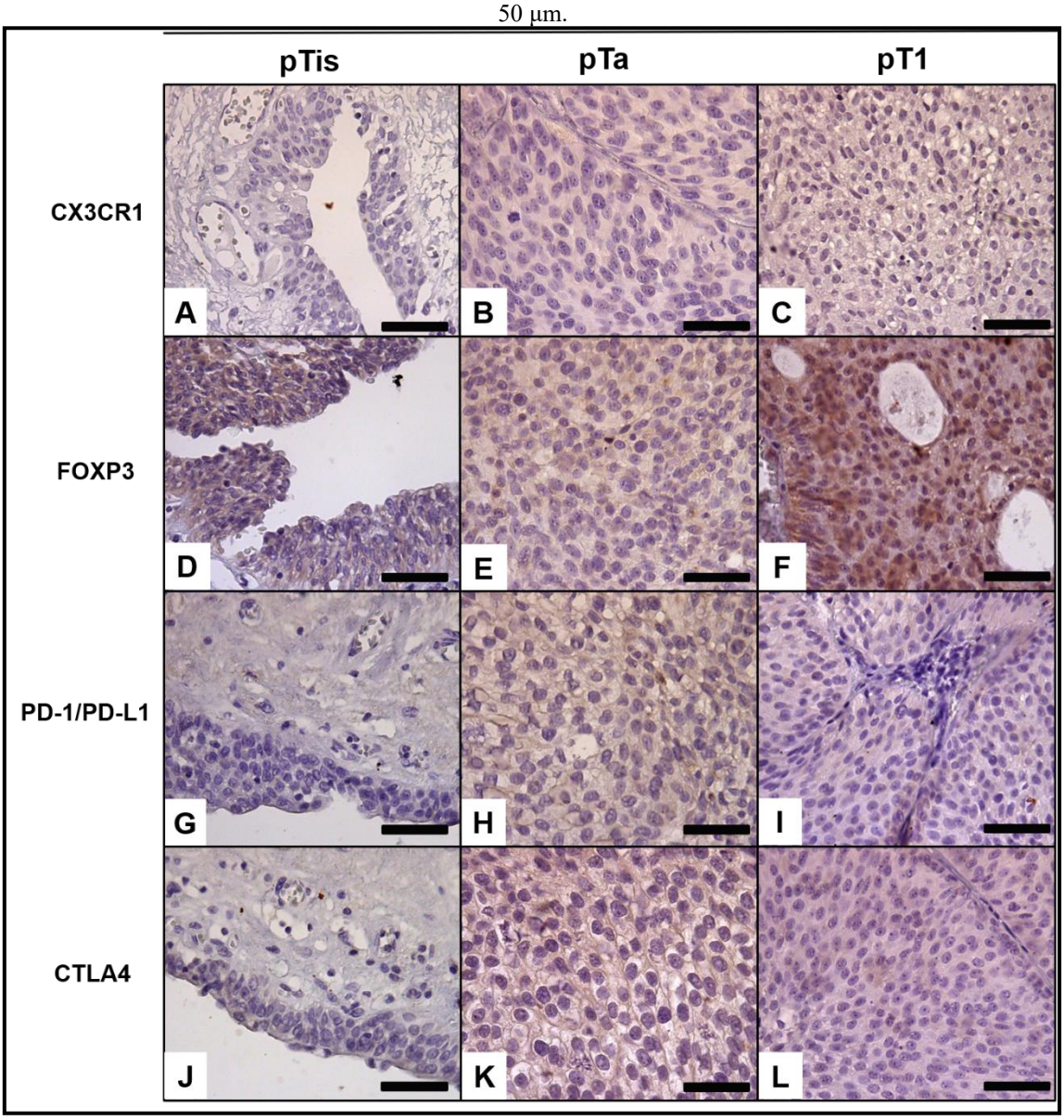


Figure 2: Immunostaining of the antigens in the urinary bladders: CX3CR1 (A - C); FOXP3 (D - F); PD-1/PD-L1 (G - I); CTLA4 (J - L). pTis group (A, D, G, J); High-grade pTa (B, E, H, K); pT1 group (C, F, I, L). Bars = 50 μm.



4 CONCLUSIONS

Our data demonstrated that pTis stage was characterized by an immunosuppressive microenvironment in comparison with pTa and pT1 stages, showing decreased TLR4-mediated interferon signaling pathway and low activation of $CX3CR1^{+}CD8^{+}$ T-cells, which implies in low sensitivity to immunotherapy. The larger number of $FOXP3^{+}$ Treg cells in pTa and pT1 was correlated with intensified immune checkpoints immunoreactivities, indicating higher sensitivity to immunotherapy. Finally, these biomarkers may be useful in the clinical management of patients with NMIBC.

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