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Multi-parameter flow cytometric analysis of uterine immune cell fluctuations over the murine estrous cycle

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ABSTRACT

Investigating immune cell populations within various reproductive tissues commonly utilises flow cytometric methods. With advances in fluorophore technology and equipment capabilities, multiple cell types from a single tissue sample can be identified by using different combinations of cell surface markers to distinguish specific cell populations. Here a protocol optimized for mouse uterine tissue was used to show the proportional changes in dendritic cells, monocyte/macrophages, T and B cells, NK and NK T cells, and the granulocytes, neutrophils and eosinophils at each of the four stages of the estrous cycle. Importantly, we demonstrate that use of anti-SiglecF or assessment of FSC/SSC plots could be used to differentiate monocyte/macrophage and eosinophil populations that otherwise cannot be distinguished by use of the common combination of antibodies against F4/80 and CD11b. Our results clearly indicate that within the uterus a dynamic population of immune cells resides, with many cell types reaching peak abundance at estrus and metestrus phases of the cycle, consistent with their importance in the response to paternal antigens and/or pathogens encountered after insemination.

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1. Introduction

The murine reproductive cycle is controlled by the endocrine system via the ovarian steroid hormones 17-β-estradiol and progesterone, and the non-steroid hormones, the pituitary gonadotropins, luteinizing and follicle stimulating hormones, and the luteotropic hormone prolactin. Distinct oscillations in the circulating levels of these hormones over 4–5 days delineate the four main stages of the murine estrous cycle: proestrus, estrus, metestrus, and diestrus (Wood et al., 2007), and are thought to be associated with changes in behavior and function of immune cells during these times (Butts et al., 2010). Changes in the uterine tissue itself also happen throughout this period, with continuous growth and development of the endometrium occurring at estrus in readiness for ovulation and mating receptivity. In the absence of mating and fertilization, subsequent wide spread atrophy follows with the cycle continuously repeating itself until pregnancy ensues (Curry and Osteen, 2001; Wood et al., 2007). As a type I mucosal tissue, it is well established that the uterus harbors a diverse immune cell population, of which certain cell types have been shown by varying methods to be increased or decreased at different stages of the estrous cycle (Wira et al., 2005; Schumacher et al., 2014).

Immunohistochemistry has been the most widely used method of studying cells of the reproductive system and has been invaluable in advancing knowledge of these complex tissues, particularly as spatial location is an important parameter. However, with ongoing investigations of specific immune cell populations revealing different subsets with different capabilities, for instance tissue-resident memory T cells and T regulatory cells (Schumacher and Zenclussen, 2014; Turner and Farber, 2014; Thome and Farber, 2015), it is becoming increasingly apparent that techniques which enable the detection of multiple cell markers on the same cell are required. Flow cytometry represents an increasingly valuable tool in the interrogation of immune cell types by its ability to detect several cell surface and intracellular markers on the same individual cell rapidly, reproducibly and with high accuracy. A greater application of this method, along with the ability to sort individual cell populations from a mixed cell suspension, would significantly advance our understanding of the role that different types of immune cells play over the course of the peri-conception and peri-implantation

Abbreviations: FSC, forward scatter; SSC, side scatter; NK, natural killer; DC, dendritic cell; NETs, neutrophil extracellular traps.

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periods and potentially help us understand how perturbations at this early time can lead to later pregnancy complications and/or impact on infant and adult health (Hanson and Gluckman, 2014).

To our knowledge, there is no comprehensive analysis incorporating all of the broad range of immune cell types located within the uterine tissue across different stages of the murine estrous cycle. Therefore in this study, we developed and implemented a flow cytometric protocol to clearly identify distinct uterine immune cell populations, allowing interrogation of how the proportions of these cells change over the murine estrous cycle. The protocol and panel of antibodies were optimized specifically for use with murine uterine tissue to maximize cell recovery and visual separation of populations by flow cytometric analysis. Multiple staining panels were used to differentiate closely aligned cell populations, and were designed to be adaptable to the majority of flow cytometers in standard use. The concise nature of the panels also allows for the addition of more specific cell markers to further delineate cell subsets depending on the particular cells of interest.

Our results demonstrate that most immune cell populations assessed peak in number at estrus, and that innate immune cells, particularly granulocytes, make up the majority of cells at this time. Overall these immune cells are likely to play an important role in priming of the maternal immune response induced by seminal fluid against paternal antigens (Moldenhauer et al., 2009), promoting the necessary remodeling of the uterine tissue required for subsequent embryo implantation (Robertson et al., 2000; Care et al., 2013; Sun et al., 2013), as well as regulating the growth and development of the semi-allogeneic conceptus and placenta. Innate immune cell accumulation also coincides with the possibility of acquiring an infection at the time of coitus (Wiesenfeld et al., 2002; Butts et al., 2010). Therefore, the influx of innate immune cells during this period associated with coitus and fertilisation is likely to play a crucial role in the development and maintenance of a successful pregnancy.

2. Materials and methods

2.1. Mice

Female C57Bl/6 mice were utilized for all experiments which were conducted with dual approval of both SA Pathology/CHN and University of Adelaide Animal Ethics Committees. Mice were housed in individually ventilated cages under standard specific-pathogen free animal facility conditions with food and water provided *ad libitum*.

2.2. Vaginal washouts

To determine the stage of estrous cycle, vaginal smears were performed. Briefly, the vagina was repeatedly flushed three times with a single volume of 50 μL endotoxin-free PBS using standard laboratory micropipette and pipette tip. After the final flush, the exudate was deposited onto a microscope slide and a coverslip applied. Visualization of vaginal cell proportions by microscopic analysis allowed determination of the stage of estrous cycle. For clarity of figures, some exudates were cytospun onto slides, air dried, fixed and differentially stained as described in section 2.7 below. Proestrus is the pre-ovulatory stage, and is identified by the presence of nucleated epithelial cells among the anucleated cornified epithelial cells (Supplementary Fig. 1A). The number of cells obtained from vaginal exudates at proestrus is often low. Estrus is associated with ovulation, and is readily identified by the presence of abundant cornified epithelial cells in clusters (Supplementary Fig. 1B). Vaginal exudates that contain leukocytes as well as nucleated and cornified epithelial cells are indicative of metestrus (Supplementary Fig. 1C). Lastly, exudates that indicate a close to homogeneous population of leukocytes in large numbers are indicative of diestrus (Supplementary Fig. 1D).

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jri.2015.11.005.

2.3. Uterine digestion

The procedure for obtaining single cell suspensions was loosely based on previous published protocols for obtaining splenic dendritic cells (Vremec 2010), and further refined empirically. Uterine tissue from female mice at specific stages of the estrous cycle was dissected and placed into cold RPMI containing 2% FCS (RPMI-FCS) on ice. Fat and connective tissue was removed prior to transfer into 1 mL digest mix (1 mg/mL (210U/mg) collagenase type I [Life Technologies]; 4U DNase I [NEB] in RPMI-FCS) equilibrated to RT. Sharp pointed scissors were used to macerate the tissue into very small pieces, then fragments were digested with gentle shaking for 30 min at 37 degrees. For the last 5 min, 100 μL 0.1 M EDTA pH7.22 was added (1/10th volume of the digest mix) and mixed well. The cell suspension was then gently ground between the frosted ends of two microscope slides to release remaining loosely associated cells, and was filtered through a 70 μM cell strainer followed by 2×1 mL washes with 2% FCS/RPMI. The resultant 3 mL cell suspension was underlayed with 0.5 mL cold FCS-EDTA (FCS containing 0.01 M EDTA pH7.22) and spun at $1000 \times g$ for 7 mins, 4 degrees. The resultant pellet was resuspended in cold BSS-EDTA-FCS (150 mM NaCl; 3.75 mM KCl; 5 mM EDTA; 25 nM HEPES; 2% FCS) and placed on ice for subsequent staining.

2.4. Antibodies

Antibodies were purchased from BD Biosciences or eBioscience and used in staining combinations as listed in Table 1. Additional antibodies were occasionally substituted for that listed in Panel 3 to definitively confirm eosinophil populations.

2.5. Flow cytometric staining and analysis

The cell pellet resulting from the uterine digestion was resuspended in 50 μL of 1/100 dilution of FcBlock (CD16/CD32) and incubated for 15 min before distribution into the appropriate number of tubes for staining. Panels of directly conjugated antibodies as indicated in Table 1 were subsequently added and incubated on ice for a further 30 min. Cells were washed twice with BSS-EDTA-FCS (2×3 mL) and resuspended in endotoxin-free PBS for immediate assessment on a FACSCanto II flow cytometer (BD Biosciences). The results were analysed using FACS Diva software (BD Biosciences), gating only on viable cells and excluding red blood cells.

2.6. Cell sorting

Uteri were digested as above, with resultant cell suspensions pooled and stained with antibodies against CD11b, F4/80, Gr-1 and MHC class II (Table 1) prior to washing and resuspension in BSS-EDTA-FCS. Cells were then sorted three ways at low speed to maximize purity at the Detmold Imaging Facility, Hanson Institute, Adelaide, Australia, utilising a FACS Aria II flow cytometer (BD Biosciences).

2.7. Cytospins and cytological staining

Cytospins were generated by resuspending cells obtained from vaginal smears or cell sorting in 150 μL PBS/30% FCS and loading into cytocentrifuge cuvettes (Shandon; ThermoScientific) followed by centrifugation at 300 rpm for 8 min. Slides were carefully

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