

Original Article

Distribution and expression profiles of dendritic cell subpopulations in human bladder cancer

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Abstract: Bladder cancer is one of the most common cancers worldwide, with transitional cell carcinoma (TCC) being the predominant form. Dendritic cells (DCs) are a heterogeneous population of antigen-presenting cells that play a crucial role in the regulation of the immune response and immunological tolerance. The aim of this study was to investigate the expression and characteristic of different DC subsets in non-muscle-invasive TCC (NMI-TCC), muscle-invasive TCC (MI-TCC) and controls. Our study demonstrated that normal human bladder contained only immature CD1a⁺, CD11c⁺, and CD209⁺ DCs whereas CD123⁺ pDCs and CD83⁺ mature DCs were virtually absent. The number of both CD11c⁺ IL-23⁺ mDCs and CD123⁺ pDCs were significantly elevated in NMI- and MI-TCC, and the mDC populations of CD1a⁺, CD209⁺ immature DCs and CD83⁺ mature DCs also increased in NMI- and MI-TCC. Except for a relatively low number of CD83⁺ mature DCs, the numbers of CD11c⁺, CD123⁺, CD1a⁺, CD209⁺ DCs were also significantly increased in MI-TCC compared with NMI-TCC. Taken together these results suggest that an imbalance in the distribution of tumor infiltrating DCs in the tumor area seems to be involved in the development or progression of bladder cancer.

Keywords: Dendritic cells, bladder cancer, progression, immune response

Introduction

Bladder cancer is one of the most common cancers worldwide, with transitional cell carcinoma (TCC) being the predominant form [1]. Bladder cancer represents a heterogeneous disease, with two distinct subtypes of TCCs: ~75% of the affected individuals have superficial non-muscle-invasive TCCs (NMI-TCCs), which tend to recur but not life-threatening, and ~25% of patients have muscle-invasive TCC (MI-TCCs), which are associated with a high risk of death from distant metastases [2]. Previous studies demonstrate that the presence of tumor-infiltrating lymphocytes was associated with increased disease-free survival in MI-TCCs [3, 4]; whereas an earlier study found that the density of tumor infiltrating lymphocytes was positively correlated to a short recurrence-free survival in NMI-TCCs [5]. Recent studies reported that a high level of CD83⁺ mature DC infiltration of NMI-TCC patients do not respond as well to bacillus Calmette-Guerin (BCG) immunotherapy [6]; subsequently, their studies showed

that the presence of CD83⁺ mature DC was found associated with a significant risk of progression to MI-TCCs [7]. However, DCs are a heterogeneous population of antigen-presenting cells, and the distribution and characterization of DC subpopulations remain to be elucidated in human bladder cancer.

DCs are thought to be crucial regulators of immune response and immunologic tolerance in numerous organs, which comprise two major classes: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) [8]. pDCs express CD123, CLA and BDCA-2, lack expression of CD11c in human, and sense the environment for nucleic acids with the help of pattern-recognition receptors in the context of viral and microbial infections as well as autoimmune diseases [9]. mDCs also called conventional DCs have a strong capability to capture antigens which enables them to stimulate T cells. These major antigen presenting and activating cells also comprise a very heterogeneous subset of cells expressing high levels of human leukocyte antigens (HLA) class

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Table 1. Summary of primary antibodies used in this study

Antibody against	Dilution	Clone	Source
CD1a	1:200	O10	Santa Cruz, Mouse Monoclonal Ab
CD11c	1:100	5D11	Novocastra, Mouse Monoclonal Ab
CD83	1:100	HB15e	AbD seroTec, Mouse Monoclonal Ab
CD123	1:100	10B8E7	LSBio, Mouse Monoclonal Ab
CD209	1:200	h209	LSBio, Rat Monoclonal Ab
IL-23p19	1:1000	Polyclonal	Abcam, Rabbit Polyclonal Ab

II molecules and integrin CD11c on their cell surface. Immature DCs express CD1a, adhesion molecules (such as CD209), and low level of HLA class II. After antigen uptake and processing, mDCs undergo maturation with coordinate up-regulation of HLA class II molecules, co-stimulatory molecules (CD80, CD86), CD83 and migrate to lymphoid tissue where they elicit adaptive Th1/Th17 type immunity [10, 11].

Thus, unraveling the characterization and prognostic value of tumor-infiltrating DC subsets are helpful for analyzing the immunopathological mechanisms of bladder cancer and understanding the mechanisms of immune escape and prognosis of bladder cancer. The purpose of the present study was to identify the distribution of DC subpopulations in NMI- and MITCCs to elucidate the contribution of TIDCs to the immune escape and clinical prognosis of human bladder cancer.

Materials and methods

Sample collection

Samples were obtained from 20 patients with urothelial tumors of the urinary bladder (2 women, 18 men). The mean age was 61 years (range 44-77 years) in patients with a non-muscle invasive tumor ($n=10$) and 63 years (range 45-79 years) in patients with a muscle invasive tumor ($n=10$). The patients were treated at the Department of Urology, Da Ping hospital, from 2012 to 2014. Tumors were diagnosed and classified according to the International Union Against Cancer TNM Classification criteria. The grading of these tumors was assessed according to the WHO Classification of Tumors. The exclusion criteria were the following: 1) non-urothelial bladder cancer, 2) any intravesical instillations (BCG or chemotherapy) or systemic chemotherapy prior to study inclusion, and 3) insufficient tissue material available for histological re-review and immunohistochemistry. All samples were collected after getting the

informed consents from patients. The study was conducted with the approval of the local ethics committee and clinical investigation was performed according to the principles expressed in the Declaration of Helsinki.

Immunohistochemistry (IHC)

The IHC technique was performed in a standard manner as previously described [12]. Briefly, IHC was performed on 4 μ m sections of the archival epididymal tissue biopsies, which had been fixed in formalin's fixative and embedded in paraffin. The sections were deparaffinized, hydrated, washed in phosphate-buffered saline (PBS, pH 7.2). Antigen retrieval was done with Dako Target Retrieval Solution (pH 6.0, Dako, Glostrup, Denmark), and then the sections were applied with 3% H₂O₂ solution to block endogenous peroxides at room temperature (RT) for 10 min, treated with 10% normal goat serum containing 5% bovine serum albumin (BSA) at RT for 30 min (Jackson ImmunoResearch, West Grove, USA), and then incubated with the primary antibodies at the appropriate optimal dilutions overnight at 4°C in a humidified chamber (Table 1). Maixin MaxVision™ HRP-Polymer anti-Mouse/Rabbit IHC Kit (Maixin, Fuzhou, China, Code K5010) was applied for detection of the primary antibody binding. 3,3'-Diaminobenzidine (DAB) was carried out with Substrate-Batch and the sections were counterstained with Mayer's hematoxylin. In addition, the negative controls were obtained by substitution of the primary antibodies with isotype control IgG₁ or IgG_{2a} respectively in the staining procedure. All slides were mounted in Microscopy Kaiser's glycerol gelatine (Merck, Darmstadt, Germany, HX807791), and images were acquired with Leica Application Suite (Version 4.2.0, Oberkochen, Germany) under a Leica DM 4000B microscope.

Immunofluorescence (double staining)

Paraffin-embedded slides were deparaffinized by immersion in xylene, followed by dehydration in ethanol. Antigen retrieval was done with Dako Target Retrieval Solution (pH 6.0, Dako, Denmark). Following a 5-minute wash with Tris-buffered saline-Tween 20 (TBS-T), sections were incubated for 30 min at RT with 10% nor-

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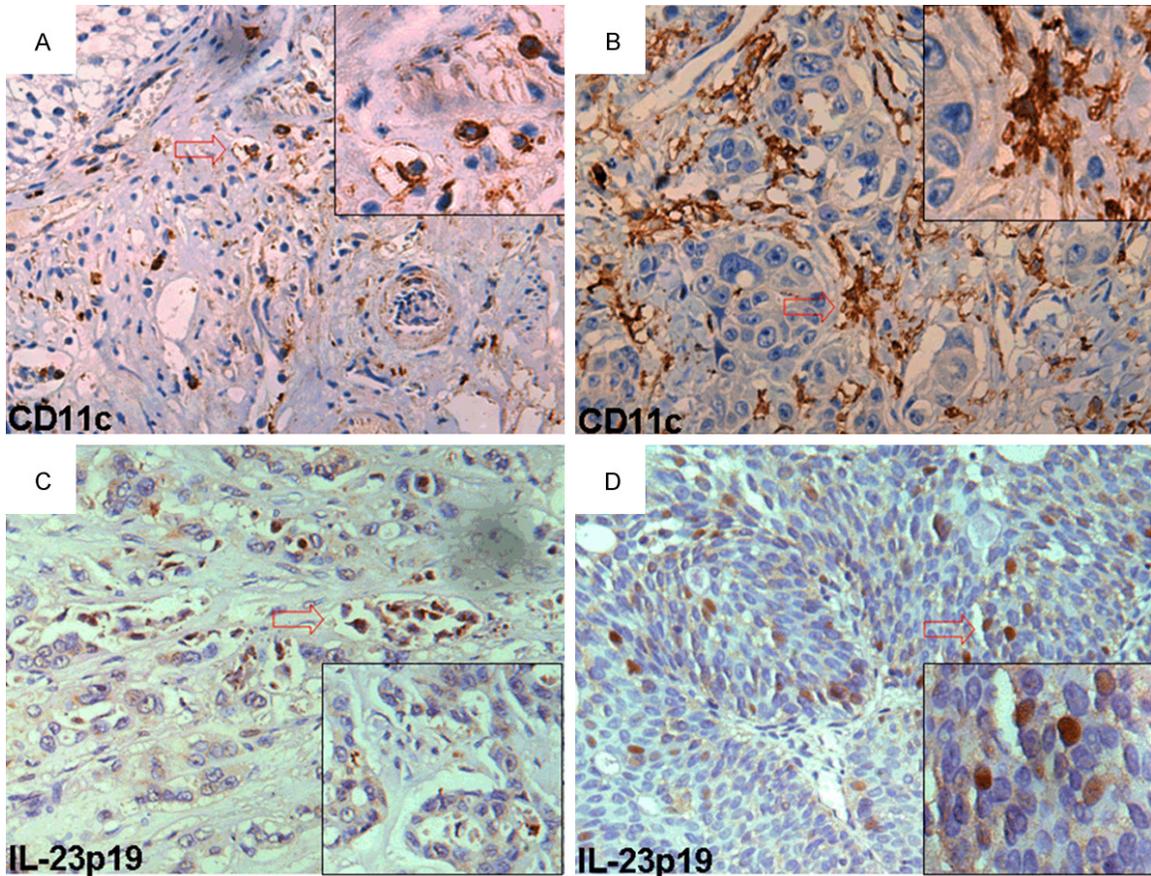


Figure 1. Distribution of CD11c⁺ mDCs and IL-23p19 in NMI- and MI-TCCs. CD11c⁺ mDCs were observed in the stroma and within tumors (A, B). For comparison, a stronger CD11c⁺ mDC infiltration was observed in MI-TCC (B) in contrast to NMI-TCC (A). Immunostaining for IL-23p19 could be observed in NMI- (C) and MI-TCC (D) (original magnification, $\times 200$; Inset: $\times 400$).

mal goat serum. Tissue sections were incubated overnight at 4°C with the mixture of two primary antibodies (CD11c⁺ IL-23p19 and CD4⁺ IL-17A). Following a 5-minute wash with TBS-T, slides were incubated for 30 min at RT with the mixture of two secondary antibodies (Cy2-conjugated affinipure goat anti-mouse IgG and Cy3-conjugated affinipure goat anti-rabbit IgG; Jackson ImmunoResearch, USA), and counterstaining was performed with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Merck, Germany). Again, sections of psoriatic skin served as positive control, while negative controls were obtained by substitution of the primary antibodies with mouse IgG₁ or rabbit serum in the staining procedure. All slides were mounted with Fluoromount-G (Southern Biotech, Birmingham, USA) to preserve fluorescence. The filters were used for fluorescence microscopy described as follows: Cy2, filter I3; Cy3, filter Y3; DAPI, filter A. Images were acquired with a Leica DM4000B fluorescence

microscope with a JVC digital device camera and collected by Leica Application Suite (Version 4.2.0).

Stereological techniques and statistical analysis

The immunohistochemical image data were analyzed and quantified by two blinded observers using stereological image processing software Image-Pro Plus (Version 6.0; Media Cybernetics, Inc, Rockville, USA). Five random microscope fields were selected from each slide ($\times 200$), and the expression intensity of immunopositive cells was represented by mean optical density value, which equals Integrate Optical Density (IOD) summary/Area summary. The Number of immunopositive cell/field was counted by means of the five fields. Data are expressed as mean \pm SEM. For the statistical evaluation of significances, the value of data obeyed normal distribution and Pearson test was performed using SPSS software (Version

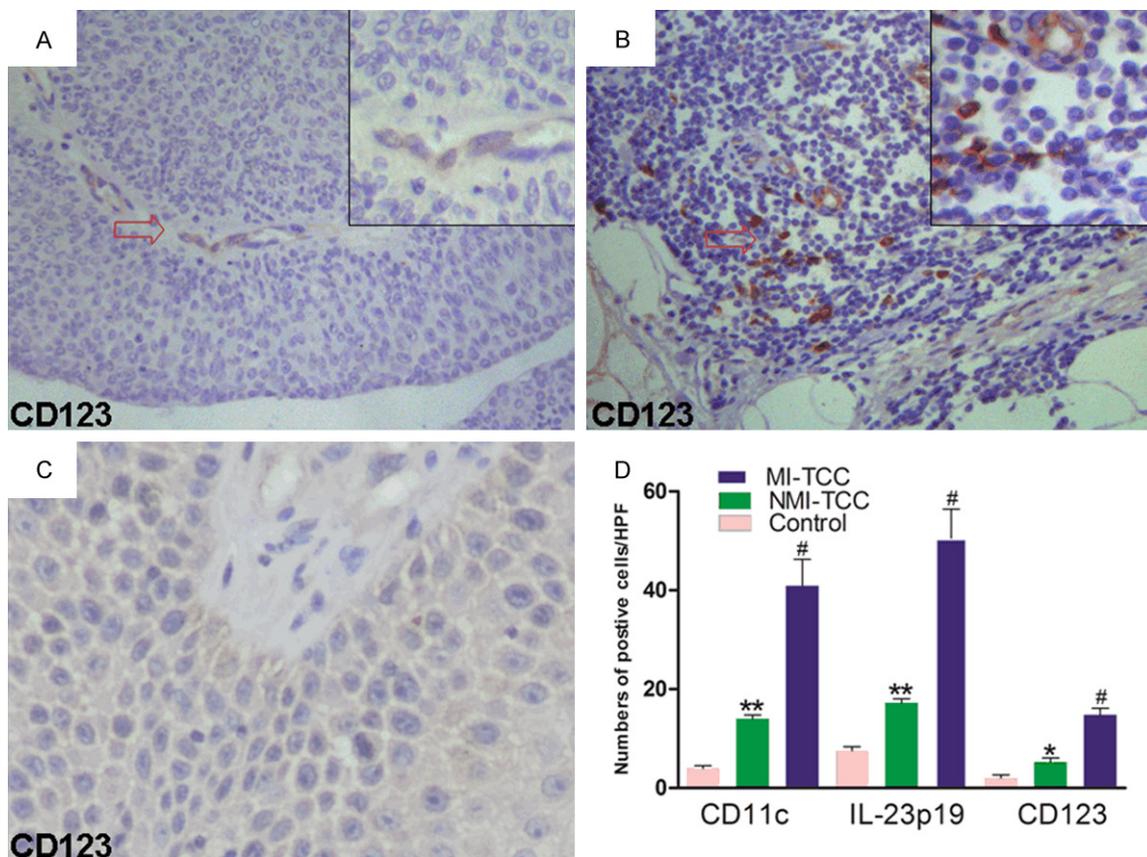


Figure 2. Distribution of CD123⁺ pDCs in NMI-, MI-TCCs and controls. CD123⁺ pDCs were detected in the vast majority of NMI- (A) and MI-TCC (B), whereas they were virtually absent from the control (C). This population of DCs was observed around the small vessels or as single cells in NMI-TCC (A) and principally localized within the leukocyte infiltration, arranged as clusters in MI-TCC (B). The results showed that the number of CD123⁺ pDCs significantly elevated in NMI- and MI-TCC compared with the control (D) (*compared with control, $P < 0.05$; **compared with control, $P < 0.01$; #compared with NMI-TCC, $P < 0.01$; data are expressed as the mean \pm SEM) (original magnification, $\times 200$; Inset: $\times 400$).

17.0, SPSS GmbH Software, an IBM Company, Shanghai, China). P -value < 0.05 was considered to indicate a statistically significant difference.

Results

Increased numbers of CD11c⁺ IL-23p19⁺ mDCs in NMI- and MI-TCC

As little is known about the expression of CD11c⁺ mDCs in human bladder cancer, the results showed that CD11c⁺ mDCs were observed in the stroma and within tumors (Figure 1A, 1B). The number of CD11c⁺ mDCs was low in control (Figure 2D), and a significant increase of CD11c⁺ mDC number can be detected in NMI- and MI-TCC compared with that in control (Figure 2D). Moreover, a stronger CD11c⁺ mDC infiltration was observed in MI-TCC in contrast to NMI-TCC (Figure 1A, 1B).

There was a base line expression of IL-23p19⁺ cells in control (data not shown), and an increased number of IL-23p19 expression could be observed in NMI- and MI-TCC (Figure 1C, 1D). In addition, the double staining of immunofluorescence revealed that the abundant numbers of CD11c⁺ IL-23p19⁺ mDCs were found in NMI- and MI-TCC. The results demonstrated that CD11c⁺ IL-23p19⁺ mDCs were the predominant component in bladder cancer and abundantly infiltrated into the stroma and tumors especially in MI-TCC.

The abundance of CD123⁺ pDC recruitments in NMI- and MI-TCC

pDCs represent the natural IFN- α -producing cells; of note, within 6 h of activation by a virus, human pDCs secrete 200-1000 times more IFNs than any other blood cell type [9]. However, the possible involvement of CD123⁺ pDCs in

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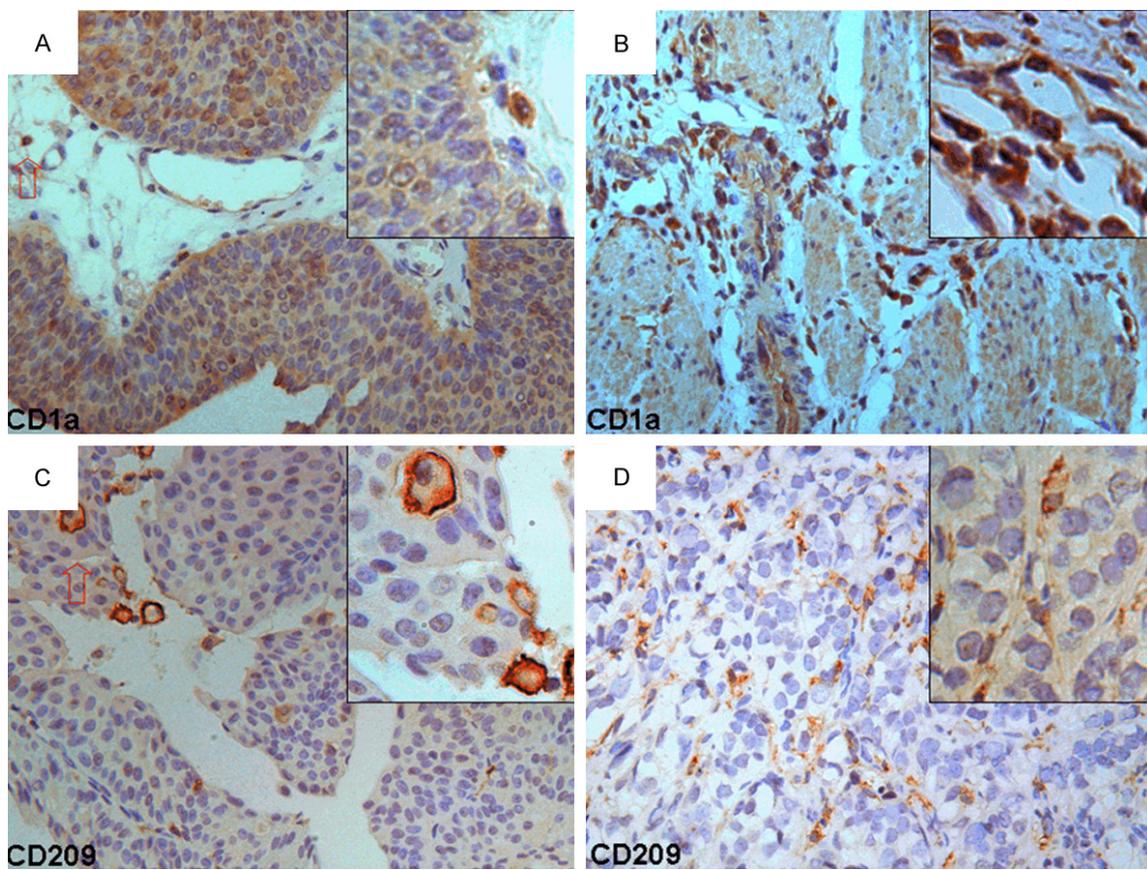


Figure 3. Distribution of CD1a⁺ and CD209⁺ immature DCs in NMI- and MI-TCCs. CD1a⁺ DCs were found mostly in the epithelium of the control (data not shown). Compared with normal counterparts, the numbers of CD1a⁺ DCs were significantly increased in the stroma of NMI- (A) and MI-TCC (B). Moreover, the abundance of CD1a⁺ DCs was observed mostly in the stroma but rarely within tumors in MI-TCC (B). The numbers of CD209⁺ DCs in NMI- (C) and MI-TCC (D) were also significantly increased in comparison to control and were frequently located in the stroma and occasionally as small aggregates in approximately half of MI-TCC (D) (original magnification, ×200; Inset: ×400).

bladder cancer has not been reported. The results showed that CD123⁺ pDCs were detected in the vast majority of NMI- and MI-TCC (Figure 2A, 2B), whereas they were virtually absent from the control (Figure 2C). This population of DCs was observed around the small vessels or as single cells in NMI-TCC (Figure 2A) and principally localized within the leukocyte infiltration, arranged as clusters in MI-TCC (Figure 2B). The results showed that the number of CD123⁺ pDCs significantly elevated in NMI- and MI-TCC compared with the control (Figure 2D).

Increased numbers of CD1a⁺, CD209⁺ immature DCs and CD83⁺ mature DCs in NMI- and MI-TCC

CD1a⁺ DCs are known to represent an immature subset of DCs and the low number of

CD1a⁺ DCs was found mostly in the epithelium of the control (data not shown). Compared with normal counterparts, the numbers of CD1a⁺ DCs were significantly increased in the stroma of NMI- and MI-TCC (Figures 3A, 3B, 4D). Moreover, the abundance of CD1a⁺ DCs was observed mostly in the stroma but rarely within tumors in MI-TCC (Figure 3B).

CD209⁺ immature DCs were sparsely distributed in the interstitial of control (data not shown), but there were virtually no CD83⁺ mature DCs in control (Figure 4C). The numbers of CD209⁺ DCs in NMI- and MI-TCC were also significantly increased in comparison to control and were frequently located in the stroma and occasionally as small aggregates in approximately half of MI-TCC (Figure 3D). Staining for CD83 showed a significant number of CD83⁺ cells in NMI- and MI-TCC and they were identified as

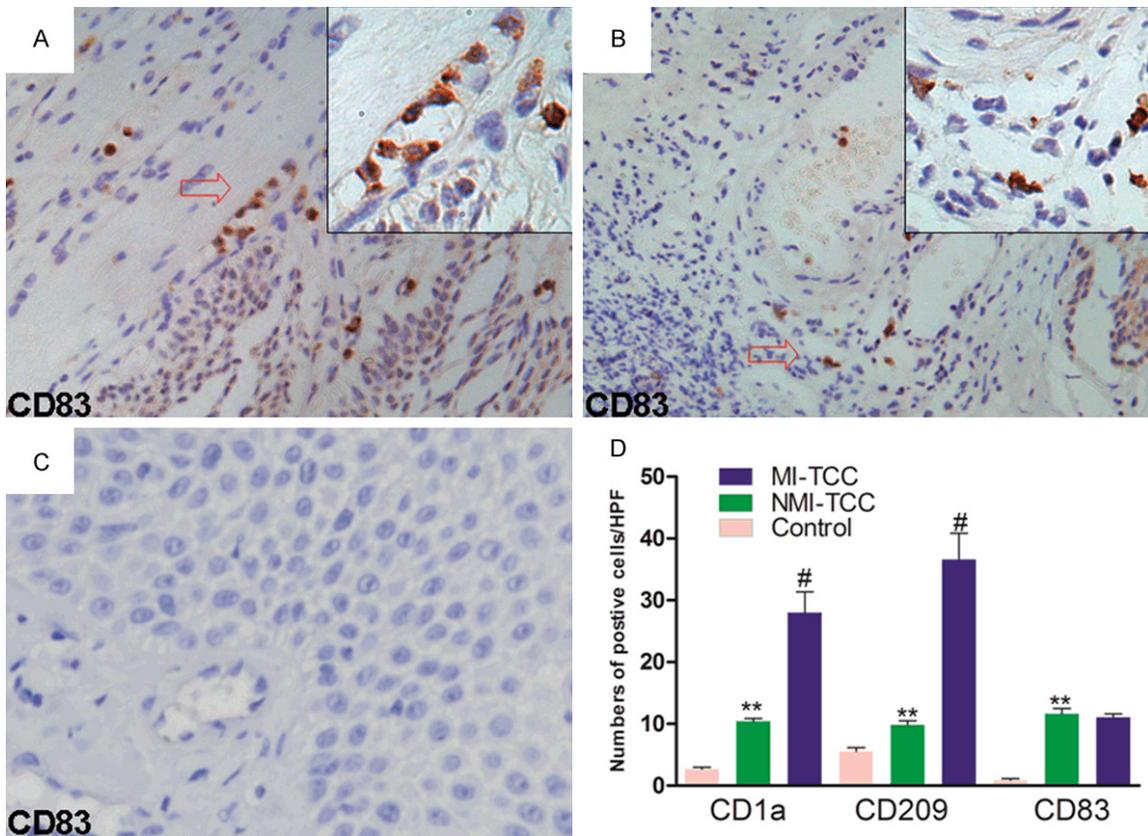


Figure 4. Distribution of CD83⁺ mature DCs in NMI-, MI-TCCs and controls. CD83⁺ mature DCs were distributed in the underlying stroma, rarely within tumors and frequently in lymphoid aggregates (A, B), but there were virtually no CD83⁺ mature DCs in control (C). Interestingly, a relatively low number of CD83⁺ DCs was observed in MI-TCC compared with NMI-TCC (D) (*compared with control, $P < 0.05$; **compared with control, $P < 0.01$; #compared with NMI-TCC, $P < 0.01$; data are expressed as the mean \pm SEM) (original magnification, $\times 200$; Inset: $\times 400$).

mature DCs (Figure 4A, 4B). CD83⁺ mature DCs were distributed in the underlying stroma, rarely within tumors and frequently in lymphoid aggregates (Figure 4A, 4B). Interestingly, a relatively low number of CD83⁺ DCs was observed in MI-TCC compared with NMI-TCC (Figure 4D).

Discussion

DCs are specialized APCs that are key player in antigen presentation and T cells activation exerting pathogenic role in tumor immunology, but the characteristics, distribution and function of DC subsets in bladder cancer have not been well defined in the past. In this study, we demonstrated for the first time that (I) normal human bladder contained only immature CD1a⁺, CD11c⁺, and CD209⁺ DCs whereas CD123⁺ pDCs and CD83⁺ mature DCs were virtually absent; (II) the number of both CD11c⁺ IL-23⁺ mDCs and CD123⁺ pDCs were significantly elevated in NMI- and MI-TCC; (III) the

mDC populations of CD1a⁺, CD209⁺ immature DCs and CD83⁺ mature DCs also increased in NMI- and MI-TCC; (IV) except for a relatively low number of CD83⁺ mature DCs, the numbers of CD11c⁺, CD123⁺, CD1a⁺, CD209⁺ DCs were also significantly increased in MI-TCC compared with NMI-TCC.

The infiltration of DCs in various cancers has been reported as an important prognostic parameter, either favorable or unfavorable and that often depends on both cell localization and degree of cell maturation [6, 13]. Previous studies revealed that CD1a⁺ and CD209⁺ DCs played key roles in the capture of antigens and subsequent processing for presentation to T cells [14, 15]. Moreover, immature DCs capture apoptotic cells and cross-present antigens derived from internalized dying cells on MHC I class molecules for recognition by CD8⁺ T cells [16]. Therefore, the strategic localization of immature CD1a⁺ DCs and CD209⁺ DCs sug-

gests that they might have an important role in the afferent arm of the adaptive immune response in bladder tissue, by taking up antigens from apoptotic cells and pathogens. On the other hand, cancer cells actively suppress steady state DCs (also called tumor-infiltrating DCs, TIDCs) and keep them in the favorable immature state [17]. This results in the promotion of tumor development because immature DCs are less capable of initiating T lymphocyte activation resulting in T cell anergy.

Our study showed that the large numbers of CD11c⁺ cDCs were observed in NMI- and MI-TCC compared with that in control. Similarly, the number of IL-23p19⁺ cells also increased in bladder cancer. In accordance with this finding, previous studies demonstrated that the abundance of CD11c⁺ DCs were probably derived from the monocytes that were first to recruited to the inflammatory site and marked the critical step in the formation of inflammatory lesion and tumor development [18, 19]. IL-23p19 produced by APCs is thought to play a pivotal role in terminal differentiation and survival of Th17 cells associated with the development of autoimmunity and inflammatory response [15]. The termed "inflammatory DCs" are recently identified as CD11c⁺ IL-23⁺ mDCs and probably differentiated from monocytes that are first to recruit to the site of inflammation and induce Th17 differentiation [20]. Our observations indicate that CD11c⁺ DCs probably represent the main source of IL-23, thereby initiating the inflammatory cascade and promoting Th17 development.

Interestingly, this study demonstrates that pDCs are found in the vast majority of bladder cancer, whereas they were virtually absent from the controls. pDCs represent the main IFN- α producers among leucocytes and this cytokine might induce cytotoxicity, activating NK/T cytotoxic cells or FasL-mediated apoptosis [21]. Cytotoxic T cells as the main lymphocyte subpopulations have been reported in muscle invasive urothelial carcinoma, which were strongly associated with longer overall survival [3]. Fas/FasL system played crucial roles in cytotoxic T lymphocyte and natural killer cell mediated cytotoxicity against cancer cancers; meanwhile, circulating soluble Fas antagonizes cell-surface Fas function and may interfere with immune surveillance against autologous tumors [22]. Therefore, the recruitment of pDCs in bladder cancer might facilitate

and amplify the cytotoxic T cell functions acting as a barrier to prevent invading pathogens and eliminate malignant cells. Although pDCs have been implicated in the initiation and maintenance of various cancers, it remains unclear whether pDCs are dispensable during the onset and development of bladder cancer.

Previous studies suggest that a high level of CD83⁺ mature DC infiltrations decrease the risk of recurrence in patients with bladder cancer and were associated with a significant risk of progression to muscle-invasive cancer [6, 7]. Interestingly, our study demonstrates that a relatively low number of CD83⁺ mature DCs are observed in MI-TCC compared with NMI-TCC but there are virtually no CD83⁺ DCs in control. Only mature DCs are able to stimulate T cells, hereby increasing their proliferation and secretion of IFN- γ , which are often considered to be surrogate indicators of a productive immune stimulation [23]. On the other hand, abundance of CD1a⁺, CD209⁺ immature DCs are found in NMI- and MI-TCC, which suggest this may be the result of tumor cells inhibiting the release of DC stimulation and maturation factors. Therefore, an imbalance in the distribution of TIDCs in the tumor area seems to be involved in the development or progression of bladder cancer. Taken together, an understanding of the heterogeneity and function of different TIDC subsets in bladder cancer is critical to design of better strategies for the treatment of bladder cancer immunotherapy.

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Disclosure of conflict of interest

None.

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