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Comprehensive overview of murine epididymal mononuclear phagocytes and lymphocytes: Unexpected populations arise

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ABSTRACT

Despite increasing evidence that epididymal immune disorders can lead to infertility, the cells and mechanisms underlying epididymal immunity remain poorly understood. In this study, we propose a rather exhaustive overview of innate and adaptive immune cells present in the murine *caput* and *cauda* epididymis. Using flow cytometry and a wide set of markers, we screened the broadest panel of immune cells ever, in this organ. For the first time, we unequivocally quantified the innate populations of monocytes, macrophages, and dendritic cells subtypes. We also revealed the presence of B cells, gamma delta T cells, and double negative T cells in the murine epididymis. They were localized by immunofluorescence stainings, and appeared to be all present in the interstitium and epithelium along the organ, but with respective preferential regional distribution. Altogether, these findings provide new insights on the actors and potential mechanisms involved in the immune responses against genital tract ascending pathogens and in the setting and maintenance of tolerance toward the sperm cells.

1. Introduction

Immune disorders, including infections and autoimmune reactions, represent up to 15% of known cases of male infertility and might be involved in the 30% of idiopathic cases (Haidl et al., 2008; Jungwirth et al., 2012). Studies regarding male reproductive tract immunity mainly focus on testis, originally thought to confer immunity to the whole tract while very little is known on the accessory organs, like epididymis. However, the incidence of cancers and inflammatory diseases vary in these organs, partly due to the tissues structural organization. While the blood-testis barrier is efficient at isolating most meiotic and all haploid germ cells from immune cells, the blood-epididymis barrier is not that impenetrable, suggesting that epididymal immune control is different from that of the testis (Hedger, 2011).

The epididymis has a vital role in managing the influx of both ascending pathogens and "foreign" spermatozoa. Sperm cells are produced at puberty, long after the setting of self-tolerance, and thus express germ-cell specific antigens that appear as foreign to the adult immune system which have yet to develop a tolerance to them. At the same time, the male genital tract, in contact with the external environment, is also frequently challenged by ascending pathogens. The epididymis is especially sensitive to these pathogens as epididymitis, mostly caused by sexually transmitted bacteria in young men, is the most frequent inflammatory disease of the male urogenital tract and can lead to transient or permanent infertility if not properly treated (Michel et al., 2015). While there are abundant studies on epididymal innate immunity, and especially beta defensins (for review, see Ribeiro et al., 2016), the relatively scarce literature on cellular innate and adaptive immunity may account for the poor understanding of the complex mechanisms involved in sperm tolerance and concomitant active protection against pathogens.

Cellular adaptive immunity relies on the lymphoid B and T cell populations. To date, B lymphocytes have been described only in rats while $CD4^+$ T lymphocytes were mainly found in the interstitium, and cytotoxic $CD8^+$ T lymphocytes mainly in the epithelium of mouse, rat and human epididymis (Nashan et al., 1989; Ritchie et al., 1984; Serre and Robaire, 1999; Yakirevich et al., 2002). However since these studies were published, the adaptive compartment has proven to be much

Abbreviations: APC, antigen-presenting cell; BEB, blood-epididymis barrier; CD, cluster of differentiation; DC, dendritic cell; cDC1, conventional type 1 dendritic cell; cDC2, conventional type 2 dendritic cell; DN T cells, double negative T cells; IDO, indoleamine 2,3-dioxygenase; Ig, immunoglobulin; MPS, mononuclear phagocyte system; NKT cell, natural killer T cell; O/N, overnight; pDC, plasmacytoid dendritic cell; PFA, paraformaldehyde; TCR, T cell receptor; Treg, regulatory T cell; WT, wild type

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more complex than originally thought, especially among the T cell populations.

Among the innate immune cells, macrophages have long been described as the major leukocyte population of the interstitium and peritubular layer of mouse epididymis from the *caput* to the *cauda* regions (Nashan et al., 1989). These cells may phagocytose senescent and excess sperm but this remains a controversial issue (Cooper et al., 2002; Tomlinson et al., 1992). Recent studies also identified mouse and human epididymal dendritic cells. While few or no mature cells (defined as $CD83^+$ dendritic cells) populate the interstitium of human epididymis, dendritic cells form a dense network in the basal region of the mouse epididymal epithelium, and extend dendrites toward the lumen through the apical tight junctions in the initial segment (Da Silva et al., 2011; Duan et al., 2016).

Mouse dendritic cells were also shown to be able to present antigens, at least *in vitro* (Da Silva et al., 2011). Nevertheless, the authors could not exclude that these cells were macrophages and further referred to them as mononuclear phagocytes in their subsequent works (Shum et al., 2014; Smith et al., 2014).

In fact, the mononuclear phagocyte system (MPS) gathers three innate immune cell types as it was long thought that monocytes were the precursors of both macrophages and dendritic cells. Since the first description of the MPS, technical advances, and especially flow cytometry, has allowed the characterization of multiple distinct monocyte, macrophage and dendritic cell subsets (Guilliams et al., 2014).

In this study, we propose an overview of the mononuclear phagocyte and lymphocyte populations present in the murine *caput* and *cauda* epididymis at steady state. Using a wide set of markers and flow cytometry, we screened six populations of mononuclear phagocytes and seven populations of lymphocytes. We finally localized the newly discovered cell populations using immunofluorescence and confocal imaging on tissue sections from the initial segment, *caput*, *corpus*, and *cauda* epididymis. This study gives reliable clues to the spatial distribution and organization of murine epididymal immune cells. It is a first mandatory step to decipher these cells interactions and mechanisms of action to set and maintain tolerance to spermatozoa and/or immune response to pathogens.

2. Materials and methods

2.1. Animals

Six month-old WT BALB/cAnNCrl male mice (Charles River Laboratories) were housed in an animal facility with controlled environment and fed *ad libitum* (Teklad global diet_2016, Envigo). Mouse housing and manipulation were approved by the Regional Ethic Committee in Animal Experimentation (C2E2A, authorization CE 99-12). Mice were killed by cervical dislocation and epididymides were collected.

2.2. Single cell suspension preparation

Pooled organs were divided into *caput* and *cauda* epididymides (*corpus* was discarded) and processed for the preparation of single cell suspensions. Briefly, the conjonctive capsule was mechanically removed before the tissues were digested in HBSS medium (Sigma-Aldrich®) containing 1.25 mM $CaCl_2$, 0.4 mM $MgSO_4$, and 1 mg/ml type II collagenase (Sigma-Aldrich®) for 30 min (*caput* epididymides) or 15 min (*cauda* epididymides) at 37 °C under gentle shaking. Digested tissues were washed in PBS with 2.5 mM EDTA and 0.5% BSA, and filtered through 30 µm Partec CellTrics® filters (Sysmex).

2.3. Flow cytometry analyses

All Abs and kits were purchased from BioLegend® unless otherwise stated. Viability was assessed using the Zombie UV™ Fixable Viability Kit according to the manufacturer's instructions. Cells were then fixed in 1% PFA (paraformaldehyde, Sigma-Aldrich®) for 15 min at 4 °C, washed, and incubated in a blocking buffer (PBS, 2.5 mM EDTA, 0.5% BSA, 5 µg/ml anti-CD16/CD32) for 10 min at 4 °C. Cells were incubated for 20 min at 4 °C with combinations of conjugated anti-mouse Abs or corresponding isotypes: PE/Cy7 or A700-TCRβ (H57-597; 2 or 5 µg/ml), PE-CD3 (17A2; 2 µg/ml), A488-CD4 (GK1.5; 5 µg/ml), PerCP-CD8α (53-6.7; 2 µg/ml), APC-CD19 (6D5; 2 µg/ml), PE/Cy7-TCRγδ (GL3; 2 µg/ml), A488- or Pacific Blue™-CD49b (DX5; 10 µg/ml), PE-CD25 (3C7; 2 µg/ml), PE/Dazzle™ 594-CD64 (X54-5/7.1; 4 µg/ml), A700-CD24 (M1/69; 10 µg/ml), PerCP-CD11b (M1/70; 4 µg/ml), PE-CD11c (N418; 2 µg/ml), PE/Cy7-I-A/I-E (M5/114.15.2; 2 µg/ml), A700-Ly6C (HK1.4; 5 µg/ml), A488-CX3CR1 (R&D Systems; 1/50), APC- or A488-F4/80 (BM8; 4 or 10 µg/ml), A647-CD103 (2E7; 10 µg/ml), APC-Siglec H (551; 4 µg/ml). To detect FOXP3-positive cells, the A647 anti-mouse/rat/human FOXP3 Flow kit was used according to the manufacturer's instructions. Finally, cells were washed and kept at 4 °C until processing. Data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using the FACSDiva™ Software (v6.1.3, BD Biosciences). The cells were first selected based on their size (FSC) and granularity (SSC) characteristics (Fig. 1, P1 gate). The residual sperm cells were excluded from the analysis thanks to the FSC/SSC criteria. Indeed the sperm cells are smaller and less granular than the epididymal somatic cells and consequently appear as a distinct population on the FSC/SSC dot plot. The quantification is thus only based on somatic epididymal cells. Next, the singlets were selected (P2 and P3 gates). Finally, live cells were gated based on their negative staining for the viability marker before the cells were selected for the various markers tested. The percentages presented for each cell population are reported as the positivity minus the corresponding isotype control.

2.4. Immunofluorescence staining

Whole epididymides were fixed for 1 h (TCRγδ staining only) or O/N in 4% PFA at 4 °C, before being cryoprotected in PBS with 30% sucrose (weight/volume) for 6 h at 4 °C, embedded in cryosection medium (Richard-Allan Scientific™ Neg-50™, Microm Microtech) and sectioned (10 µm) in a cryostat (HM 560, Microm Microtech). Sections were fixed for 15 min in 4% PFA, washed and endogenous peroxidases were inhibited with 0.3% H_2O_2 for 30 min at room temperature. Sections were further saturated for 1 h in PBS with 5% normal goat serum (Vector Laboratories), and incubated with the following purified primary anti-mouse Abs alone or combined, O/N at 4 °C: anti-CD4 (GK1.5; 5 µg/ml), anti-CD8 (53–6.7; 5 µg/ml), anti-CD19 (6D5; 10 µg/ml), anti-TCRγδ (GL3; 10 µg/ml), all purchased from BioLegend®. Then, various conditions were used as follows. To detect double negative (DN) T cells, the CD4 and CD8 double staining was followed by a first incubation with A488 goat anti-rat IgG (2 µg/ml; ThermoFisher Scientific) and a second incubation with A594-CD3 Ab (17A2; 10 µg/ml; BioLegend®). Concerning the CD19 staining, slides were incubated with the ImmPRESS™ reagent (anti-rat Ig, Vector Laboratories) and an A555-labeled tyramide (ThermoFisher Scientific), according to the manufacturer's instructions. Concerning the TCRγδ staining, sections were incubated with a biotin-goat anti-hamster IgG (1 µg/ml; BioLegend®), before an A555 Tyramide Signal Amplification kit was used (ThermoFisher Scientific), according to the manufacturer's instructions. Finally, all slides were counterstained with 1 µg/ml Hoechst 33342 (Sigma-Aldrich®), mounted in Tris-MWL 4-88 solution (Citifluor™) and observed through a Leica SPE confocal microscope. Digital images were processed with the OMERO open-source software (OME consortium).

2.5. Statistical analyses

Mann-Whitney *U* tests were performed (GraphPad Prism software v6.01) to analyze the differences in immune cell proportions between *caput* and *cauda* epididymides. P values $< .05$ were regarded as statistically significant.