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Original Article

Identification and function analysis of macrophages subsets in chronically inflamed human epididymis

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Abstract: Chronic epididymitis is amongst the leading causes of male infertility. The main clinical manifestations of chronic epididymitis included oligozoospermia and asthenospermia. Our previous studies showed that over-expression of dendritic cells (DCs) and secreting cytokines were found in chronically inflamed human epididymis. They were thought to play a key role in the immune response of chronic epididymitis. The object of this study was to investigate the expression and characteristic of macrophage subsets in chronic epididymitis and its controls. Our study demonstrated that both inducible nitric oxide synthase (iNOS) +CD68+ M1 and CD163+CD206+ M2 macrophages were significantly increased in the chronic inflammation of human epididymis compared with its controls. Moreover, CD68+iNOS+ M1 and CD163+CD206+ M2 macrophages were observed to capture spermatozoa in the lumen of cauda epididymidis. The CD68+ M1 macrophages in chronically epididymitis expressed cytokines IL-6 and IL-23p19; meanwhile, CD163+ M2 macrophages expressed transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF) and IL-23p19. In addition, Th1 (CD3+IFN-γ+) cells were predominantly distributed in the chronically epididymitis. Taken together, our results indicate that epididymal macrophages may play an important role in the development of chronic epididymitis and induce oligozoospermia and asthenospermia of male infertility patients.

Keywords: Chronic epididymitis, male infertility, M1/M2 macrophages, oligozoospermia and asthenospermia

Introduction

Chronic infection and inflammatory conditions of the male reproductive tract is one main pathogeny of male sterility. Etiologies of chronic inflammation in male reproductive tract are numerous and include chronic orchitis, epididymitis, prostatitis, seminal vesiculitis and urethritis [1, 2]. Compared to the testis or seminal vesicles, the epididymis is not only considered to be the most common site of intrascrotal inflammation but also a significant cause of male infertility [3]. The incidence of chronic epididymitis is more prevalent than orchitis and which very often accompanied with epididymo-orchitis, as the elaborate blood-testis barrier (BTB) appears to be more restrictive compared to the blood-epididymis barrier (BEB) [4]. Although earlier studies have showed that T cells, dendritic cells and macrophages were detected in the interstitial compartment and epithelium area of epididymis, but their immunological characters remain to be clarified especially under inflammatory conditions of human epididymitis [5-7].

Macrophages are a heterogeneous population of antigen presenting cells (APCs) that are crucial regulators of innate immune and adaptive immune system, which can be classified into two major types: M1 macrophages and M2 macrophages [8]. The classically activated macrophages (M1 macrophages) are pro-inflammatory and play a pivotal role in host defense against infection which are associated with iNOS and IL-23 production, and their cell surface expressed CD80/86 or HLA-DR that attract killer cells like neutrophils and/or direct Th1 (cytotoxic) responses and stimulate further M1-type responses [9]. Meanwhile, the alternatively activated macrophages (M2 macrophages) are associated with the respon-

ses to anti-inflammatory reactions as well as tissue remodeling which are linked with transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) production, and cell surface expressed CD163 or CD206 that stimulate Th2 (antibody-mediated) type responses and promote further amplification of M2-type responses [10].

The post-testicular and, especially, the epididymis environment contain an intricate network of macrophages and DCs; but the immunophysiology of this highly specialized and intriguing mucosal system remains poorly defined [11]. The identification and removal of potentially defective sperm or the possible presence of spermatozoa "mass control" mechanisms is one of the most controversial aspects of epididymal physiology [12-15]. Previous studies have showed that the obstruction of male genital tract is linked with macrophages undergoing spermatophagy in the lumen of human epididymis [16-18]. Although "spermiophagy" or "spermatophagy" has been previously reported [13, 16], but there has no convictive evidence presented, until nowadays, that macrophage, DCs and other white blood cells (WBCs) type play a significant role in the identification or destruction of defective sperm in the normally functioning epididymis of human.

Therefore, unraveling the characterization and function of macrophage subsets are helpful for understanding the mechanisms of immune tolerance to mature sperm or spermatozoa "mass control" in the normal environment of epididymis and illustrating the pathological mechanisms of chronic inflamed epididymis. The purpose of this study was to identify the distribution and intensity of macrophage of various phenotypes in the chronically inflamed human epididymis and its control to elucidate the contribution of macrophages to the spermatozoa "mass control", immune tolerance and autoimmunity of human epididymis. To address this question, we firstly characterized the subtype of the macrophages in chronic epididymitis and normal epididymis; Secondly, we detected the macrophages, DCs and T lymphocyte in the lumen of cauda epididymidis and analyzed the relationship between those immune cells and spermatozoa; Subsequently, we investigated the cytokine profile of macrophages under chronic inflammation of human epididymis; Finally, we assessed the balance between Th1 (CD3+IFN-γ) and Th2 (CD3+IL4+) which associated with M1/M2 paradigm.

Materials and methods

Antibodies for immunohistochemistry and immunofluorescence staining

For morphology (immunohistochemistry and immunofluorescence) staining, the following antibodies were used as primary antibodies: polyclonal rabbit anti-human CD3 (ab5690, IgG; Abcam, Cambridge, UK), monoclonal mouse anti-human CD4 (ab67480, IgG; Abcam), monoclonal mouse anti-human CD68 (66231-1-Ig, IgG1; ProteinTech, Wuhan, CHN), polyclonal rabbit anti-human CD163 (ab87099, IgG; Abcam), monoclonal mouse anti-human CD163 (MAB-0206, IgG; MaiXin-Bio; CHN), monoclonal mouse anti-human HLA-DR (ab8085, IgG2a; Abcam), polyclonal rabbit anti-human IL-23p19 (ab45420, IgG; Abcam), polyclonal rabbit anti-human TGF-β (ab66043, IgG; Abcam), polyclonal rabbit anti-human iNOS (ab-15326, IgG; Abcam), polyclonal rabbit anti-human IL-4 (ab9622, IgG; Abcam), polyclonal rabbit anti-human IFN-γ (ab56070, IgG; Abcam), polyclonal rabbit anti-human IL-6 (21865-1-AP, IgG; ProteinTech), polyclonal rabbit anti-human CD206 (ab64693, IgG; Abcam), polyclonal rabbit anti-human VEGF (ab46154, IgG; Abcam), monoclonal mouse anti-human CD11c (60258-1-Ig, IgG2a; ProteinTech), polyclonal rabbit anti-human DC-SIGN (LS-B479, IgG; Life Span, WA Seattle, USA), monoclonal mouse anti-human CD123 (LS-C311924, IgG1; Life Span), polyclonal rabbit anti-human IL-10 (20850-1-AP, IgG; ProteinTech), 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) 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.

Epididymal tissue specimens

Chronic epididymitis and matched specimens were obtained from patients undergoing urological work-up between 2005 and 2015 at the Peking University Shenzhen Hospital. Biopsies were immediately fixed in Bouin's fixative and

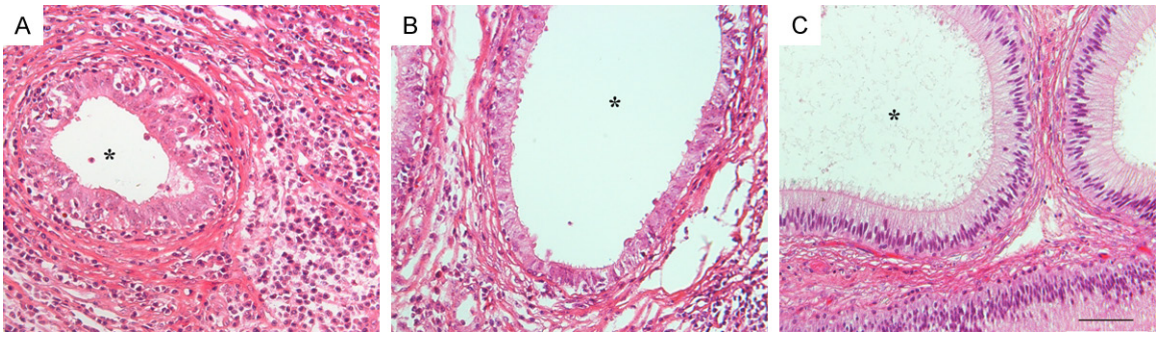

Figure 1. Microphotographs of haematoxylin and eosin stained in inflamed and control epididymis. Both the caput (A) and cauda (B) of inflamed epididymis, the histopathological changes were characterized by mononuclear cell infiltrates in the interstitial area. Furthermore, peritubular fibrosis and the disordered morphology of the epithelial epithelium were formed. Control epididymis without mononuclear cell infiltrates (C). *represent the lumen of epididymis. Bar =100 μm.

Table 1. Primary antibodies for immunohistochemistry and immunofluorescence staining

Against	Dilution	Specificity	Isotype	Source
DC-SIGN	1:800	DC/Macrophage	IgG	Rabbit
CD4	1:200	Th cell	IgG	Mouse
CD68	1:350	Macrophage	IgG1	Mouse
CD163	1:500	M2 Macrophage	IgG	Rabbit
CD163	1:200	M2 Macrophage	IgG	Mouse
HLA-DR	1:800	DC/Macrophage	IgG2a	Mouse
iNOS	1:500	M1 Macrophage	IgG	Rabbit
CD206	1:800	M2 Macrophage	IgG	Rabbit

Th cell: T helper cell; DC: Dendritic cell.

embedded in paraffin for immunofluorescence and immunohistochemistry staining studies. Inclusion criteria for chronic epididymitis were previous history of bacterial epididymitis, clinical symptoms of continuous epididymal pain (over 6 weeks), increased numbers of dendritic cells and/or macrophages in semen, diagnosis of epididymitis by scrotal Doppler ultrasonography, and the patients has strongly willingness to receive epididymectomy. On the other hand, the exclusion criteria were the genitourinary tract infection, prostatitis, previous vasectomy or scrotal surgery, chronic pelvic pain syndrome, and concurrent diseases such as a granuloma or an epididymal cyst both of them can have scrotal pain symptom. According to the histopathological evaluation, ten patients with both signs of inflammation and impaired epididymal structure were included in this study (**Figure 1A**, **1B**). For comparison, ten non-inflamed epididymal samples (**Figure 1C**) 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 which expressed in the declaration of Helsinki.

Immunohistochemistry

The immunohistochemical staining was performed by using a standard three-step technique. 4-μm sections of the epididymal biopsies were deparaffinized in dimethylbenzene and rehydrated in alcohol gradient. Antigen retrieval was performed with EDTA (pH 9.0). Pho-phate-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 (**Tables 1**, **2**) and overnight at 4°C refrigerator in a humidified chamber. After washing steps, the slides were treated with the 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 hematoxylin. 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 obtai-

Table 2. Dilution of antibodies used for immune-detection of cytokines

Against	Dilution	Isotype	Source	Against	Dilution	Isotype	Source
IL-23p19	1:800	IgG	Rabbit	TGF-β	1:200	IgG	Rabbit
IL-4	1:500	IgG	Rabbit	IL6	1:500	IgG	Rabbit
IFN-γ	1:800	IgG	Rabbit	VEGF	1:500	IgG	Rabbit
IL-10	1:200	IgG	Rabbit				

ned by substitution of the primary antibodies with isotype control IgG, IgG1 or IgG2 respectively in the procedure of staining. 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)

Briefly, 4-μm sections of the epididymal biopsies were deparaffinized in dimethylbenzene and rehydrated in alcohol gradient. Antigen retrieval was performed with EDTA (pH 9.0). PBS 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 7.5% normal bovine serum at 37°C incubator for 30 minutes. Tissue sections were incubated respectively with the mixture of two primary antibodies overnight at 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. 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 cells/mm^2 were used to represent the numbers of immune-positive cells and mean optical density summary was used to represent the expression intensity 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 a *p*-value less than 0.05 was considered to indicate a statistically significant difference.

Results

Increased numbers of iNOS+HLA-DR+CD68+DC-SIGN+ M1 macrophages and CD163+CD206+ M2 macrophages in inflamed epididymis

As little know about the expression of M1 and M2 macrophages in normal or inflamed epididymis, we, therefore, firstly characterized the subtype of macrophages in chronic epididymitis and its controls. The results of our study showed that mostly of CD68+, iNOS+ macrophages and HLA-DR+ cells were found close and under the epithelium area of the control epididymis (**Figure 2A**, **2D**, **2G**), meanwhile, DC-SIGN+ cells were localized in the interstitial compartment (**Figure 2J**). As expected, CD68+, iNOS+ macrophages and HLA-DR+, DC-SIGN+ cells were detected within both the interstitial area and the lumen of the epididymitis and the number were significantly higher compared with control epididymis (**Figure 2B**, **2C**, **2E**, **2F**, **2H**, **2I**, **2K-N**). In addition, our double staining of immunofluorescence studies showed that iNOS was co-localized with CD68, HLA-DR and DC-SIGN respectively (**Figure 3A-L**). In this study, CD68 was co-localized with DC-SIGN (**Figure 3M-P**). Therefore, we speculate that M1 macrophages in the inflamed epididymis were specificity identified as iNOS+HLA-DR+CD68+DC-SIGN+ macrophages and play an important role in the pathological process of chronic inflamed epididymis.

Unlike M1 macrophages, M2 were specificity identified as CD163+ and CD206+ cells. Mostly of CD163+ macrophages were found in the

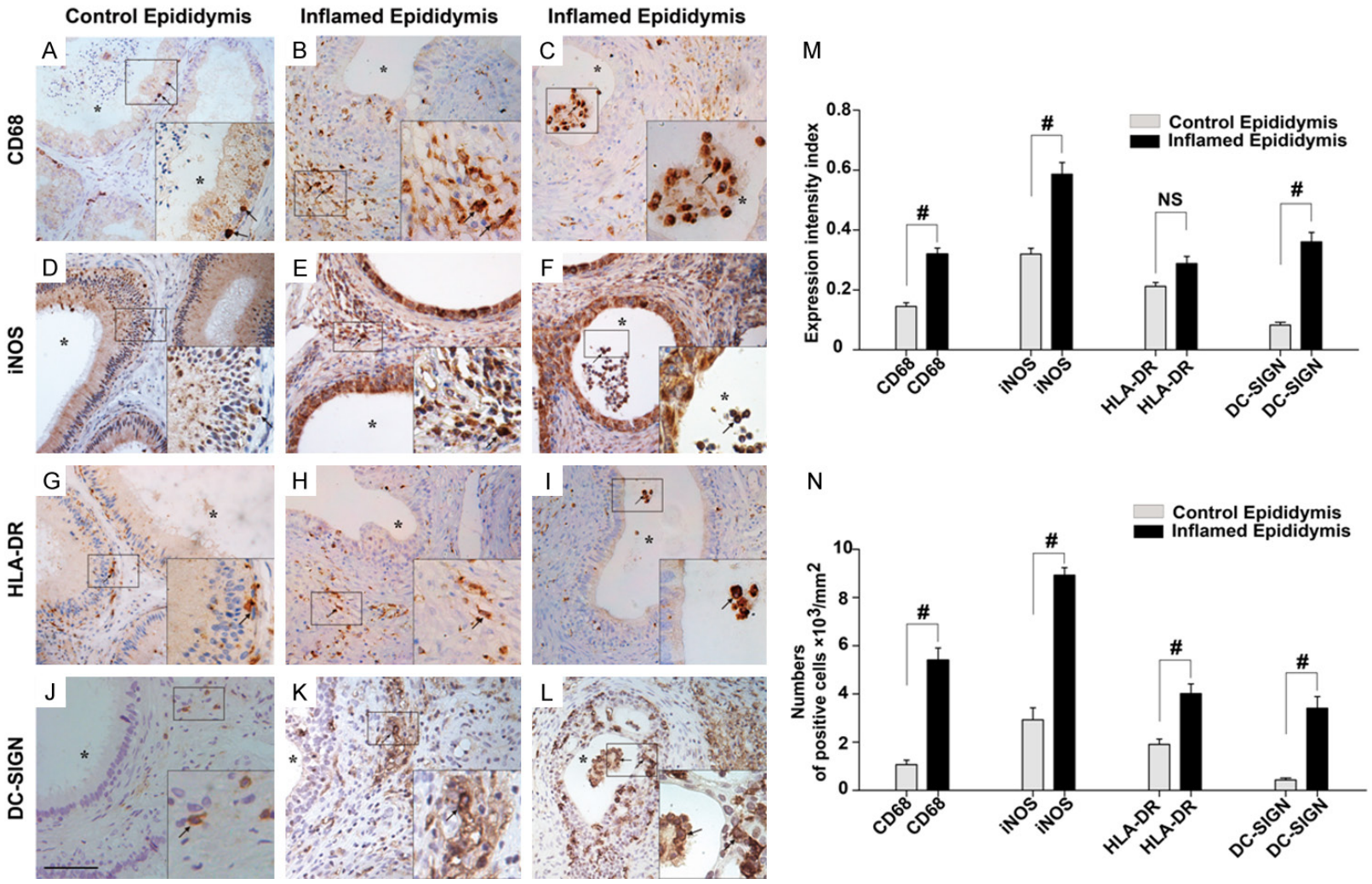

Figure 2. Distribution of CD68+, iNOS+, HLA-DR+ and DC-SIGN+ cells in the inflamed epididymis and its controls. Mostly of CD68+, iNOS+ macrophages and HLA-DR+ cells were found close and under the epithelium area of the control epididymis (A, D, G). Abnormal large amount of CD68+, iNOS+ and HLA-DR+ cells were detected within both the interstitial area and the lumen of the epididymitis (B, C, E, F, H, I). DC-SIGN+ cells were sparsely localized in the interstitial area of the control epididymis (J). Abnormal large amount of DC-SIGN+ cells were detected within both the interstitial area and the lumen of the inflamed epididymis (K, L). Comparison of the expression intensity and numbers of CD68+, iNOS+, HLA-DR+ and DC-SIGN+ cells in controls and inflamed epididymis (M, N). Quantitative enumeration was performed at the single cell level in immunohistochemically stained sections and 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 the positive cells. *represent the lumen of epididymis. #represent $P<0.05$. Bar =100 μm. (Original magnification, ×200; Inset: ×400).

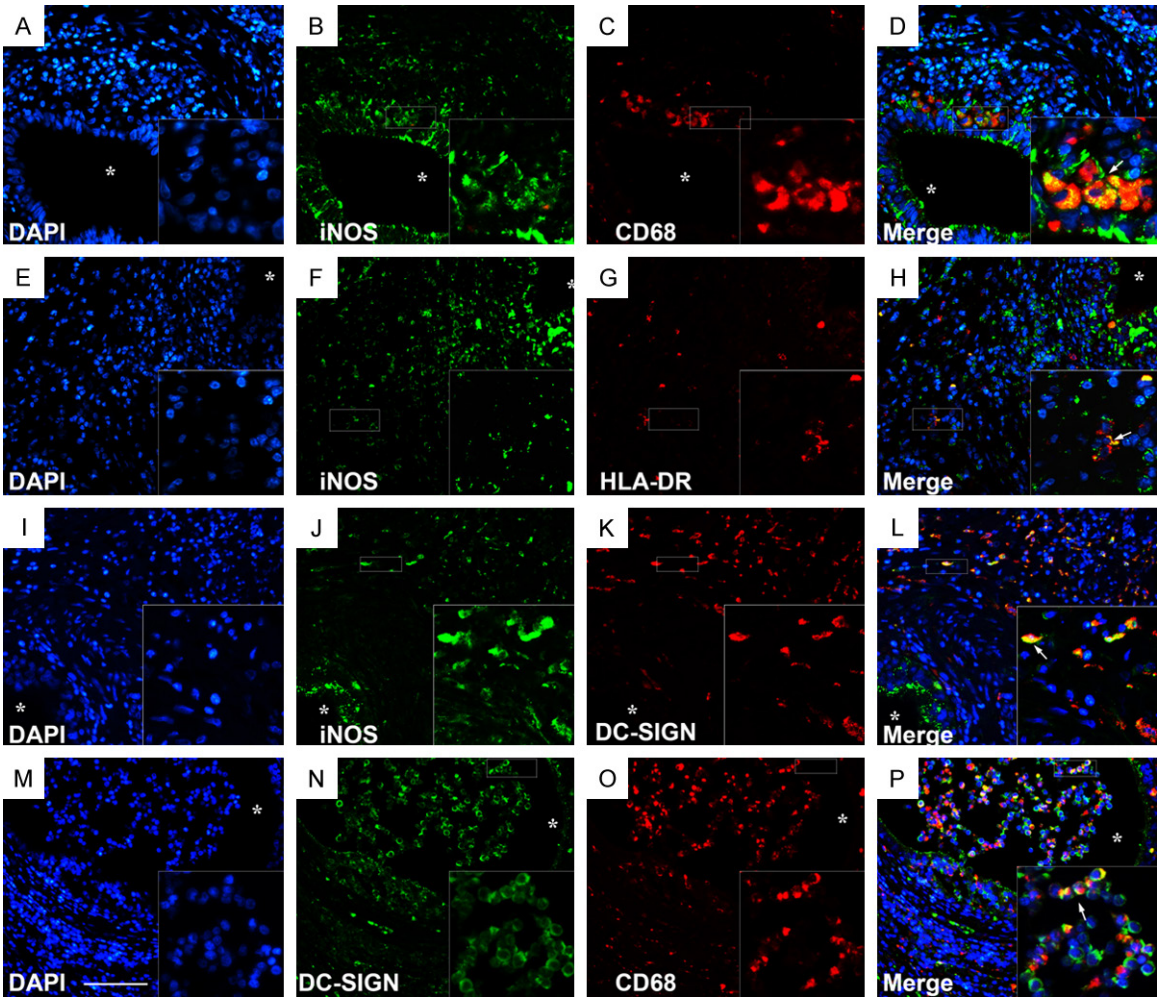

Figure 3. Immunofluorescence double staining of iNOS+CD68+, iNOS+HLA-DR+, iNOS+DC-SIGN+, CD68+DCSIGN+ M1 macrophages in the inflamed epididymis. Two color immunofluorescence was used to determine iNOS+CD68+, iNOS+HLA-DR+, CD68+DC-SIGN+, iNOS+DC-SIGN+ macrophages expression: CD68, HLA-DR and DC-SIGN, CY3, red fluorescence (C, G, K, O); iNOS and DC-SIGN, CY2, green fluorescence (B, F, J, N). iNOS+CD68+ M1 macrophages, merged (D); iNOS+HLA-DR+ M1 macrophages, merged (H); iNOS+DC-SIGN+ macrophages, merged (L); CD68+DC-SIGN+ M1 macrophages, merged (P); Nuclei were labeled with DAPI (A, E, I, M); respectively. Arrows represent positive cells; *represent the lumen of epididymis; Bar =100 μm. (Original magnification, ×200; Inset: ×400).

interstitial compartment of the control epididymis (**Figure 4A**), meanwhile, CD206 were detected both within the interstitial and epithelium area of the control epididymis (**Figure 4D**). Interesting, epithelial cell of control epididymis strongly expressed CD206 (**Figure 4D**), which has not previously been reported. Compared with the controls, significantly increased numbers of CD163+, CD206+ cells were localized both the interstitial compartment and the lumen of the inflammatory epididymis (**Figure 4B**, **4C**, **4E-H**). Furthermore, immunofluorescence studies of double staining show that CD163 was co-localized with CD206, meanwhile, did not co-localized with CD68, HLA-DR and DC-SIGN (**Figure 5A-P**). The results showed that the alternatively activated CD163+CD206+ M2 macrophages were the predominant component of the inflammatory epididymis and abundantly infiltrate into the epididymal lumen under chronic inflamed conditions.

iNOS+CD68+ M1 and CD163+CD206+ M2 macrophages capturing spermatozoa in the lumen of cauda epididymidis

As M1 and M2 macrophages were abundantly infiltrate into the epididymal lumen under chronic inflamed conditions, we, therefore, investigated the relationship between those macrophages and spermatozoa. Interestingly, we obs-

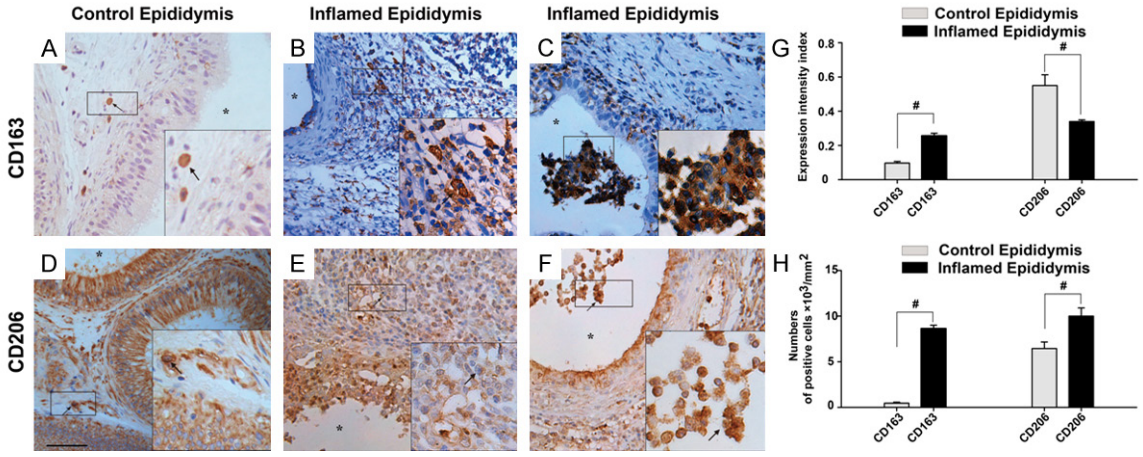

Figure 4. Distribution of CD163+, CD206+ cells in the inflamed epididymis and its controls. CD163+ cells were sparsely localized in the interstitial area of control epididymis (A). Abnormal large amount of CD163+ cells were detected within both the interstitial area and the lumen of the epididymitis (B, C). CD206 were detected both within the interstitial and epithelium of the control epididymis (D). Abnormal large amount of CD206+ cells cells were detected within both the interstitial area and the lumen of the epididymitis (E, F). Comparison of the expression intensity and numbers of CD163+ and CD206+ cells in controls and inflamed epididymis (G, H). Quantitative enumeration was performed at the single cell level in immunohistochemically stained sections and 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 epididymis; #represent P<0.05; Bar =100 μm. (Original magnification, ×200; Inset: ×400).

erved large numbers of spermatozoa-attached cells in the lumen of inflamed cauda epididymis which indicated that sperm were captured and degraded by those cells (**Figure 6A**, **6B**). Previous studies have showed that oligozoospermia and asthenospermia are associated macrophages undergoing spermatophagy in the semen of infertility patients [19, 20]. Accordingly, we detected M1 and M2 macrophages in the lumen of inflamed cauda epididymis by using immunofluorescence and immunohistochemistry staining. Both the M1 and M2 macrophages were found capturing spermatozoa in the lumen of cauda epididymidis (**Figure 7A-P**). In this study, the negative controls were obtained by substitution of the primary antibodies with isotype control IgG, IgG1 or IgG2 respectively in the procedure of immunohistochemistry and immunofluorescence staining (**Figure 8A-L**).

The inflammatory infiltrate is characterized by the recruitment of IL-6, IL23p19 producing M1 macrophages and TGF-β, VEGF, IL-23p19 producing M2 macrophages in inflamed epididymis

Previous study indicated that IL-23p19 producing CD11c+ and CD68+ 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 [21]. Moreover, our recent studies demonstrated that DCs through secreting cytokine IL-23p19 induce an increased recruitment of Th17 cells under chronic inflammation of human epididymis [22]. The results of immunohistochemical and immunofluorescence (double staining) studies revealed that an increased number of IL-23p19+ cells could be detected in epididymitis tissue (**Figure 9A**, **9L**) and its expression could be localized to epididymal CD68+ and CD163+ macrophages (**Figure 10A-H**).

In this study, we also test the expression profiles of other cytokines such as VEGF, TGF-β1, IL-10 and IL-6 in the inflamed epididymis and its controls. IL-6+ cells were detected mostly in the epididymal epithelium of the controls (**Figure 9B**), in addition, the subepithelial IL-6+ cells formed a closely network in the epithelium area as well as CD1a+ DCs with a satellite morphology and exhibits a dense network in normal human epididymis [22]. Meanwhile, in the inflamed epididymis, the IL-6+ cells were detected within both the interstitial and

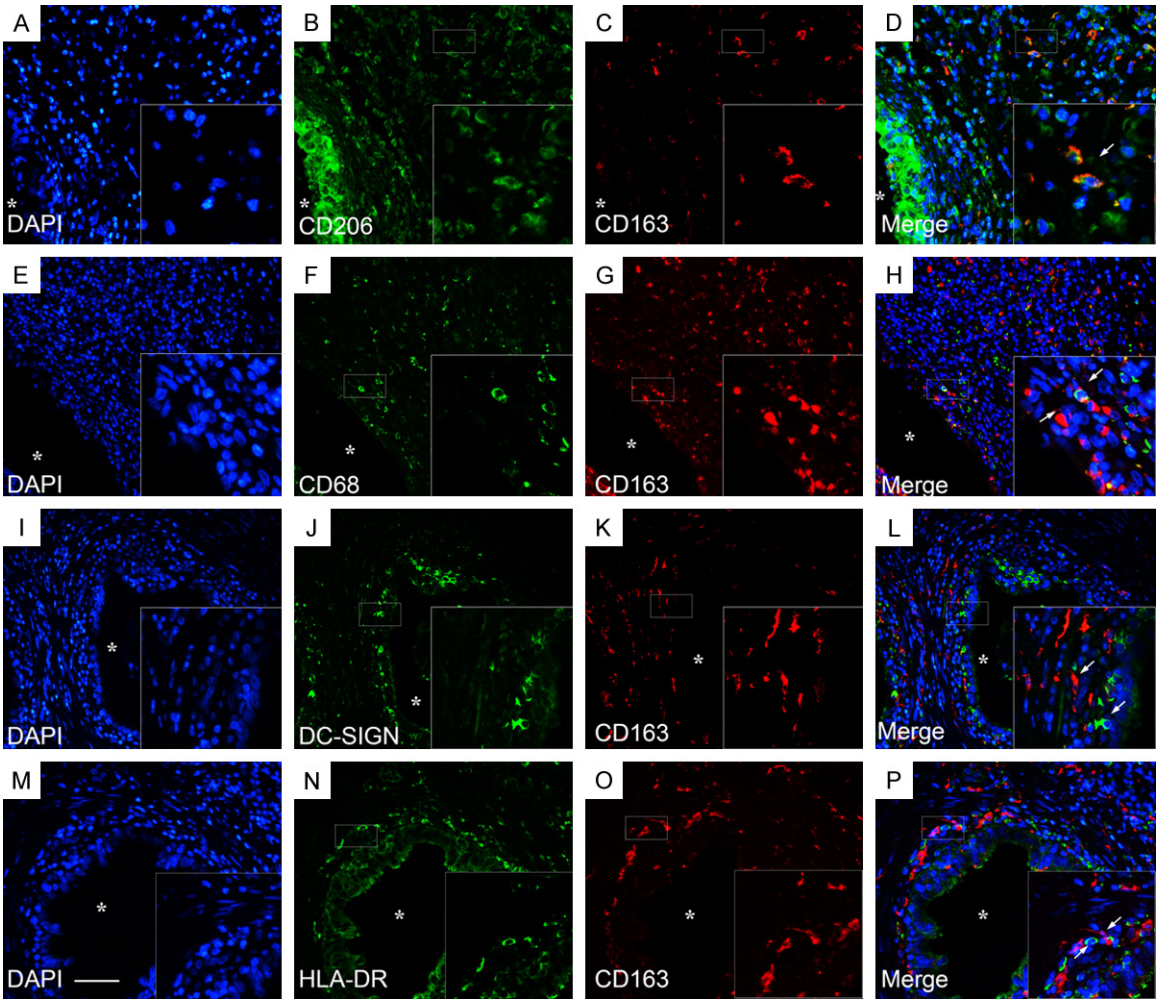

Figure 5. Immunofluorescence double staining of CD163+CD206+, CD163+CD68-, CD163+DC-SIGN- and CD163+HLA-DR- M2 macrophages in the inflamed epididymis. Two color immunofluorescence was used to determine CD163+CD206+, CD163+CD68-, CD163+DC-SIGN- and CD163+HLA-DR- M2 macrophages expression: CD163, CY3, red fluorescence (C, G, K, O); CD206, CD68, DC-SIGN and HLA-DR, CY2, green fluorescence (B, F, J, N). CD163+CD206+ M2 macrophages, merged (D); CD163+CD68- M2 macrophages, merged (H); CD163+DC-SIGN- M2 macrophages, merged (L); CD163+HLA-DR- M2 macrophages, merged (P); Nuclei were labeled with DAPI (A, E, I, M); respectively. Arrows represent positive cells. *represent the lumen of epididymis. Bar =100 μm. (Original magnification, ×200; Inset: ×400).

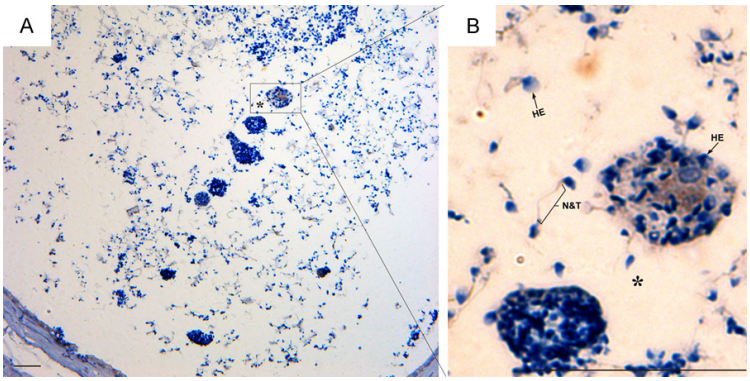

Figure 6. Spermatozoa-attached cells in the lumen of inflamed cauda epididymis. Magnification: ×200 (A), ×600 (B). HE represent the head of spermatozoa. N&T represent the neck and tail of spermatozoa. *represent the lumen of epididymis. Bar =100 μm.

epithelium area of inflamed epididymis (**Figure 9G**) and its expression could be localized to epididymal CD68+ (**Figure 10 I-L**) but not CD163+ macrophages (data not show).

Previous studies have showed that M2 macrophages mediate

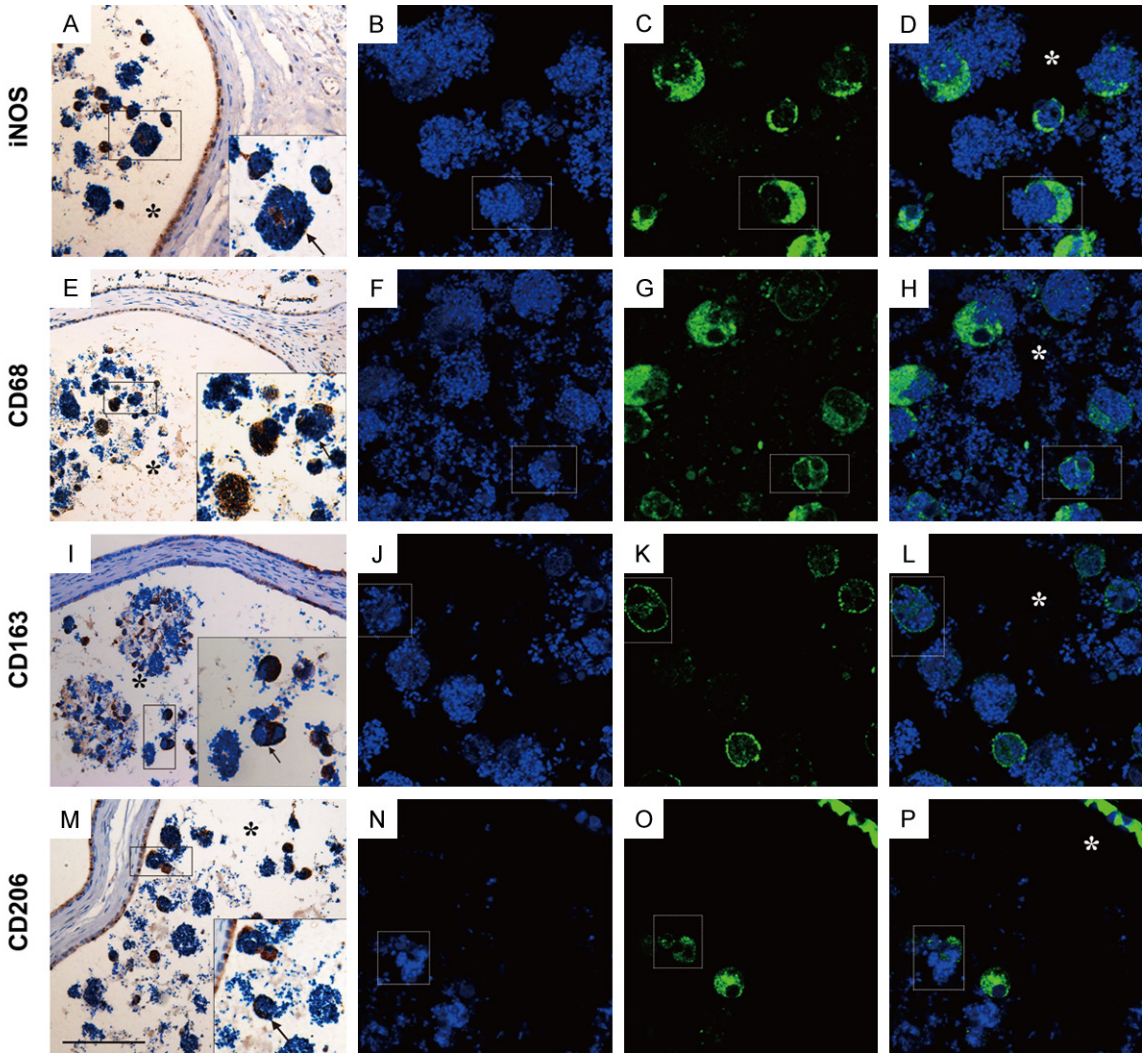

Figure 7. iNOS+CD68+ M1 and CD163+CD206+ M2 macrophages capturing spermatozoa in the lumen of cauda epididymidis. Immunohistochemical studies showed that iNOS+ (A), CD68+ (E), CD163+ (I) and CD206+ (M) macrophages capturing spermatozoa. Immunofluorescence staining of iNOS+, CD68+, CD163+ and CD206+ cells in the inflamed epididymis: iNOS (C), CD68 (G), CD163 (K) and CD206 (O) CY2, green fluorescence; iNOS+ M1 macrophages, merged (D); CD68+ M1 macrophages, merged (H); CD163+ M2 macrophages, merged (L); CD206+ M2 macrophages, merged (P); Nuclei were labeled with DAPI (B, F, J, N); respectively. Arrows represent the positive cells. *represent the lumen of epididymis. Bar =100 μm. (Original magnification, ×200; Inset: ×400).

anti-inflammatory reactions and tissue remodeling is associated with TGF-β, IL-10 and VEGF under chronic inflamed environment [10, 23-25]. In this study, TGF-β+ cells were found mostly in the epithelium and sparsely distributed in the interstitial compartment of control epididymis (**Figure 9C**). In addition, the subepithelial TGF-β+ cells formed a closely network in the epididymal epithelium of controls which consisted with IL-6+ and CD1a+ cells in normal epididymis. On the other hand, the numbers of TGF-β+ cells were significantly elevated in inflamed epididymis (**Figure 9H**, **9L**) and its expression could be localized to epididymal CD163+ M2 macrophages (**Figure 10M-P**). Unlike TGF-β, the expression of VEGF was specially found in the intraepithelium area of control epididymis (**Figure 9D**). Meanwhile, the expression of VEGF was greatly increased in the inflamed epididymis (**Figure 9I**, **9K**, **9L**) and double staining of immunofluorescent studies showed that VEGF could be localized to epididymal CD163+ M2 macrophages (**Figure 10Q-T**). In this study, IL-10 was detec-

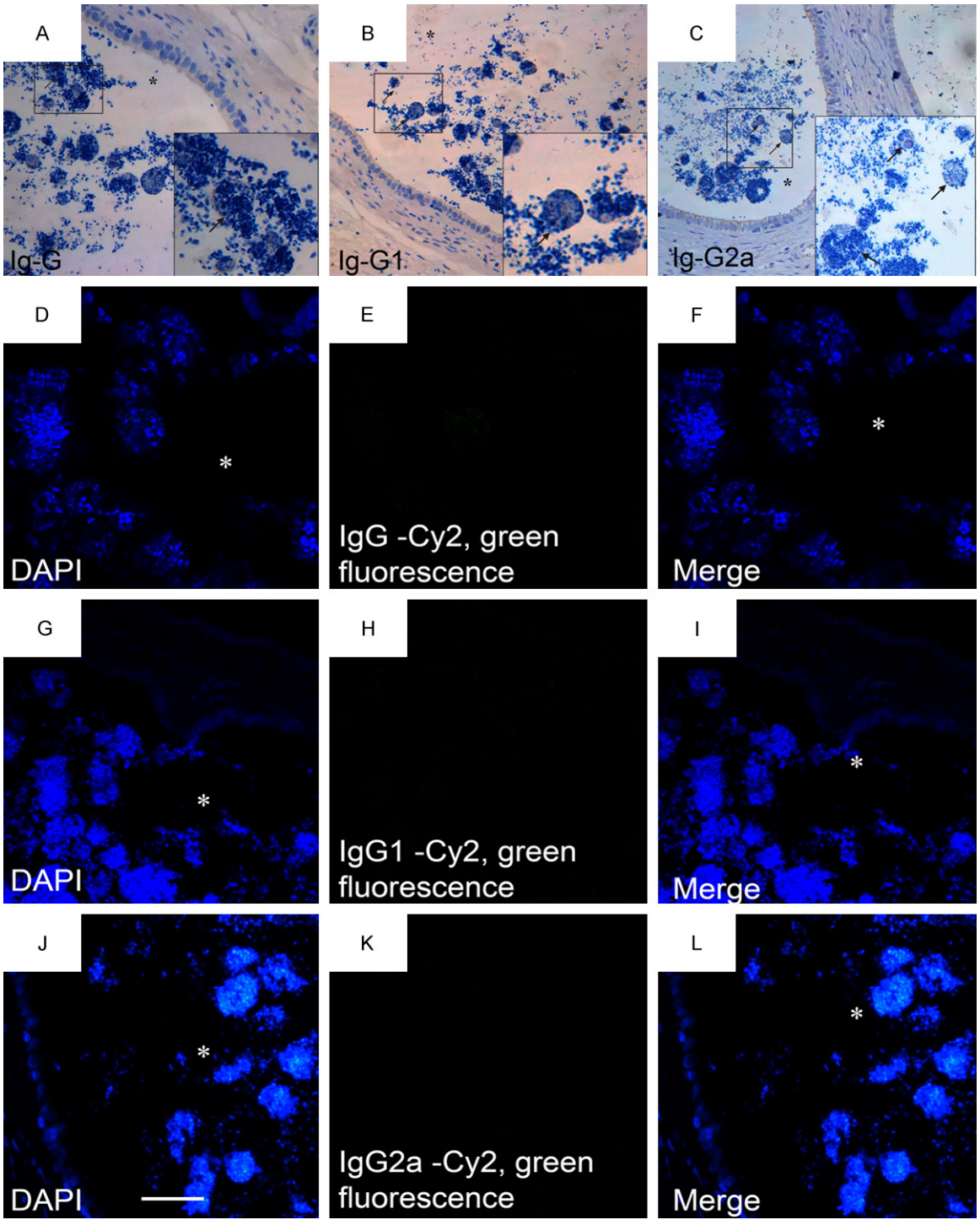

Figure 8. Appropriate isotype control of immunohistochemical and immune-fluorescence staining. The staining in the inflamed epididymis of human: IgG (A), IgG1 (B), IgG2a (C); IgG, IgG1 and IgG2a, Cy2 green fluorescence (E, H, K); DAPI+IgG merged (F); DAPI+IgG1 merged (I); DAPI+IgG2a merged (L); Nuclei were labeled with DAPI (D, G, J). *represent the lumen of epididymis. Arrows represent the positive cells. Bar =100 μm. (Original magnification, ×200; Inset: ×400).

ted in the principal cells of epididymal epithelium rather than immune cells of normal control epididymis (**Figure 9E**). On the other hand, IL-10+ cells were sparsely distributed in the epididymal interstitial compartment of inflamed epididymis (**Figure 9J**).

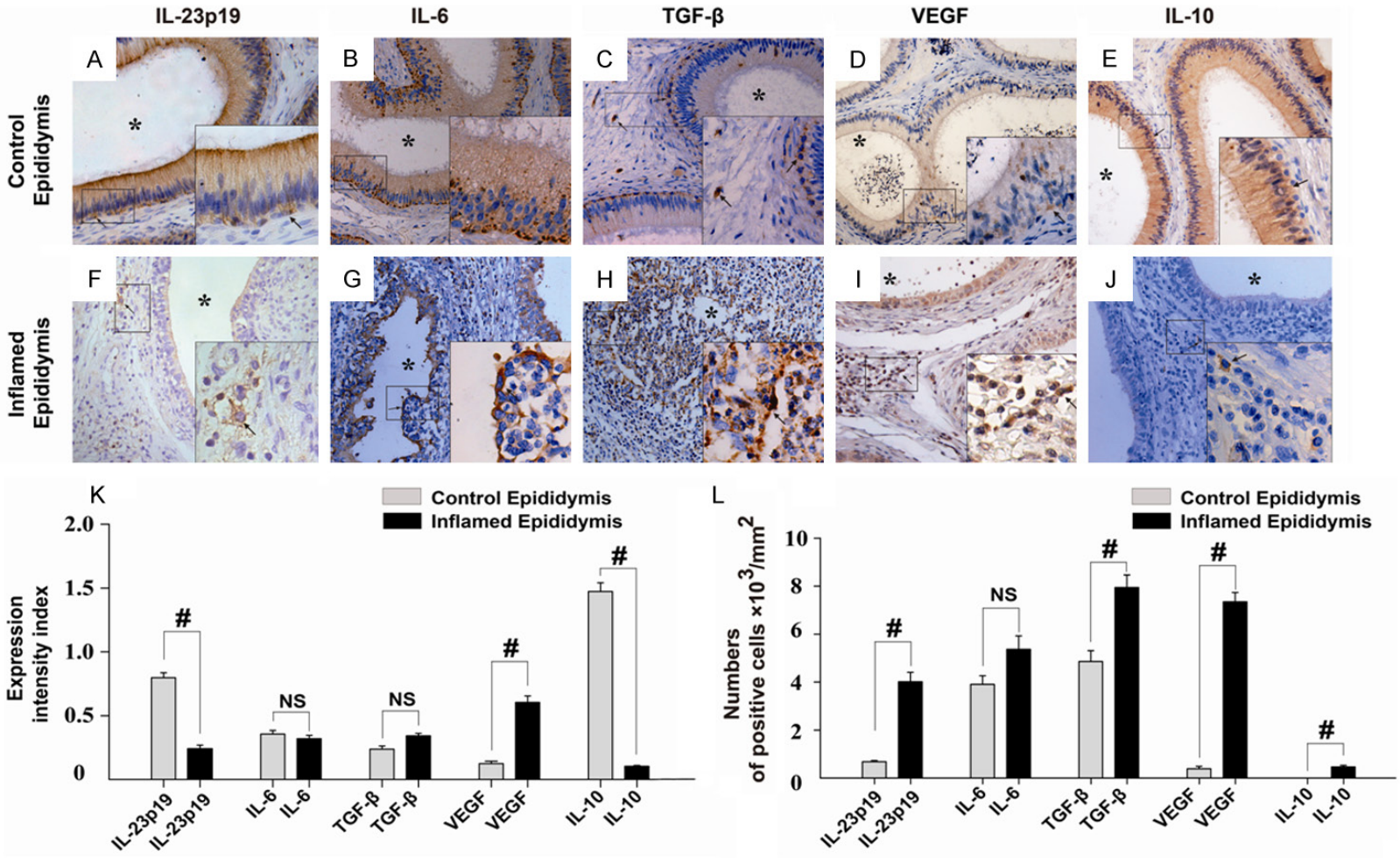

Figure 9. Distribution of IL-23p19+, IL-6+, TGF-β+, VEGF+ and IL-10+ cells in the inflamed epididymis and its controls. IL-23p19+ cells were found close and under the epithelium area of the normal control epididymis (A). Abnormal large amount of IL-23p19+ cells were detected within the interstitial area of inflamed epididymis (F). IL-6+ cells were detected mostly in the epididymal epithelium of the control epididymis (B). IL-6+ cells were detected within both the interstitial and epithelium area of inflamed epididymis (G). The subepithelial TGF-β+ cells formed a closely network in the epididymal epithelium of the control epididymis (C). Abnormal large amount of TGF-β+ cells were detected within both the interstitial and epithelium area of inflamed epididymis (H). VEGF was specially found in the intraepithelium area of normal control epididymis (D). Abnormal large amount of VEGF + cells were detected within both the interstitial and epithelium area of inflamed epididymis (I). IL-10 is expressed in the principal cells of epididymal epithelium (E). On the other hand, IL-10+ cells were sparsely distributed in the epididymal interstitial compartment of inflamed epididymis (J). Compared the expression intensity and numbers of IL-23p19+, IL-6, TGF-β+, VEGF+ and IL-10+ cells in controls and inflamed

epididymis (K, L). Quantitative enumeration was performed at the single cell level in immunohistochemically stained sections and 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 the positive cells. *represent the lumen of epididymis. #represent P<0.05. NS represent no significance. Bar =100 μm. (Original magnification, ×200; Inset: ×400).

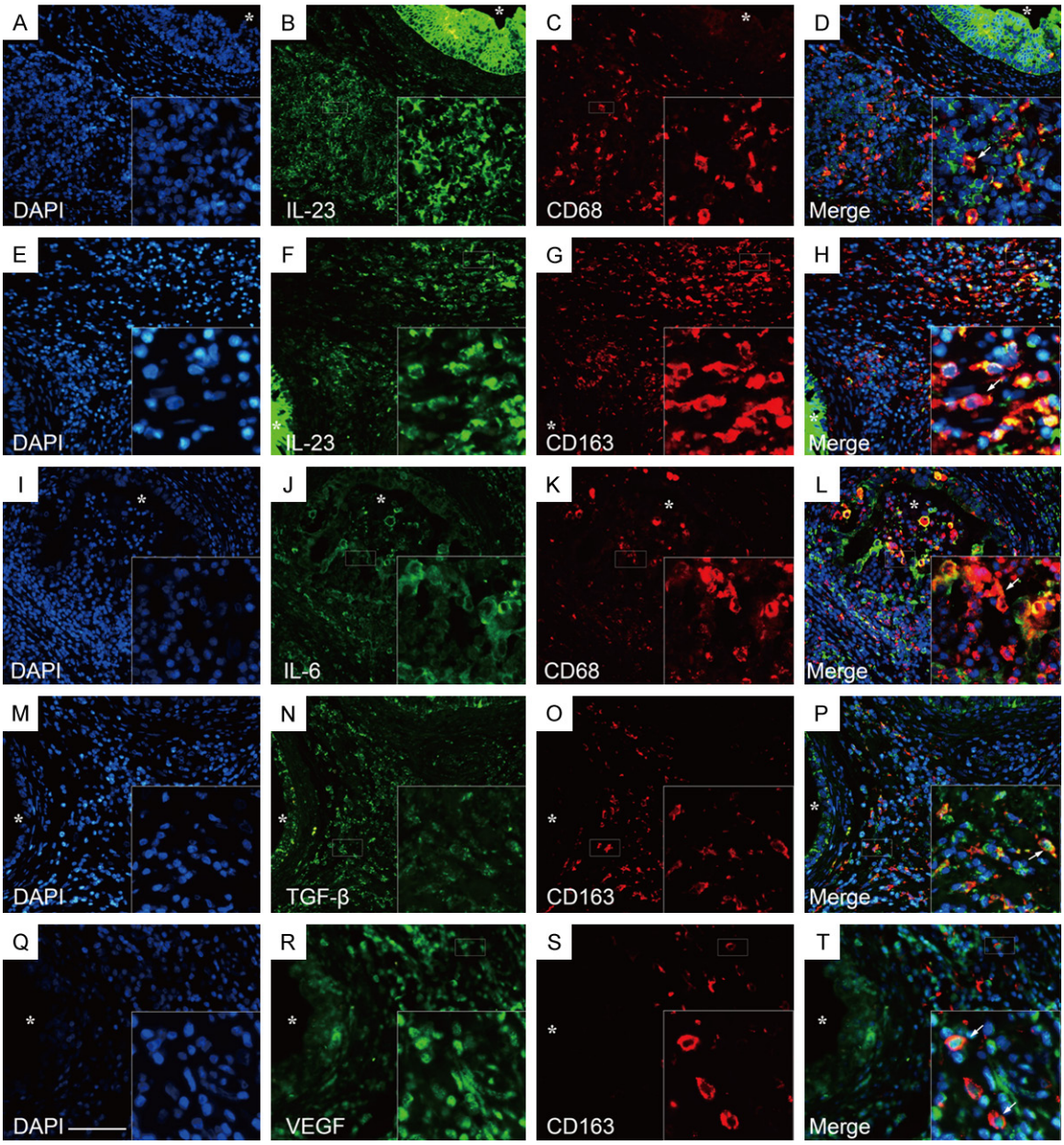

Figure 10. Immunofluorescence double staining of CD68+IL-23+, CD68+IL-6+, CD163+IL-23+, CD163+TGF-β+ and CD163+VEGF+ macrophages in the inflamed epididymis. Two color immunofluorescence was used to determine CD68+IL-23+, CD68+IL-6+, CD163+IL-23+, CD163+TGF-β+ and CD163+VEGF+ macrophages expression: CD68, CD163, CY3, red fluorescence (C, G, K, O, S); IL-23, IL-6, TGF-β and VEGF, CY2, green fluorescence (B, F, J, N, R). CD68+IL-23+ M1 macrophages, merged (D); CD163+IL-23+ M2 macrophages, merged (H); CD68+IL-6+ M1 macrophages, merged (L); CD163+TGF-β+ M2 macrophages, merged (P); CD163+VEGF+ M2 macrophages, merged (T); Nuclei were labeled with DAPI (A, E, I, M, Q); respectively. Arrows represent positive cells. *represent the lumen of epididymis. Bar =100 μm. (Original magnification, ×200; Inset: ×400).

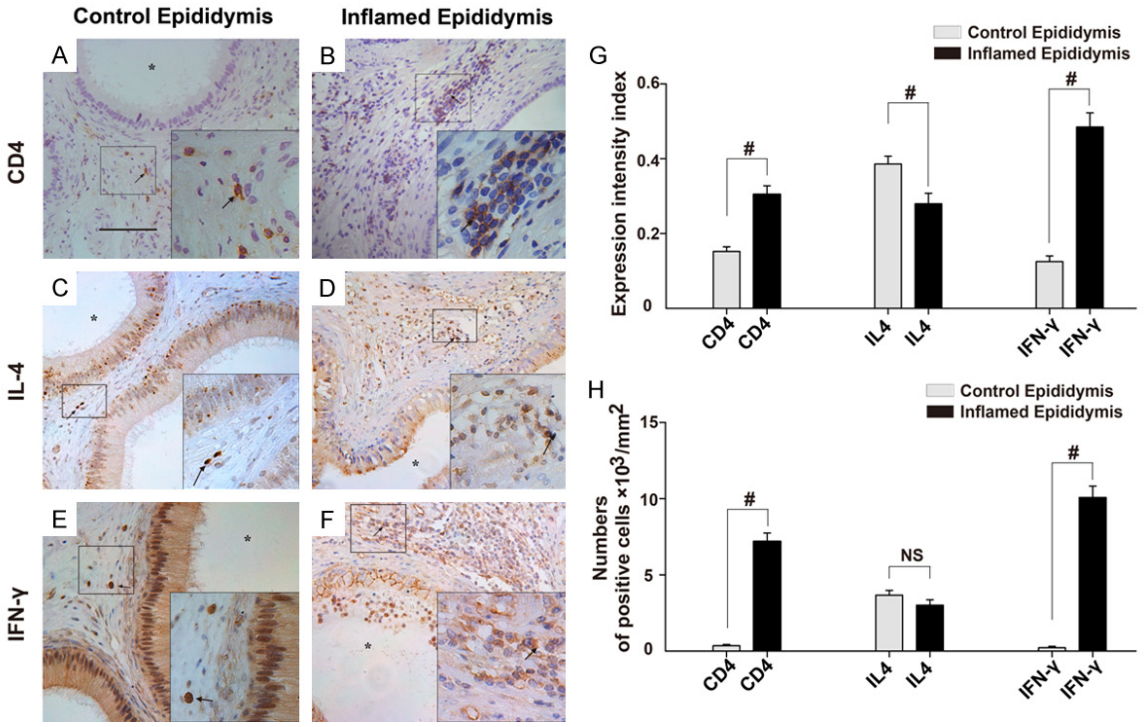

Figure 11. Distribution of CD4+, IL-4+ and IFN-γ+ cells in the inflamed epididymis and its controls. CD4+ cells were sparsely localized in the interstitial area of normal control epididymis (A). Abnormal large amount of CD4+ cells were detected within the interstitial area of the epididymitis (B). IL-4+ cells were detected both within the interstitial and epithelium of the control epididymis (C). IL-4+ cells were detected within the interstitial area of the epididymitis (D). IFN-γ+ cells were sparsely localized in the interstitial area of control epididymis (E). Abnormal large amount of IFN-γ+ cells were detected within both the interstitial area and the lumen of the epididymitis (F). Comparison of the expression intensity and numbers of IL-4+ and IFN-γ+ cells in controls and inflamed epididymis (G, H). Quantitative enumeration was performed at the single cell level in immunohistochemically stained sections and 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 the positive cells. *represent the lumen of epididymis. NS represent no significance. Bar =100 μm. (Original magnification, ×200; Inset: ×400).

Th1 (CD4+IFN-γ+) cells were predominantly chronically epididymitis

It is well known that M1 and M2 macrophages can stimulate antigen-specific T cells and induce Th1/Th2 type responses respectively through surface molecules and secreting cytokines [25-27]. CD3+ and CD4+ T cells have been found in the epididymis for a very long time [28], However, Th1 and Th2 cells were characterized recently as a distinct lineage of IFN-γ and IL-4-producing CD4+ T cells respectively remain to be elucidated.

The populations of CD4+ T cells were low in control epididymis, and most of them were detected in the interstitial compartment (**Figure 11A**). For comparison, the numbers of CD4+ T cells were significantly increased in peritubular areas of inflamed epididymis and also concentrated in the inflammatory infiltrate (**Figure 11B**, **11H**).

Our immunohistochemical study suggests that the cytokines IFN-γ is expressed in controls at low levels (**Figure 11E**). On the other hand, the expression of IFN-γ was significantly increased in inflamed epididymis (**Figure 11F-H**) and its expression could be localized to epididymal CD4+ T lymphocytes (**Figure 12E-H**). From this part of experiments, we speculate that M1 macrophages could induce an increased recruitment of Th1 cells under chronic inflammation of human epididymis.

Discussion

Macrophages are versatile controller between innate and adaptive immunity system which play a critical role in non-specific defense, and

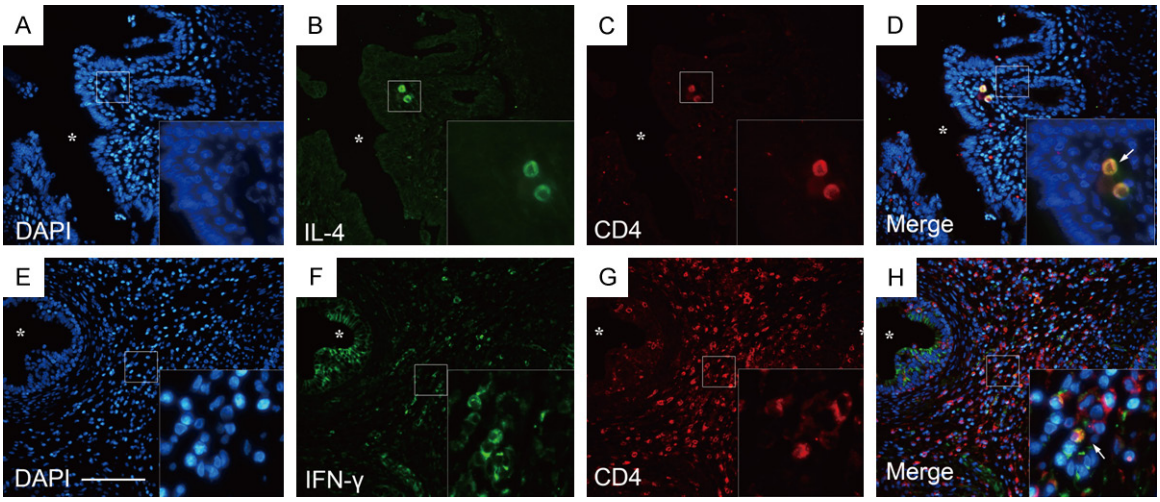

Figure 12. Two color immunofluorescence was used to determine CD4+IL4+ and CD4+IFN-γ+ cells expression: CD4, CY3, red fluorescence (C, G); IL-4 and IFN-γ, CY2, green fluorescence (B, F). CD4+IL-4+ Th2 cells, merged (D); CD4+IFN-γ+ merged (H); Nuclei were labeled with DAPI (A, E); respectively. Arrows represent the positive cells. *represent the lumen of epididymis. Bar =100 μm. (Original magnification, ×200; Inset: ×400).

also promote initiate specific defense mechanisms by recruiting other immune cells such as T lymphocytes [29]. Along with DCs specialized APCs, macrophages are foremost among the cells which present antigens, exerting a pivotal role in initiating an immune response, but the distribution, characteristics and function of macrophages subsets in the normal and inflamed epididymis have not been well defined in the previous study. Recently, we observed that a high population of CD11c+, CD123+, CD209+ DC subsets and Th17 (CD4+ IL-17A) cells in the inflamed epididymis [22]. In this study, we demonstrated for the first time that: (i) iNOS+, HLA-DR+, CD68+, CD163+ and CD206+ cells were found in the normal epididymis of human. (ii) The number of both iNOS+HLA-DR+CD68+DC-SIGN+ M1 and CD-163+CD206+ M2 macrophages was significantly increased in inflamed epididymis. (iii) iNOS+, CD68+ M1 macrophages and CD163+, CD206+ M2 macrophages capturing spermatozoa in the lumen of cauda epididymidis. (iV) IL-6, IL-23p19 producing M1 macrophages and TGF-β, VEGF, IL-23p19 producing M2 macrophages were significantly elevated in inflamed epididymis. (V) Th1 (CD4+ IFN-γ+) cells were predominantly distributed in the inflamed epididymis.

A majority of macrophages which are known as the mononuclear phagocyte system are stationed at strategic sites where accumulation of foreign particles or microbial invasion is likely to occur. Under a certain condition, the BEB and immune cells in the epithelium of normal epididymis may cooperate to prevent auto antigens from entering the circulation and initiating a pathological immune reaction against sperm [30]. Hence, the strategic localization of CD68+, iNOS+, HLA-DR+, DC-SIGN+, CD163+, CD206+ macrophages/DCs indicate that they may play a pivotal role in the afferent arm of the adaptive immune reaction in normal epididymis, by taking up luminal antigens from pathogens and apoptotic cells.

Our studies showed that abnormal large amount of CD68+ macrophages were found in the inflammatory infiltrates as well as concentrated in the lumen of inflamed epididymis, which were significantly correlated with the population of iNOS+ cells. Furthermore, immunofluorescence studies of double staining show that CD68 was co-localized with iNOS, meanwhile, did not co-localized with CD163 which specificity expressed in M2 macrophages. It is well known that “killer” M1 macrophages are activated by IFN-γ and LPS, and secrete high levels of inflammatory cytokines such as IL23p19, IL-6 and low levels of anti-inflammatory cytokines like IL-10 [31, 32]. Accordingly, our observations showed that epididymal CD68+iNOS+ M1 macrophages express high level of MHC class II molecular (HL-DR) and represent inflammatory cytokines IL-23

and IL-6 under chronic inflammation of human epididymis. Collectively, these results indicate that a pro-inflammatory response involving M1 macrophages might play an important role in the cascade of events which leading to epididymis rupture.

Interestingly, we found that CD163+CD206+ M2 macrophages were significantly increased and expressed tolerance cytokines VEGF and TGF-β in the inflamed epididymis, which suggest that the anti-inflammatory activities of M2 may have a role the constructive processes such as tissue repair, and further turn off damaging immune activation by secreting anti-inflammatory cytokines like VEGF and TGF-β. On the other hand, our study showed that both CD68+ M1 and CD163+ M2 macrophages in inflamed epididymis represent pro-inflammatory cytokines IL-23. It is relevant to know that IL-23 produced by antigen presenting cells was thought to play an important in role in terminal differentiation of Th17 cells which associated with the development of inflammatory response and autoimmunity [33]. Our previous study indicated that IL-23 producing CD11c+ DCs could induce an immunodeviation of T helper (Th cell) cell towards a Th17 immune response associated with epididymis damage were detected in inflamed epididymis [22]. Therefore, we speculate that both CD11c+ DCs and M1/M2 macrophages represent the main source of IL-23 which initiating and promoting inflammatory cascade and survival of Th17 cells. Furthermore, unlike the classical "repair" designation, M2 macrophages with pro- and anti-inflammatory cytokine profiles may enact both stimulatory and inhibitory effects on the development of chronic inflamed epididymis.

Our data showed that IFN-γ was represented not only in control but also in inflamed epididymis with elevated number; meanwhile, the expression intensity of IL-4 was significantly decreased. IFN-γ secreted by CD4+ T cells is a pro-inflammatory cytokine that acts as a potent mediator in initiating and promoting inflammatory cascade of M1 macrophages, and via positive feedback, M1 macrophages can stimulate antigen-specific T cells and induce IFN-γ (Th1) type response [34]. IFN-γ also inhibits the production of cytokines IL-4, an important cytokine associated with the Th2 type response, and thus it also acts to preserve its own response [35]. These studies provide credence for indication that macrophages induce an increased recruitment of Th1 cells under inflamed condition of chronic epididymitis and leading to epididymis rupture.

The most remarkable of our study is that both M1 and M2 macrophages were found capturing spermatozoa in the lumen of cauda epididymidis. In accordance with this finding, earlier studies demonstrated that in the patients with chronic accessory gland infections or occurrence of antibody-coated spermatozoa in their semen, the presence of macrophages "spermatophagy" activity on ejaculated sperm is significant [36]. Previous studies have showed that the inhibitory power of seminal plasma, such as PGE1, 19-OH-PEG1 and PGE2, protected spermatozoa from capture by APCs [37]. It is relevant to know that the inflamed epididymis does not store the seminal plasma and under a pro-inflammatory conditions. Taken together, we speculate that the excessively activated macrophages under chronic inflamed epididymis probably make the spermatozoa "mass control" mechanisms out of control which induce oligozoospermia and asthenospermia of male infertility patients.

In summary, our data suggests that the chronic inflamed epididymis is characterized by the recruitment of different macrophage subsets, such as iNOS+, CD68+, HLA-DR+ M1 and CD163+, CD206+ M2 macrophages, and which capture spermatozoa in the chronic inflamed epididymis. These cell populations, by means of cell interactions and their secretory products, may play an important role in the initiation of immune reaction and autoimmune response which leading to epididymis rupture. In the future, unraveling the characterization, function and regulation of epididymis macrophage subsets in inflamed epididymis is critical to the design of better strategies for the treatment of immunological male infertility.

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Disclosure of conflict of interest

None.

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