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Original Article

Immature CD11c+ myeloid dendritic cells with inflammatory and regulatory cytokine profile in human seminoma

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Abstract: Our previous study indicated that IL-23 (interleukin-23) producing antigen presenting cells (APCs) which could induce an immunodeviation of T helper (Th cell) cell towards a Th17 immune response associated with testicular damage were detected in azoospermic testis with chronic inflammation. Unlike inflammatory microenvironment, tumor cells have means of altering the tumor microenvironment which hijack APCs function and recruit immune suppressive dendritic cells (DCs). The objective of this study was to investigate the expression and characteristic of different DC subsets cells in the seminoma and its controls. Our study demonstrated that CD11c+ myeloid DCs (mDCs) developed into two different statues: tumor infiltrating and aggregate unit forming DCs. Aggregate unit forming CD11c+ mDCs were predominantly distributed in the seminoma and most of them expressed surface molecules (CCR6, DC-SIGN), regulatory cytokines (VEGF, IL-4, indoleamine 2,3-dioxygenase (IDO)) and inflammatory cytokines (IL-23, IL-6). Moreover, the numbers of CD3+, CD4+, IL4+, IL17+ and Foxp3+ cells were significantly increased in the seminoma. Taken together, our results indicate that the CD11c+ mDCs in seminoma might play a vital role in the development of seminoma and are critical to design of DCs vaccine therapy.

Keywords: CD11c+ mDC, aggregate unit, immature DC, cytokine profile, seminoma

Introduction

The complete BTB was comprised of three components: anatomical (physical), physiological, and immunological [1]. The tight junctions (TJs) (Sertoli cells-Sertoli cells tight junctions) from the anatomical barrier of animal testis that protect the passage of cytotoxic molecules and cells from entering into the lumen [2, 3]. The physiological barrier of testis is comprised of transport molecules that regulate the movement of substances in or out of the lumen, which play an important role in the proper development and maturations of germ cells (GCs) [4]. Under the physiology condition, the immunological barrier constructed an overall immune-privilege of the testis, which protects GCs from autoimmune attack [5, 6]. Whereas, in the inflammatory conditions, immune tolerance is disrupted and increased number of immune cells, such as mature dendritic cells (DCs), macrophages, CD4+ and CD8+ T cells, and their pro-inflammatory mediators respond to spermatogenic cells self antigens, inducing damage of the spermatogenic epithelium [7]. It has been reported that the level of transcripts associated with inflammation, such as inflammatory cytokines, correlated positively with the severity of mans infertility and perhaps reflecting a gradual breakdown of the status of testis immune privilege [8]. Our previous study indicated that IL23 producing CD11c+ and CD68+ APCs which could induce an immunodeviation of T helper (Th cell) cell towards a Th17 immune response associated with testicular damage were detected in azoospermic testis with chronic inflammation [9].

Compared with chronic inflammation, tumor cells can evade the immune system by inhibit-

ing the function of APCs, which induce T cells tolerance and inactive immune surveillance in the process of tumorigenic [10]. Testicular cancer had the highest rates among young men aged 20-34 years, and most of testicular tumors (more than 95%) are germ cell tumors (TGCT). TCGT of young and adolescents can be divided into two kinds: seminomas and non-seminomas. Seminomas are histologically homogeneous and it represents 50%-70% of the TGCT, while non-seminomas are heterogeneous include embryonic carcinoma, yolk sac tumor, teratoma and choriocarcinoma [11].

Patients with seminoma have been reported to have increased frequency of DCs and Th cells in the tumor tissues of testis [12]. It is relevant to know that tumor cells have means of altering the tumor microenvironment which hijack antigen presenting cells (APCs) function and recruit immune suppressive DCs [13]. In the meantime, the immune suppressive DCs and paralyzed tumor infiltrating DCs suppress both innate and adaptive immune effectors by secreting immunosuppressive cytokines, which contribute to tumor escape [14]. In addition, tumor cells promote the differentiation of DCs producing IDO, IL-10, TGF-β and VEGF that induce the expansion of CD4+CD25+ regulatory T cells which have reported contribute to tumor escape [15]. Thus, unraveling the characterization and function of DC subsets in the seminoma tissues are helpful for analyzing the mechanisms of immune tolerance and tumor escape. However, the characterization and function of DC subsets in seminoma are yet to be defined in detail.

The purpose of this study was to identify the distribution and intensity of DCs of various phenotype in the seminoma and its control to elucidate the contribution of DCs to the immune tolerance and tumor escape. To address this question, we firstly characterized the subtype of the infiltrating DCs in the testis with overt classical seminoma, because this cancer type of testis tumor is greatly associated with a prominent immune cells infiltrate [16]. Subsequently, we analysis DCs maturation in seminoma by detecting the expression intensity of specific marker CD83 (mature), HLA-DR (mature) and CCR6 (immature), as tumor cells can release factors that reverse or inhibit tumor infiltrating DCs maturation and normal function. We also investigated the cytokine profile of CD11c+ mDCs in the testis biopsies from seminoma patients, because CD11c+ mDCs were the predominant component of the seminoma in this study. Finally, we analyzed the expression of Th1 (IFN-γ+), Th2 (IL4+), regulatory T (Foxp3+) and Th17 (IL-17A+) cells producing cytokines in the seminoma as DCs in the tumor microenvironment possesses potent T cell suppressive functions and induce a balance shift by secreting immunosuppressive cytokines.

Materials and methods

Antibodies for immunohistochemistry and immunofluorescence staining

For morphology (immunohistochemistry and immunofluorescence) staining, the following antibodies were used as primary antibodies: monoclonal mouse anti-human CD3 (ab699, IgG; Abcam, Cambridge, UK), polyclonal rabbit anti-human CD4 (ab133616, IgG; Abcam), monoclonal mouse anti-human CD1a (ab708, IgG1; Abcam), monoclonal mouse anti-human CD11c (60258-1-Ig, IgG2a; ProteinTech, Wuhan, CHN), polyclonal rabbit anti-human CD68 (25747-1-AP, IgG1; ProteinTech), polyclonal rabbit anti-human CD163 (ab87099, IgG; Abcam), monoclonal mouse anti-human CD207 (sc-271272, IgG1; Santa Cruz, California, USA), polyclonal rabbit anti-human DC-SIGN (LS-B479, IgG; Life Span, WA Seattle, USA), polyclonal rabbit anti-human BDCA-2 (17941-1-AP, IgG; ProteinTech), monoclonal mouse anti-human CD123 (LS-C311924, IgG1; Life Span), polyclonal rabbit anti-human IL-17A (ab136668, IgG; Abcam), polyclonal rabbit anti-human IL-23p19 (ab45420, IgG; Abcam), polyclonal rabbit anti-human Foxp3 (ab10563, IgG; Abcam), polyclonal rabbit anti-human TGF-β (ab66043, IgG; Abcam), polyclonal rabbit anti-human IL-4 (ab9622, IgG; Abcam), polyclonal rabbit anti-human IL-6 (21865-1-AP, IgG; ProteinTech), polyclonal rabbit anti-human TNF-α (ab6671, IgG; Abcam), polyclonal rabbit anti-human VEGF (ab46154, IgG; Abcam), polyclonal rabbit anti-human IFN-γ (ab56070, IgG; Abcam), polyclonal rabbit anti-human IDO (ab55305, IgG; Abcam), mouse anti-human IgG (A57H, IgM; Gene Tex, California, USA), rabbit anti-human IgG (GTX22410; Gene Tex), mouse anti-human IgG1 (GTX76251, IgG2a; Gene Tex)

Table 1. Summary of clinicopathological features of seminoma

Pt No.	Age/years	Grade	Stage	Pt No.	Age/years	Grade	Stage
1	22	I	T2N0M0	13	42	II	T2N1M0
2	39	I	T2N0M0	14	29	I	T2N0M0
3	53	I	T1N0M0	15	22	II	T3N3M0
4	37	II	T3N1M0	16	46	I	T2N0M0
5	51	I	T2N0M0	17	39	I	T2N0M0
6	38	I	T1N0M0	18	38	I	T1N0M0
7	48	II	T2N1M0	19	36	I	T2N1M0
8	37	I	T2N0M0	20	28	I	T2N0M0
9	43	I	T1N0M0	21	37	II	T2N2M0
10	35	I	T2N0M0	22	54	II	T2N1M0
11	35	II	T2N2M0	23	61	II	T3N1M0
12	38	I	T2N0M0				

Note: Pt No. patient number; Grade the World Health Organization 2004 classification; Stage the American Joint Committee on Cancer TNM classification.

and mouse anti-human IgG2a (code X0943; DaKo, Copenhagen, DEN) were served as negative control. Mouse anti-human Vimentin (ab8978, IgG1; Abcam) were severed as positive control.

Study population and sample collection

Seminoma and matched pericarcinous biopsies were obtained from twenty-three patients undergoing urological work-up between 2008 and 2014 at the Peking University Shenzhen Hospital (**Table 1**). Biopsies were fixed in Bouin's fixative and embedded in paraffin for immunofluorescence and immunohistochemistry staining. Hematoxylin-eosin stained sections from all paraffin-embedded samples were reviewed by pathologist to confirm the diagnoses according to World Health Organization criteria [17]. For comparison, ten normal testicular biopsies were collected from patients who underwent testectomy for castration therapy of prostate cancer. Written informed consent was obtained from all study subjects and received institutional review board approval from Peking University Shenzhen Hospital and Shenzhen PKU-HKUST Medical Center Research Ethics Committee according to the principles expressed in the declaration of Helsinki.

Immunohistochemistry

The immunohistochemical staining was performed by using a standard three-step technique. 4-um sections of the testicular biopsies were deparaffinized in dimethylbenzene and rehydrated in alcohol gradient. Antigen retrieval was performed with Tris-EDTA (pH 9.0). Phosphate-buffered saline (PBS, pH 7.45) was severed as wash buffer. Endogenous peroxidase activity of biopsies was blocked by 3% H_2O_2 solution at room temperature for 12 minutes and non-specific binding of the secondary antibody was decreased by 7.5% normal bovine serum at 37°C incubator for 30 minutes. Tissue sections were treated with the primary antibodies at the optimal dilutions and overnight at 4°C refrigerator in a humidified chamber (**Tables 2**, **3**). After washing, the slides were treated with secondary antibodies and streptavidin-peroxidase solutions at 37°C incubator for 30 minutes. The staining reactions were developed brown by using DAB Kit (DAB-0031, MaiXin-Bio; CHN) and nuclear counterstaining was performed with Mayer's haematoxylin. The sections of lymph node and psoriatic skin were severed as the positive control. In addition, the primary antibodies were replaced with anti-vimentin antibodies. The negative controls were obtained by substitution of the primary antibodies with isotype control IgG, IgG1 or IgG2 respectively in the procedure of staining (Data not show). The slides were visualized and photographed with Leica Application Suite (Version 4.2.0, Oberkochen, Germany) under a Leica DM 4000B microscope.

Immunofluorescence (double staining)

The immunofluorescence staining was performed as previously describe [9]. Briefly, 4-um sections of the testicular biopsies were deparaffinized in dimethylbenzene and rehydrated in alcohol gradient. Antigen retrieval was performed with Tris-EDTA (pH 9.0). PBS (pH 7.45) was severed as wash buffer. Endogenous peroxidase activity of biopsies was blocked by 3% H_2O_2 solution at room temperature for 20 minutes and non-specific binding of the secondary antibody was decreased by 10% normal bovine serum at 37°C incubator for 30 minutes. Tissue sections were incubated respectively with the mixture of two primary antibodies overnight at

Table 2. Dilution and specificity of antibodies used for immunodetection of testicular immune cells

Antibody against	Dilution	Specificity	Reference
CD11c	1:1000	Myeloid dendritic cells (mDC)	Rival *et al.,* 2006
CD123	1:200	Plasmacytoid dendritic cells (pDC)	Andrade *et al.,* 2015
BDCA-2	1:250	Plasmacytoid dendritic cells (pDC)	Boiocchi L *et al.,* 2013
DC-SIGN	1:800	Stromal dendritic cells	Colmenares *et al.,* 2002
CD1a	1:150	Langerhans cells (LCs)/Dendritic cells	Longley *et al.,* 1989
CD207	1:200	Langerhans cells (LCs)	Valladeau *et al.,* 2000
CD68	1:350	Macrophages	Falini *et al.,* 1993
CD163	1:500	Macrophages	Lau *et al.,* 2004
CD83	1:100	Mature dendritic cells	Lechmann *et al.,* 2001
CCR6	1:500	Immature dendritic cells	Dieu *et al.,* 1998
HLA-DR	1:800	Major histocompatibility complex II	Klitz *et al.,* 2003
CD3	1:100	T cells	Mason *et al.,* 1989
CD4	1:200	T cells	Rudd *et al.,* 1988

Table 3. Dilution of antibodies used for immunodetection of cytokines

Antibody against	Dilution	Antibody against	Dilution
VEGF	1:500	IDO	1:200
IL23p19	1:800	IL4	1:500
IL6	1:500	Foxp3	1:800
IFN-γ	1:800	IL17A	1:1000
TGF-β	1:200		

4°C in a humidified chamber. After washing with PBS three times, the slides were incubated with the mixture of two secondary antibodies (CY2 conjugated goat anti-rabbit IgG and CY3 conjugated goat anti-mouse IgG; Jackson Immunoresearch) for 45 min at 37°C incubator. The nuclear of tissues were mounted with Hoechst 33342 (H3570, Life Technologies; OR, USA). The tissues of lymph node and psoriatic skin were severed as positive control. The negative controls were obtained by substitution of the primary antibodies with isotype in the procedure of staining (Data not show). The slides were visualized and photographed with Leica Application Suite (Version 4.2.0, Oberkochen, Germany) under a Leica DM 4000B microscope.

Quantitative analysis

The images data of immunohistochemical were analyzed and quantified by Image-Pro Plus (Version 6.0) using manually handwork and segmentation for color image. Ten random fields of microscopic were selected from each slide (×200) and quantified by two blinded observers with results variations less than 5%. Positive cell numbers/HPF was used to represent the numbers of immune-positive cells and mean optical density (IOD) summary was used to represent the expression intensity (EI) of immune-positive cells. Data are expressed as mean ± SD and were analyzed by SPSS software (version 16.0). Statistical significance was assessed using Independent Samples t-test and One Way ANOVA (LSD-t test was used for multiple comparison analyze). A *p*-value less than 0.05 was considered to indicate a statistically significant difference.

Results

CD11c+ mDCs forming an aggregate unit and have a predominant numbers in the seminoma

The results of immunohistochemical staining showed that CD11c+ mDCs were sparsely distributed in the interstitial area of normal testis (**Figure 1A**). Meanwhile, the CD11c+ mDCs in seminoma were developed into two different statues: tumor infiltrating DCs and aggregate unit forming DCs (**Figure 1B**), and the number of them were significantly higher compared with normal testis (**Figure 1C**).

DC-SIGN+ cells with immune phenotype were sparsely localized in the interstitial compartment of normal testis; in addition, germ cells as well as sertoli cells expressed DC-SIGN (**Figure 1D**). Compared with normal controls, the numbers of DC-SIGN+ cells were significantly increased in the seminoma tissues (**Figure 1F**). Interestingly, DC-SIGN+ cells were developed into tumor infiltrating and aggregate unit which were consisted with CD11c+ mDCs in the seminoma (**Figure 1E**). Furthermore, double staining

of immunofluorescence studies showed that CD11c+ mDCs were co-localized with DC-SIGN in the seminoma (**Figure 2Aa-Ad**), which indicated that DC-SIGN in the seminoma were mostly expressed in DCs but not macrophages.

pDCs in mouse tissues also express surface marker CD11c but not in human. For this reason, CD123 and BDCA-2 which severed as the specific marker of pDCs were investigated in this study. CD123+ and BDCA-2+ pDCs were sparsely distributed in the interstitial area of normal testis (**Figure 1G**, **1J**). Compared with normal testis, the populations of CD123+ and BDCA-2+ pDCs were increased weakly in the seminoma (**Figure 1H**, **1I**, **1K**, **1L**).

In this study, both CD207+ and CD1a+ DCs were found in the interstitial compartment of normal testis (**Figure 1M**, **1P**). Interestingly, only a small number of CD1a+ DCs were detected in the seminoma (**Figure 1N**, **1O**, **1Q**, **1R**) which indicated that CD1a+ DCs in testis were different from LCs of skin and mucosa.

We also tested the expression of macrophages in the seminoma and its controls. Both CD68+ and CD163+ macrophages were detected in the interstitial area of normal testis (**Figure 1S**, **1V**). The populations of CD68+ and CD163+ macrophages were significantly elevated in the seminoma compared with its controls (**Figure 1T**, **1U**, **1W**, **1X**). In addition, the double staining of immunofluorescence showed that CD68 did not co-localize with CD163 (data not show), which indicated that two types of macrophages expressed in the seminoma. As immunohistochemical staining showed that CD68+ macrophages in the seminoma appeared to aggregate (**Figure 1T**), we, therefore, analyzed the relationship between CD68+ macrophages and CD11c+ mDCs using double staining of immunofluorescence. Study results showed that CD68+ macrophages distributed around CD11c+ mDCs in the seminoma (**Figure 2Ba-Bd**), which indicated CD11c+ mDCs had a tendency to recruit CD68+ macrophages under tumor microenvironment.

In order to evaluate the classic of immune cells which play a dominant role in the seminoma, we analyzed the expression of above surface markers in the seminoma using One Way ANOVA (LSD-t test was used for multiple comparison study). The results indicated that CD11c+ mDCs were the predominant component of the seminoma and abundantly forming aggregate unit under tumor microenvironment of seminoma (**Figure 2C**).

CD11c+ mDCs with an immature phenotype in the seminoma

Tumor microenvironment and its tumor cells release factors that reverse or inhibit tumor infiltrating DCs (TiDCs) maturation and normal function [18]. In the meantime, immature and paralyzed TiDCs suppress both innate and adaptive immune effectors, which contribute to tumor escape [19]. Immature DCs express CD1a, Chemokine Receptor (such as CCR6), adhesion molecules (such as DC-SIGN), and low level of MHC class II (HLA-DR).

HLA-DR is an MHC class II cell surface receptor which typically found on the surface of APCs [20]. Study results of immunohistochemical staining showed that HLA-DR+ cells were located in the interstitial compartment of the normal testis (**Figure 3A**). Compared with its controls, the numbers of HLA-DR+ cells were significantly increased in the seminoma (**Figure 3B**, **3C**). However, HLA-DR did totally not co-localized with CD11c and most of them were located around CD11c+ mDCs (**Figure 3Ja-Jd**) which was consist with the distribution of CD68+ macrophages in the seminoma (**Figure 2Ba-Bd**).

CCR6 is a C-C motif chemokine receptor protein which is encoded by the CCR6 gene and preferentially expressed by memory T cells and immature DCs [21]. CCR6+ cells were occasionally found in the interstitial area of normal testis (**Figure 3D**). For comparison, the population of CCR6+ cells were significantly increased in the seminoma (**Figure 3E**, **3F**) and immunofluorescent studies of double staining showed that mostly of CD11c+ mDCs expressed CCR6 (**Figure 3Ka-Kd**).

The immunohistochemical analysis suggests that CD83 which identified as mature DCs is expressed in the seminoma at low levels (**Figure 3H**), and there were no CD83+ DCs in normal testis (**Figure 3G**). Taken together, these data suggest that CD11c+DC-SIGN+CCR6+HLA-DR-CD83- mDCs in the seminoma were in an immature phenotype and play an important role in the process of tumor immune escape.

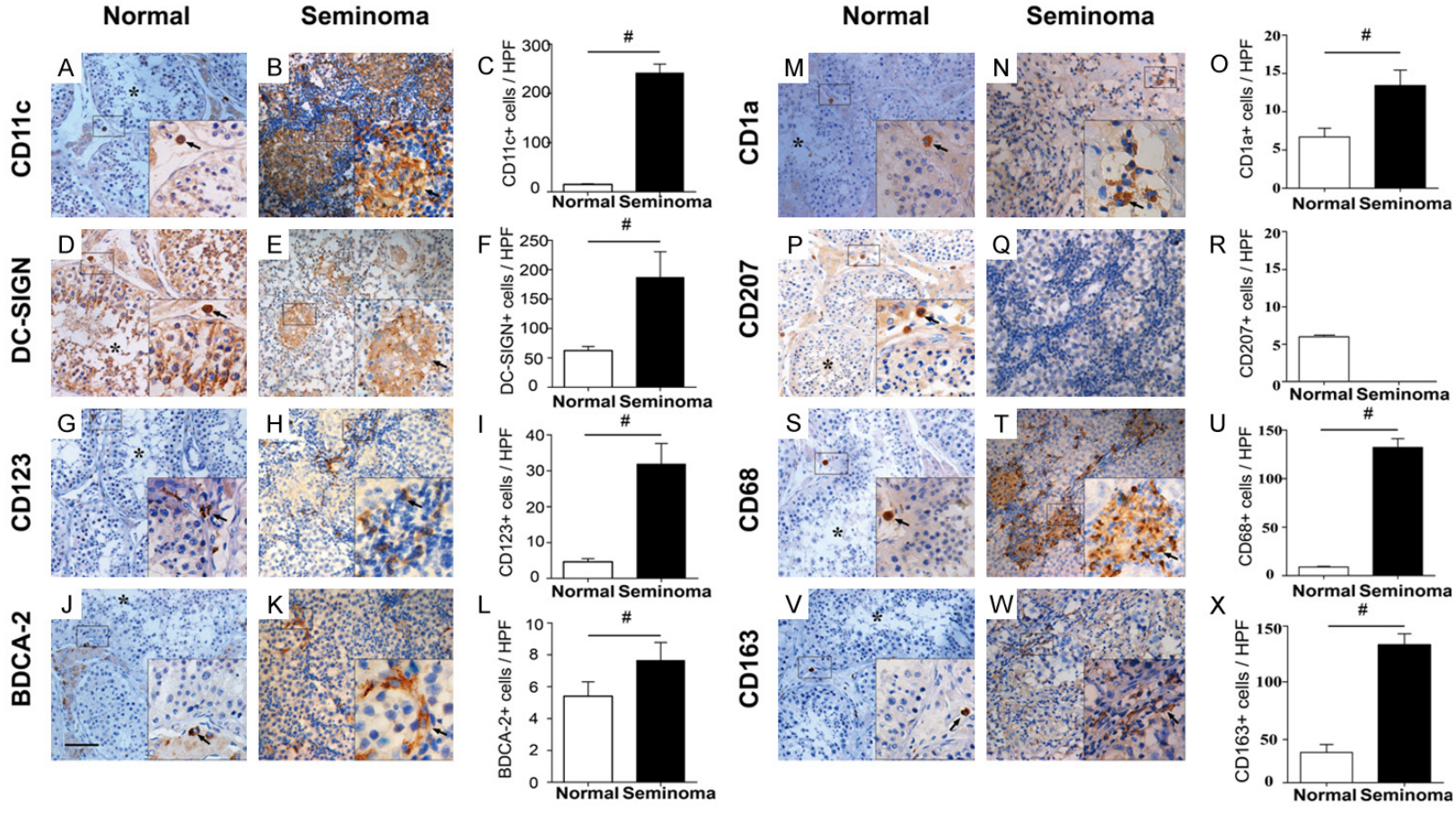

Figure 1. Distribution of CD11c+, DC-SIGN+, CD123+, BDCA-2+, CD1a+, CD207+, CD68+, CD163+ cells in normal testis and seminoma. CD11c+ mDCs were identified in the interstitial compartment of the normal testis (A). Abnormal large amount of CD11c+ mDCs were observed in the infiltrate and also concentrated as aggregate unit in the seminoma (B). DC-SIGN+ cells were sparsely localized in the interstitial area of normal testis; meanwhile, germ cells as well as sertoli cells expressed DC-SIGN (D). Abnormal large amount of DC-SIGN+ mDCs concentrated as aggregate unit in the seminoma (E). CD123+/BDCA-2+ pDCs were sparsely localized in the interstitial compartment of normal testis respectively (G, J). CD123+/BDCA-2+ pDCs were identified in the lymphocyte infiltrate area of seminoma respectively (H, K). CD1a+/CD207+ DCs were sparsely identified in the interstitial compartment of the normal testis (M, P). Abnormal large amount of CD1a+ cells in seminoma tissues (N). CD207+ DCs were absent from the controls (Q). Both CD68+ and CD163+ macrophages were identified in the interstitial compartment of normal testis (S, V). CD68+ macrophages were increased in the seminoma and mostly of them concentrated as aggregate unit were observed in the seminoma (T). Abnormal large amount of CD163+ cells in seminoma tissues (W). Comparison of the numbers of CD11c+, DC-SIGN+, CD123+, BDCA-2+, CD1a+, CD207+, CD68+, CD163+ cells in controls and seminoma (C, F, I, L, O, R, U, X). Quantitative enumeration was performed at the single cell level in immunohistochemically stained sections by in situ analysis using Image-Pro Plus software. The results are expressed as the means ± SEM; Arrows represent positive cells; *Represent the lumen of seminiferous tubule; #P<0.05; Bar = 100 μm (Original magnification, ×200; Inset: ×400).

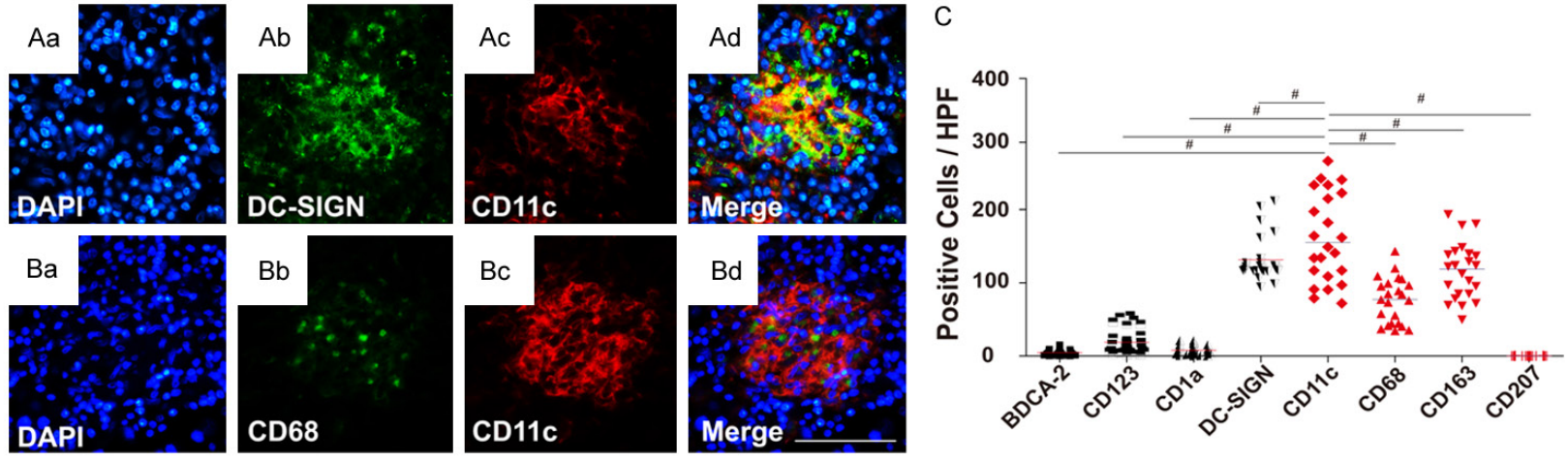

Figure 2. Immunofluorescence double staining of CD11c+DC-SIGN+ mDCs and CD68+CD11c- macrophages in the seminoma. Two color immunofluorescence was used to determine CD11c+DC-SIGN+ mDCs and CD68+CD11c- macrophages expression: CD11c, CY3, red fluorescence (Ac, Bc); CD68 and DC-SIGN, CY2, green fluorescence (Ab, Bb); CD68+CD11c- macrophages, merged (Ad); CD11c+DC-SIGN+ mDCs, merged (Bd); Nuclei were labeled with DAPI (Aa, Ba); respectively. Bar = 50 μm (Magnification: ×400). CD11c+ mDCs were the predominant component of the APCs in seminoma (C). One Way ANOVA (LSD-t test) was used for multiple comparison; Quantitative enumeration was performed at the single cell level in immunohistochemically stained sections by in situ analysis using Image-Pro Plus software. The results are expressed as the means ± SEM; #P<0.05.

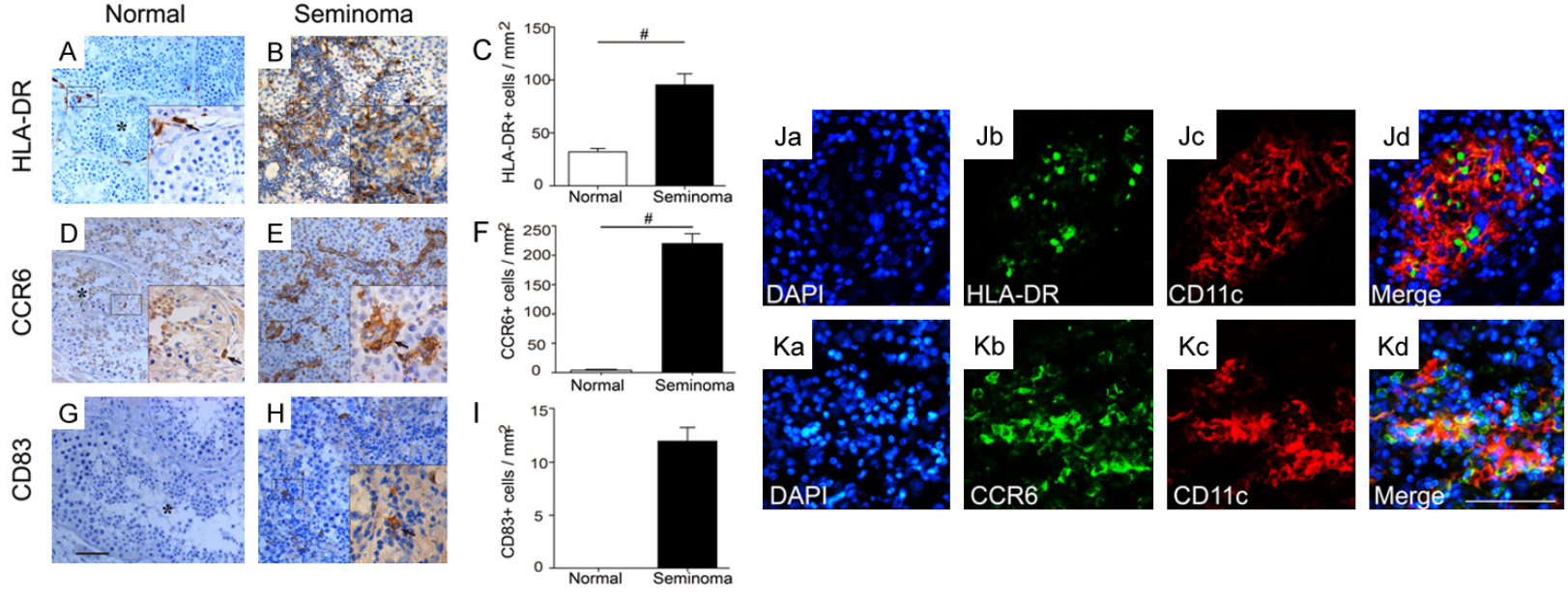

Figure 3. Distribution of HLA-DR+, CCR6+ and CD83+ cells in the seminoma and its control. Both HLA-DR and CCR6+ cells were observed in the interstitial compartment of normal testis respectively (A, D). The number of HLA-DR+ and CCR6+ cells were significantly increased in the seminoma (B, E) compared with it controls respectively (C, F). CD83+ DCs were sparsely localized in the seminoma (H) and virtually absent from the normal testis (G). The number of CD83+ cells/mm^2 in seminoma (I). Quantitative enumeration was performed at the single cell level in immunohistochemically stained sections by in situ analysis using Image-Pro Plus software. The results are expressed as the means ± SEM; Arrows represent positive cells; *Represent the lumen of seminiferous tubule; #P<0.05; Bar = 100 μm (Original magnification, ×200; Inset: ×400). Two color immunofluorescence was used to determine CD11c+HLA-DR- and CD11c+CCR6+ mDCs expression: CD11c, CY3, red fluorescence (Jc, Kc); HLA-DR and CCR6, CY2, green fluorescence (Jb, Kb); CD11c+HLA-DR- mDCs, merged (Jd); CD11c+CCR6+ mDCs, merged (Kd); Nuclei were labeled with DAPI (Ja, Ka); respectively. Bar = 50 μm (Magnification: ×400).

CD11c+ mDCs with tolerance cytokine profile in the seminoma

In a preliminary set of experiments, we observed CD11c+ mDCs with an immature phonotype playing a predominant role and abundantly forming aggregate unit in the seminoma. It is relevant to note that DCs in the tumor microenvironment possesses potent T cell suppressive functions and can induce immune tolerance by secreting immunosuppressive cytokines [22]. Therefore, we analyzed the cytokine profiles of CD11c+ mDCs in the seminoma.

Indoleamine-2,3-dioxygenase (IDO), a tryptophanmetabolizing enzyme constitutively secreted by certain subsets of DCs, which was ascribed to inhibit T-cell proliferation by depleting the essential amino-acid tryptophan in T lymphocytes [23]. Compared with normal controls, the populations of IDO+ cells were significantly increased in the seminoma and mostly distributed as clump (**Figure 4A-C**). In addition, double staining of immunofluorescent studies showed that CD11c+ DCs were co-localized with IDO in the seminoma (**Figure 5Aa-Ad**).

In this study, we also test the expression profiles of other immunosuppressive cytokines such as VEGF, TGF-β1, IL-10 and IL-4 in the seminoma and its controls. In normal spermatogenic tissue, the expression of VEGF was largely found in the sertoli cell-germ cell contact areas (**Figure 4D**). However, in seminoma tissues, the expression intensity of VEGF were greatly increased and mostly of them distributed as clump (**Figure 4E**, **4F**). In addition, double staining of immunofluorescent studies showed that CD11c+ DCs were co-localized with VEGF in the seminoma (**Figure 5Ba-Bd**).

TGF-β1 modulates many important cellular events, including cell differentiation, migration and adhesion. Furthermore, in normal testis, Leydig, Sertoli and germ cells expressed TGF-β1 (**Figure 4G**) which was involved in steroidogenesis and spermatogenesis [24]. Compared with normal testis, the expression intensity of TGF-β1 were significantly increased in the seminoma tissue (**Figure 4H**, **4I**). However, immunofluorescent studies of double staining showed that CD11c+ mDCs in tumor did not expressed TGF-β1 (**Figure 5Ca-Cd**).

Both IL-10 and IL-4 were occasionally found in the interstitial tissue of normal testis (**Figures 4J**, **6G**). Compared with normal testis, the expression intensity of IL-10 and IL-4 were significantly increased in the seminoma (**Figures 4K**, **4L**, **6H**, **6I**). Double staining of immunofluorescent techniques showed that CD11c+ mDCs were co-staining with IL-4 but not IL-10 (**Figure 5Da-Dd**, **5Ea-Ed**). Taken together, the expression intensity of IDO, VEGF, TGF-β1, IL-4 and IL-10 in the seminoma were significantly higher than in normal testis. Furthermore, CD11c+ mDCs in seminoma were found to express immune tolerance cytokines IDO, IL4 and VEGF.

CD11c+ mDCs with inflammatory cytokine profile in the seminoma

In azoospermic testis with chronic inflammation, DCs perpetuate the local activation of Th17 cells by secreting large number of IL-23p19, IL-6 and TNF-α, which results in IL-17 and IFN-γ-driven inflammation [9]. However, whether CD11c+ mDCs in the tumor microenvironment of seminoma could express those inflammatory cytokine remains unknown.

In normal testis, IL-23p19 was localized in the cytoplasm of long spermatids and sertoli cells (**Figure 4M**) and IL-6 was localized in the cytoplasm and nucleus of round spermatids (**Figure 4P**), which indicate that those proteins plays an important role during the process of spermatogenesis. Compared with normal testis, the expression intensity of IL-23p19 and IL-6 were significantly increased in the seminoma (**Figure 4O**, **4R**), and in the meantime part of them were developed into an aggregate unit (**Figure 4N**). In addition, the results of immunofluorescent double staining showed that CD11c+ mDCs were co-localized with IL-23p19 and IL-6 in the seminoma (**Figure 5Fa-Fd**, **5Ga-Gd**).

Disturbed balance between Th1, Th2, regulatory T and Th17 producing cytokines in the seminoma

The immunohistochemical studies suggests that both CD3+ T cells and CD4+ cells were expressed in the interstitial compartment of normal testis at low levels (**Figure 6A**, **6D**). Meanwhile, the number of CD3+ T cells and CD4+ cells were significantly increased in the seminoma compared with its controls (**Figure 6B**, **6C**, **6E**, **6F**). Interesting, CD4+ T cells were

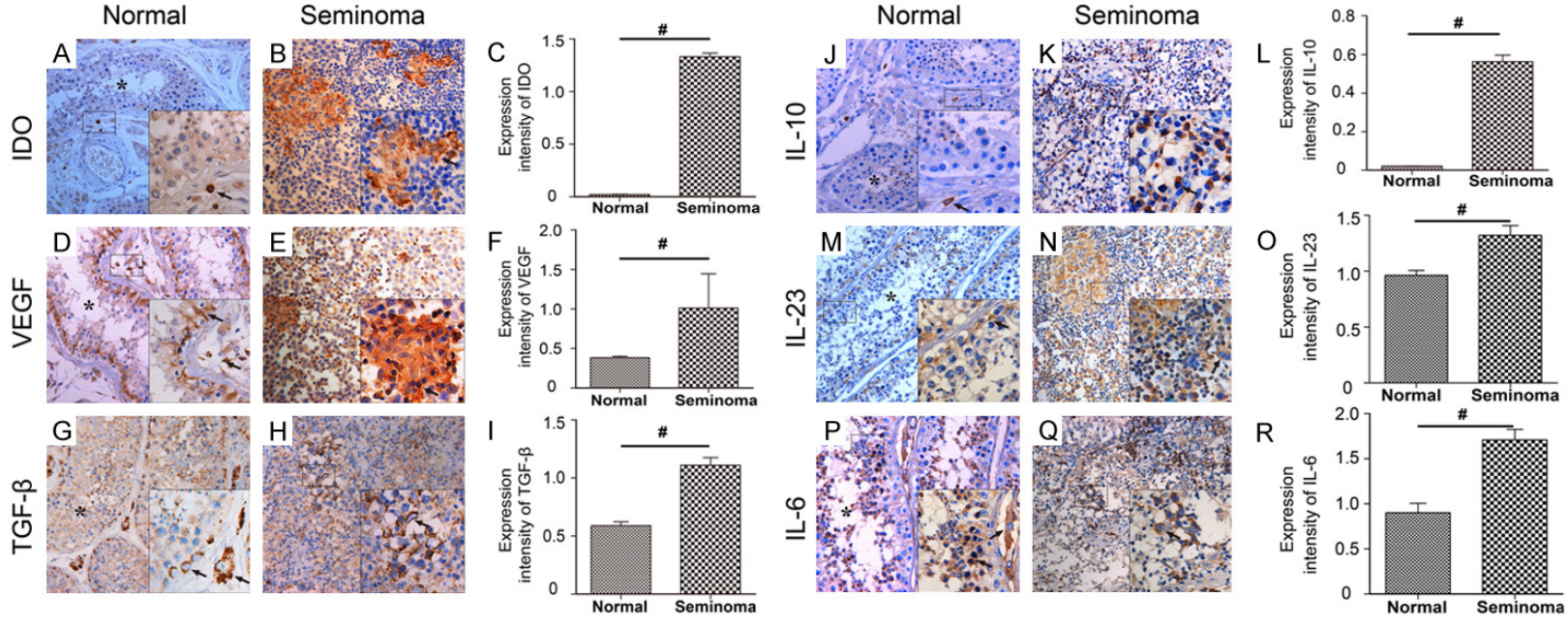

Figure 4. Distribution of IDO+, VEGF+, TGF-β+, IL-10+, IL-23+ and IL-6+ cells in the seminoma and normal testis. IDO+ cells were sparsely localized in the interstitial compartment of normal testis (A). The expression intensity of IDO were significantly increased in the seminoma (B) compared with it controls (C). The expression of VEGF was found in the sertoli cell-germ cell contact areas and interstitial compartment of normal testis (D). The expression intensity of VEGF was significantly increased in the seminoma (E) compared with it controls (F). TGF-β1 was detected in the Leydig, Sertoli and germ cells of the normal testis (G). The expression intensity of TGF-β1 were significantly increased in the seminoma (H) compared with it controls (I). IL-10+ cells were sparsely localized in the interstitial compartment of normal testis (J). The expression intensity of IL-10 was significantly increased in seminoma (K) compared with it controls (L). In normal testis, IL-23 was localized in the cytoplasm of long spermatids and sertoli cells (M) and IL-6 was localized in the cytoplasm and nucleus of round spermatids (P). The expression intensity of IL-23 and il-6 were significantly increased in the seminoma (N, Q) compared with their controls (O, R). Mean optical density summary was used to represent the expression intensity of immune-positive cells by in situ analysis using Image-Pro Plus software. The results are expressed as the means ± SEM; Arrows represent positive cells; *Represent the lumen of seminiferous tubule; #P<0.05; Bar = 100 μm (Original magnification, ×200; Inset: ×400).

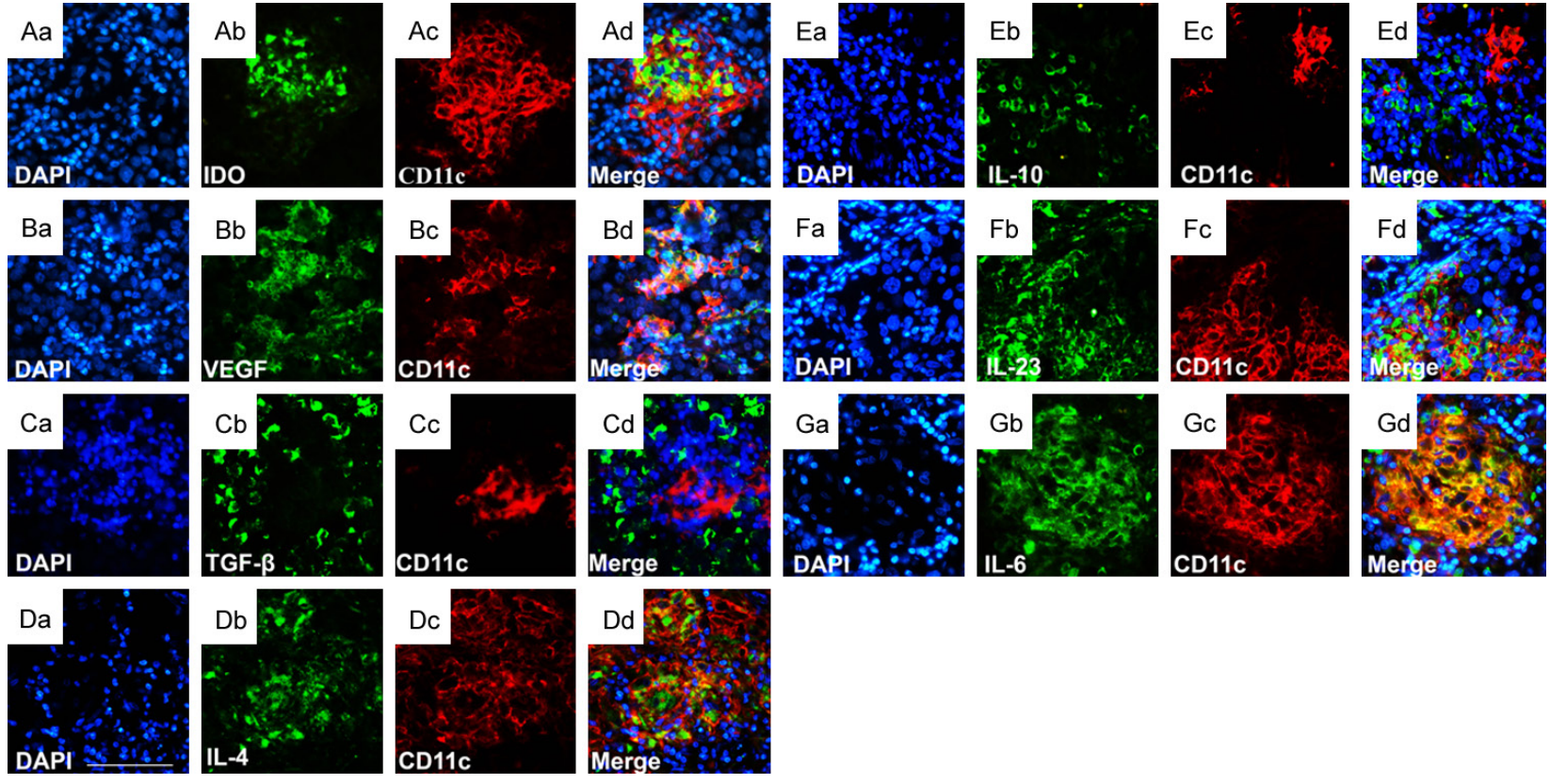

Figure 5. Immunofluorescence double staining of CD11c+IDO+, CD11c+VEGF+, CD11c+IL-4+, CD11c+IL-23+ and CD11c+IL-6+ mDCs in the seminoma. Two color immunofluorescence was used to determine CD11c+IDO+VEGF+IL-4+IL-23+IL-6+ and CD11c+IL-10-TGF-β1- mDCs expression: CD11c, CY3, red fluorescence (Ac, Bc, Cc, Dc, Ec, Fc, Gc); IDO, VEGF, IL-4, TGF-β1, IL-10, IL-23 and IL-6: CY2, green fluorescence (Ab, Bb, Cb, Db, Eb, Fb, Gb); CD11c+IDO+ mDCs, merged (Ad); CD11c+VEGF+ mDCs, merged (Bd); CD11c+ TGF-β1- mDCs, merged (Cd); CD11c+IL-4+ mDCs, merged (Dd); CD11c+IL-10- mDCs, merged (Ed); CD11c+IL-23+ mDCs, merged (Fd); CD11c+IL-6+ mDCs, merged (Gd); Nuclei were labeled with DAPI (Aa, Ba, Ca, Da, Ea, Fa, Ga); respectively. Bar = 50 μm (Magnification: ×400).

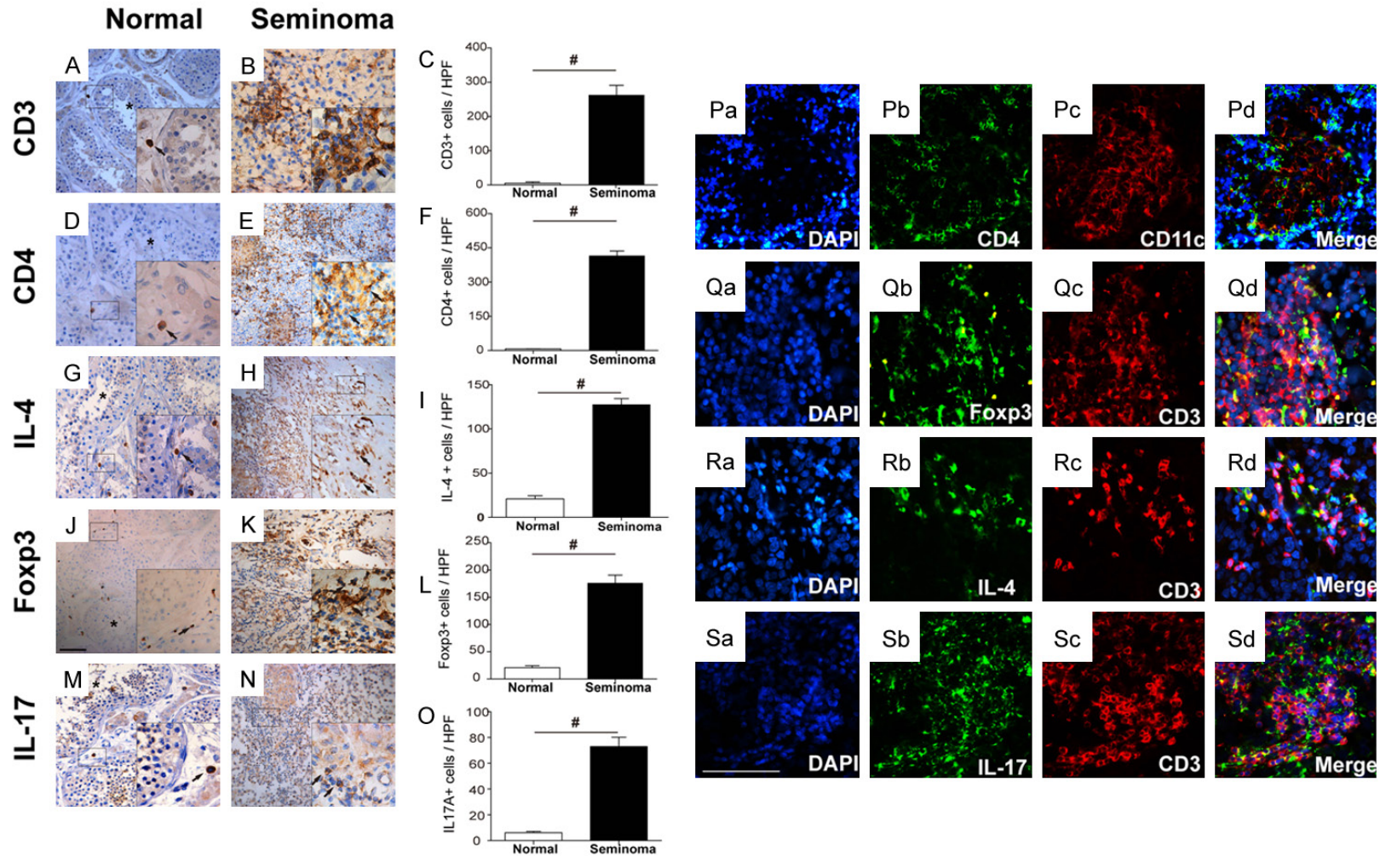

Figure 6. Distribution of CD3+, CD4+, IL-4+, Foxp3+ and IL-17+ cells in the seminoma and its control. CD3+ (A), CD4+ (D), IL-4+ (G), Foxp3+ (J) and IL-17+ (M) cells were sparsely localized in the interstitial compartment of normal testis. The numbers of CD3+, CD4+, IL-4+, Foxp3+ and IL-17+ cells were significantly increased in the seminoma (B, E, H, K, N) compared with its controls respectively (C, F, I, L, O). Quantitative enumeration was performed at the single cell level in immunohistochemically stained sections by in situ analysis using Image-Pro Plus software. The results are expressed as the means ± SEM; Arrows represent positive cells; *Represent the lumen of seminiferous tubule; #P<0.05; Bar = 100 μm (Original magnification, ×200; Inset: ×400). Two color immunofluorescence was used to determine, CD3+IL-4+ (Th2), CD3+Foxp3+ (Regulatory T) and CD3+IL-17+ (Th17) cells expression: CD11c and CD3, CY3, red fluorescence (Pc, Qc, Rc, Sc); CD4+, Foxp3+, IL-4+ and IL-17+, CY2, green fluorescence (Pb, Qb, Rb, Sb); CD4 and CD11c, merged (Pd); CD3+IL-4+ Th2 cells, merged (Rd); CD3+ Foxp3+ Regulatory T cells, merged (Qd); CD3+IL-17+ Th17 cells, merged (Sd); Nuclei were labeled with DAPI (Pa, Qa, Ra, Sa); Bar = 50 μm (Magnification: ×400).

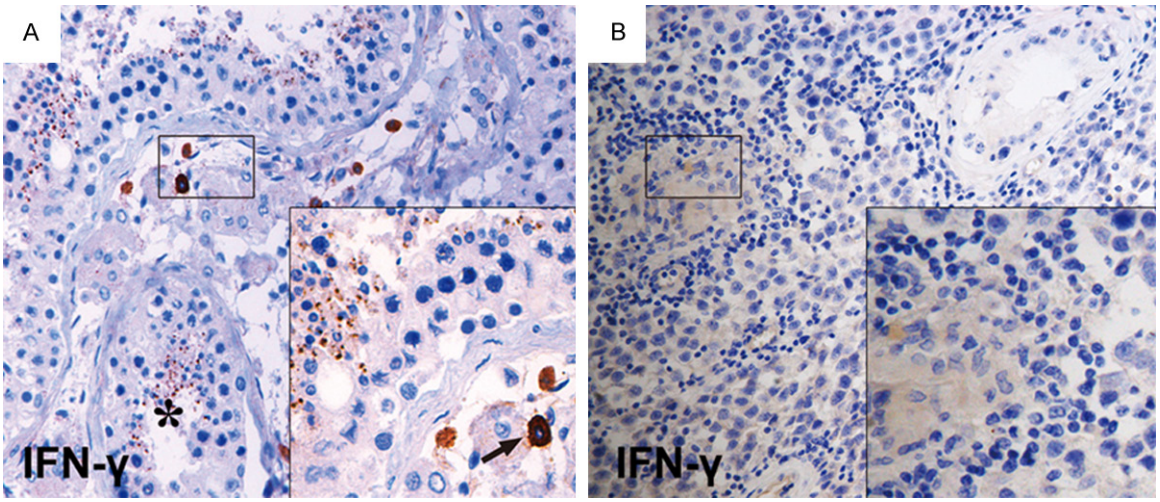

Figure 7. IFN-γ+ cells were sparsely localized in the interstitial compartment of normal testis (A); meanwhile, IFN-γ was negative in the tissues of seminoma (B). Bar = 100 μm. Arrows represent positive cells. *Represent the testis (Original magnification, ×200; Inset: ×400).

distributed around CD11c+ mDCs in the seminoma (**Figure 6Pa-Pd**), which indicated CD11c+ mDCs have a tendency to recruit T cells under tumor microenvironment.

Previous study had showed that T cells in the azoospermic testis with chronic inflammation patients show skewing from Th2 responses toward cytotoxic Th1 response, as evidenced by increased amounts of IFN-γ+ and decreased amounts of IL-4+ cells [9]. Unlike chronic inflammation microenvironment, several reports have investigated the Th1/Th2 cytokine balance in tumor microenvironment and found a strong Th2 shift [25]. The data of this study indicate that IFN-γ+ cells were sparsely distributed in the normal testis and not present in the tissue of seminoma (**Figure 7A**, **7B**). Meanwhile, the populations of IL4+ cells were significantly increased in the seminoma compared with its controls (**Figure 6G**, **6H**, **6I**, **6Ra-Rd**), which suggest that a reduced Th1 and enhanced Th2 immunity happened in the tumor microenvironment of seminoma.

Our previous studies also showed that a shift in the Foxp3+/IL-17+ balance towards Th17 cells and a related increase in the populations of IL-17+ cells were detected in the testis tissue of azoospermic patients with chronic inflammation [9]. Interestingly, both IL-17+ and Foxp3+ cells were significantly increased in the tissues of seminoma in this study (**Figure 6J-O**). Immunofluorescent studies of double staining showed that the population of Treg (CD3+ Foxp3+) and Th17 (CD4+IL-17+) cells were significantly increased in the seminoma testis (**Figure 6Qa-Qd**, **Sa-Sd**).

Discussion

DCs are versatile controller of immune system which belong to the APCs that are key player in antigen presentation and uniquely adapted to activate naïve T lymphocyte exerting a pivotal pathogenic role in autoimmune reactions and tumor immunological escape, but the distribution, characteristics and function of DC subsets in the normal testis and seminoma have not been well defined in the previous study. Recently, patients with seminoma have been reported to have increased frequency of DCs and T helper (Th) cells producing cytokines in the tumor tissues of testis [12]. Interestingly, our previous study has shown that IL-23 producing DCs were significantly increased in the azoospermic testis with chronic inflammation and could induce an immunodeviation of Th cells towards a Th17 immune response which associated with testicular damage [9]. In this study, we demonstrated for the first time that: (i) Normal human testis contained only immature CD11c+, CD1a+, CD207+, DC-SIGN+ DCs, CD123+, BDCA-2+ pDCs and CD68+, CD163+ macrophages whereas CD83+ maturity DCs were virtually absent. (ii) The number of CD11c+/CD1a+/DC-SIGN+ DCs and CD123+ BDCA-2+ pDCs were significantly elevated in

the seminoma. In addition, CD11c+ mDCs were the predominant component of the seminoma and abundantly forming aggregate unit under tumor microenvironment of seminoma. (iii) The CD11c+ mDCs in seminoma were in an immature phenotype which strongly expressed the immature markers DC-SIGN and CCR6 and weakly expressed the mature markers CD83 and HLA-DR. (iv) The CD11c+ mDCs in seminoma were in a dual cytokine profiles which expressed tolerance cytokines (IDO, VEGF, IL-4) and inflammatory cytokines (IL-23p19, IL-6). (v) Th2 (IL-4+), regulatory T (Foxp3+) and Th17 (IL-17+) producing cytokines were significantly increased under the tumor microenvironment of seminoma.

pDCs represent the main interferon-α (IFN-α) producers among leucocytes and IFN-α might help induce activating NK/T cytotoxic cells and FasL-mediated apoptosis [26]. Cytotoxic T cells as the main lymphocyte subsets have been implicated in the occurrence and development of malignant tumor [27]. However, it remains unclear whether pDCs are dispensable during the genesis and development of seminoma. This study showed that CD123+ and BDCA-2+ pDCs were increased weakly in the seminoma which indicated that pDCs are likely to have only limited effect in the occurrence and development of the seminoma.

Previous studies had revealed that immature CD1a+ and DC-SIGN+ DCs playing a pivotal role in the capture of auto antigens or exogenous antigens and subsequent processing for presentation to T lymphocyte [28, 29]. Our studies showed that the large numbers of DC-SIGN+ DCs were observed in the seminoma and also concentrated as aggregate unit which were mostly co-localization with CD11c+ mDCs. In addition, CD11c+ mDCs in the seminoma tissues expressed high level of CCR6 and low level of CD83 or HLA-DR, which were served as the immature marker and mature marker of DC respectively. Earlier studies have shown that the impaired balance between immature and immature myeloid cells (DCs/macrophages) is one of the hallmarks of malignant tumor [30]. Meanwhile, there is an increasing studies suggest that progressive cancer growth is associated with the accumulation of immature myeloid cells, and with a decreased number of mature DC in malignant tumor patients [30-32]. Therefore, the large numbers of CD11c+DC-SIGN+CCR6+ immature DCs might have the capable of inhibiting the immune surveillance and could be the major factor which is responsible for the immune escape and immune suppression of seminoma patients.

In the case of cancer, cytokines such as VEGF, TGF-β, IL-10, IDO and IL-4 secreted by tumor cells and myeloid cells leads to the profound suppression of antitumor immune responses [15, 33-36]. Our studies results showed that the expression intensity of IDO, VEGF, TGF-β1, IL-4 and IL-10 were significantly higher in the seminoma than in normal testis. In addition, CD11c+ mDCs in the seminoma were found to express the tolerance cytokines IDO, IL4 and VEGF. In vivo and vitro studies indicated that even small numbers of IDO+ DCs can have profound suppressive effects on the T lymphocyte that recognize the antigen presented by IDO+ DCs [37]. Furthermore, VEGF enhance IDO expression and activity by DCs and subsequently impacts Ag-specific and mitogen-stimulated lymphocyte proliferation [38]. So that we speculated that in seminoma testis naïve T cells might be biased by the CD11c+VEGF+IDO+ mDCs to adopt a regulatory phenotype. In some studies, IL-10 and TGF-β producing DCs induced the proliferation of CD4+CD25+Foxp3+ regulatory T cells which play a key role in tumor escape [15, 34]. However, we did not found CD11c+ mDCs in this study expressed TGF-β1 and IL-10. Hence, we speculated that high level of TGF-β1 and IL-10 secreted by seminoma cells convert the local tissue microenvironment into a tolerizing milieu and stimulate the proliferation of foxp3+ regulatory T cells by co-working with DCs in the cancer testis.

This study demonstrated that a strong shift in the Th1/Th2 balance towards Th2 producing cytokines and a related increase in the populations of IL-4+ cells were detected in the seminoma testis. An earlier study had showed that the depletion of CD11c+ DCs results in a striking impaired Th2 cytokine profile and a shift toward Th1 (IFN-γ+) immune response [39]. Interestingly, CD11c+ mDCs in this study expressed IL-4, which could establish a Th2 supportive microenvironment. Accordingly, we concluded that IL-4 producing CD11c+ mDCs in the seminoma induced the proliferation of Th2 cells which play a critical role in the immune escape and immune suppression of seminoma patients [40].

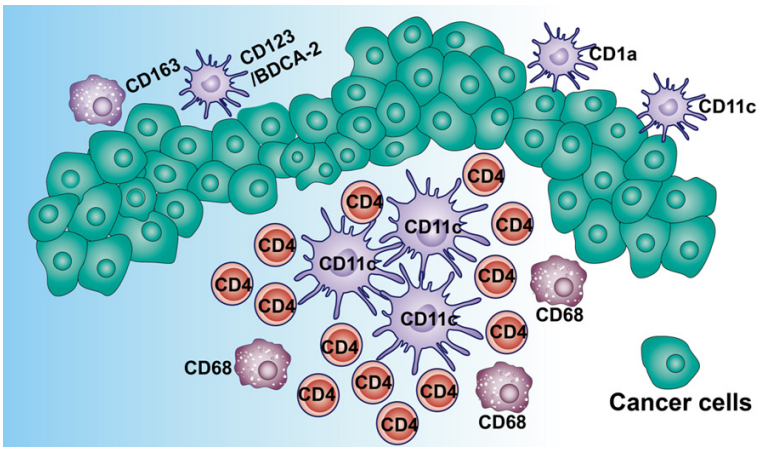

Figure 8. The working model shows the distribution of DCs, macrophages and CD4+T cells in the seminoma. Increasing number of CD11c+ mDCs which abundantly formed a aggregate unit with an immature phenotype in the seminoma testis. In addition, both CD4+ Th cells and CD68+ macrophages were distributed around CD11c+ mDCs which indicated CD11c+ mDCs have a tendency to recruit Th cells and macrophages and play an important role in the initiation of Th2 and regulatory T cells polarity shift which promoted a tolerance environment in the seminoma.

Our studies showed that the expression intensity of cytokines IL-23p19, IL-6 and the number of IL17+ cells were significantly increased in the seminoma. Interestingly, CD11c+ mDCs in the seminoma were found to express inflammatory cytokines IL-23p19 and IL-6. It is relevant to know that the "inflammatory DCs" are recently identified as CD11c+IL-23+ mDCs that are first to recruit to the site of inflammatory response and induce naïve T cells to differentiate into Th17 cells [41]. In addition, our previous study demonstrated that in patients from azoospermic testis with chronic inflammation an increased number of testicular CD11c+IL-23p19+ mDCs establish a Th17 supportive microenvironment and marked the critical step in the disease development [9]. Although the role of Th17 in the pathogenesis of chronic infectious immunity and autoimmune diseases has been well defined, the impact of these cells on the process of cancer development and metastasis is still under debate. Multiple studies have indicated that Th17 promote antitumor immunity in the occurrence and development of carcinoma [42, 43]. However, several human reports have highlighted that increased numbers of IL-17 (most of which being secreted by CD4+ T cells) directly correlated to poor prognosis in cancer patients [44, 45]. Further characterization revealed that tumor-derived IL-17-producing cells were not immune suppressive, but have the capacity to promote the tumor growth in an in vitro culture system [46]. Hence, the dichotomy function of Th17 in malignant may be related to the versatile nature of these cells depending on the underlying micro-environmental conditions. It is critical in the future to study the role of CD11c+IL-23+ DCs and Th17 cells in the seminoma and how factors that control their final lineage commitment decision which might impair the balance between their anticancer immunity versus tumor-promoting properties.

Based on these data, our hypothesis is that the occurrence and development of seminoma and its tumor escape is closely associated with the increased number of CD11c+ mDCs which abundantly forming aggregate unit with an immature phenotype in the testis of seminoma. These cells population, by means of cells interactions and their cytokines, such as IDO, VEGF, IL-4, IL-23p19 and IL-6, may play an important role in the initiation of Th2 and regulatory T cells polarity shift and promoting the tolerance environment in the seminoma (**Figure 8**). In the future, an understanding of the heterogeneity and function of CD11c+ mDCs in seminoma is critical to design of DCs vaccine therapy for the treatment of testicular cancer patients.

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Disclosure of conflict of interest

None.

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