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Immunophenotypic profile of leukocytes in hyperandrogenemic female rat an animal model of polycystic ovary syndrome☆

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ABSTRACT

The immune etiology of polycystic ovary syndrome (PCOS) is an intriguing area. However, whether there is alteration in the leukocyte populations in different tissues remain ambiguous. **Aim**: To characterize the leukocyte populations of hyperandrogenemic female (HAF) rat tissues. **Methods**: Female Sprague Dawley rats at 3 weeks of age were implanted subcutaneously with dihydrotestosterone (DHT) or placebo pellets. The rats were aged to 14–15 weeks and tissues were collected. **Results**: Peripheral blood (PB) and renal $CD4^+$ ($P < 0.03$, $P < 0.007$), Th17 ($P < 0.05$, $P < 0.002$), and $CD4^+CD28^{null}$ ($P < 0.04$, $P < 0.001$) were significantly increased in HAF rats compared to placebo, respectively, in spite of their lower percentage in the spleen. Although, the percentage of Treg T lymphocytes were significantly higher in the PB ($P < 0.001$) of HAF rats, the splenic ($P < 0.01$) and renal Treg cells ($P < 0.03$) were found to be significantly lower. Remarkably, HAF rats had higher renal mast cells ($P < 0.00009$) despite lower splenic ($P < 0.002$). The number of PB, renal, and splenic $CD8^+$ T cells and IgM^+-B cells in HAF rats remained unchanged. **Conclusion**: Results from this study 1) provide the first evidence of significant alteration of T lymphocyte subsets and different leukocyte populations profile in a rat model of polycystic ovary syndrome, 2) demonstrate alteration of the immunological niche of blood, spleen, and kidney tissues in Hyperandrogenemia state in female rats, 3) imply potential immune system dysregulation in HAF rats which may suggest a link between excess androgen, chronic inflammation, and immune-mediated diseases in polycystic ovary syndrome patients.

1. Introduction

The immune etiology of polycystic ovary syndrome (PCOS) is an intriguing area and increasing recognition has been given to the contribution of the immune system in PCOS and associated inflammation. PCOS is the most common endocrinology disorder affecting 6–10% of young women [1–4]. PCOS is a heterogeneous disorder characterized by Hyperandrogenemia (HA), chronic anovulation, and polycystic ovary [1,3]. It is associated with insulin resistance [5], type 2 diabetes (T2D), higher risk of cardiovascular disease [6,7], and chronic low-grade inflammation [8,9]. PCOS is believed to be a pro-inflammatory state [10] and chronic low-grade inflammation is considered a key contributor to the pathogenesis of PCOS. Accumulating data demonstrate increased C-reactive protein, pro-inflammatory cytokine levels of TNF-α and IL-6 [11,12] and interleukin-18 [13] in POCS patients. Furthermore, increased number of activated T cells in follicular fluid [11,14], increased white blood cells [15], an elevated peripheral blood (PB) neutrophils [16], expansion of PB cytotoxic $CD4^+CD28^{null}$ T lymphocytes [17], and higher PB Th17 cells [18] in women with PCOS imply immune dysregulation in PCOS and suggest a correlation between endocrine and immune function. Supporting evidence of expansion of PB and follicular fluid leukocytes in women with PCOS and their significant role in exacerbating inflammation by secreting pro-inflammatory cytokines indicate that leukocytes play a critical role in the pathogenesis of PCOS. However, the impact of HA on immune system in PCOS and experimental animal models is not well established.

Since, leukocytes not only have vital role in the body's defense against infectious and non-infectious agents, but also triggering inflammatory signaling pathways and contributing to a variety of chronic inflammatory diseases [19] and immune-mediated disorders therefore,

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their fluctuation in the peripheral blood and/or organs requires further examination. To the best of my knowledge, there are no studies of leukocytes profile or trafficking in spleen and kidney in human and/or animal model of PCOS. Studying splenic leukocyte profile is essential since it is heavily involved in the immune regulation in response to the pathological agents and chronic inflammation in metabolic disorder and PCOS [20]. Moreover, investigating the renal leukocyte profile is essential due to the manifestation of renal impairment in the women with PCOS [21,22].

The aim of this study was to examine leukocyte population alteration in blood, spleen, and kidney of a rat model of PCOS to shed light on how modest increase in androgen in females may fluctuate pro-inflammatory leukocyte populations in different organs. A number of animal model for PCOS have been generated by dihydrotestosterone (DHT) supplementation [23–27]. In the current study hyperandrogenemic female (HAF) rat was generated in the same fashion [23]. HAF rat mimics many of the changes that occur in women with PCOS. For example, HAF rat exhibits three fold higher plasma DHT level, similar to the elevated androgens found in women with PCOS [28], ovarian dysfunction, increased body weight, visceral fat, insulin resistance, blood glucose level, glomerular infiltration rate, and renal injury [29]. HAF rat shows elevated plasma level of leptin, cholesterol, and Tumor necrosis factor-alpha (TNF-α).

2. Materials and methods

2.1. Experimental animals

All procedures for animal use complied with the *Guidelines for the Care and Use of Laboratory Animals*, *11th edition* by the National Institutes of Health, and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center (#1495).

Female Sprague Dawley (SD) rats (50–75 g) at 3 weeks of age (Envigo, Indianapolis, IN) were housed and maintained throughout the study on standard chow in a room on 12/12-h light/dark cycle at 25 °C and 50–60% humidity with ad libitum access to food and water. After one week of acclimatization to the vivarium, rats were implanted subcutaneously with continuous-release dihydrotestosterone (DHT) pellets (7.5 mg/90 days; daily dose of 83 μg; Innovative Research, Sarasota, Florida) or 7.5 mg placebo pellets (Innovative Research, Sarasota, Florida). The dose of DHT induces a hyperandrogenic state similar to women with PCOS [23]. The HAF or placebo control rats were aged to 14–15 weeks before study and body weight was measured weekly.

2.1.1. Body weight and food intake

Rats body weight and food intake were measured twice per week for the entire period of study.

2.1.2. Plasma dihydrotestosterone assays

Blood was collected at the end of the experiment, cold centrifuged, and stored at −80 °C. Plasma DHT level was measured using (LifeSpan BioSciences - LSB Bio, Seattle, WA).

2.1.3. Peripheral blood lymphoid cell isolation

Blood was collected from rats (n = 5–7/grp) under anesthesia at 14–15 weeks of age. Mononuclear cells were isolated from blood by a density gradient medium Ficoll-Hypaque (Lymphoprep; Accurate Chemical, Westbury, NY) as per manufacturer recommendation. The blood was gently added over lymphoprep medium and centrifuged at 1600 rpm for 20 min. Peripheral blood lymphocytes were washed and suspended in Roswell Park Memorial Institute medium (RPMI-1640) (Invitrogen, Grand Islands, NY) containing 1% heat-inactivated Fetal Bovine Serum (FBS) and 0.05% sodium azide; pH 7.4 (FACS buffer). The dye exclusion test was performed using Trypan Blue to determine the number of viable cells in the cell suspension.

2.1.4. Splenic and renal lymphoid cell isolation

Rats at 14–15 weeks of age were anesthetized with isoflurane, blood and spleen were collected then kidneys were flushed with heparinized saline. The tissues were minced and incubated in a RPMI-1640 containing 125 U/ml collagenase IV (Invitrogen, Grand Islands, NY) and 200μg/ml DNAse (Sigma, St. Louis, MO) for 1 h at 37 °C. The digested tissues were washed with RPMI at 1600 rpm for 10 min. Pellets were suspended in RPMI-1640 and filtered through a 100-μm sieve (Fisher Scientific). Obtained single-cell suspensions were washed with RPMI-1640 and centrifuged at 1600 rpm for 10 min. Pellets were suspended in RPMI-1640 and layered over Ficoll-Hypaque (Lymphoprep; Accurate Chemical, Westbury, NY), centrifuged at 1600 rpm for 20 min, mononuclear cells were isolated according to the manufacturer's directions. The dye exclusion test was performed to determine the number of viable cells in the cell suspensions.

2.1.5. Flow cytometry

Isolated cells (1×10^6) were incubated with 50 μl of specific antibodies on ice for 30 min. The primary antibodies were as follow: anti-CD4, anti-CD8a, anti-CD25, anti-CD28, anti-CD45, anti-CD161, anti-mast cell, anti-CD11B-FITC-conjugated (BD Biosciences, San Jose, CA), and anti-IgM-FITC-conjugated (Southern Biotech, Birmingham, AL), followed by wash and incubation on ice with R-phycoerythrin (PE)- or Fluorescein isothiocyanate (FITC)-antibodies (Southern Biotech, Birmingham, AL) for 30 min. To detect specific Th17 ($ROR\gamma^+$) and Treg ($FoxP3^+$) cell populations; cells after extracellular staining with anti-CD4, anti-CD8a, and anti-CD25 were fixed and permeabilized according to the manufacturer's protocol with Forkhead box P3 (FOXP3) permeabilization buffer (eBioscience, San Diego, CA). Then intracellular staining of RAR-related orphan receptor-γ (ROR-γ) and FoxP3 transcription factors was performed by using anti-ROR-γ-PE-conjugated (R& D Systems) and anti-FoxP3-Allophycocyanin (APC)-conjugated antibodies (eBioscience, San Diego, CA) on ice for 30 min. Peripheral blood cells were washed and suspended in FACS buffer (RPMI-1640 + 1% heat-inactivated fetal bovine serum, 0.5% 0.5 M EDTA; pH 7.4). The splenic and renal cells after permeabilization and staining with antibodies, were washed and suspended in FACS buffer (Hanks' balanced salt solution +1% heat-inactivated fetal bovine serum, 0.5% 0.5 M EDTA; pH 7.4). Mononuclear cells were quantified using a Beckman Coulter Gallios analyzer at the UMMC Cancer Institute Flow Cytometry Core Facility. Negative control, for each individual rat, was performed using isotype controls matched to each primary antibody's host species, isotype, and conjugation format and cells were treated exactly as described above. The percent of positive staining cells above the negative control was collected for each individual rat and mean values for each experimental group (Placebo, HAF) was calculated. The Fluorescence Minus One Control (FMO) principle was used to properly interpret flow cytometry data and count for background antibody fluorescence.

2.2. Statistical analyses

Data was analyzed by Student's *t*-test for 2 groups: Placebo and HAF. Data are presented as mean ± SEM, with $P < 0.05$ considered significant.

3. Results

3.1. Body weight and food intake

The initial body weight was not significantly different in HAF rats compared to the placebo controls (Control rats: 107 ± 1 g vs. HAF rats: 114 ± 2 g, n = 6/grp). However, HAF rats body weight have significantly increased over the course of the experiment (Control rats: 252 ± 7 g vs. HAF rats: 333 ± 18 g, n = 6/grp). The HAF rats have

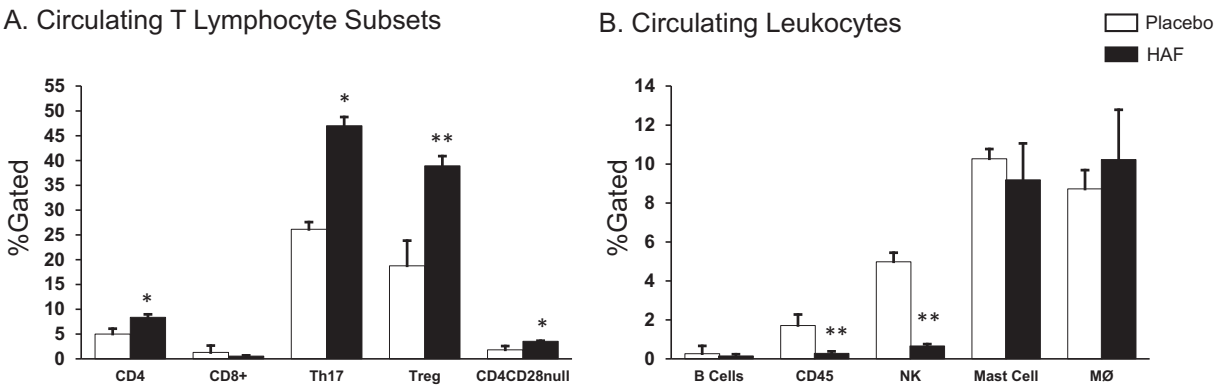

Fig. 1. Circulating T lymphocyte subsets (A) and other circulating leukocytes (B) of placebo and HAF rats. The percentage of circulating CD4+, CD8+, Th17, Treg, CD4+CD28null, IgM+, CD45+, CD161+, mast cell, and macrophage were measured using flow cytometry in Placebo and HAF rats (n = 5–7/group). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, compared to Placebo.

consumed more food compared to placebo control (Control rats: 12 ± 1 g vs. HAF rats: 15 ± 1 g, n = 6/grp).

3.1.1. Plasma dihydrotestosterone

Plasma DHT level was almost three folds higher in HAF rats compared to the placebo rats.

3.2. Immune cell populations profile

As shown in Figs. 1, 2, and 3, significant alterations in the PB, splenic, and renal immune cell populations in HAF rats were observed. Fig. 1A shows that frequency of the PB T lymphocyte subsets; $CD4^+$ (Placebo: 5 ± 1 vs. HAF: 8 ± 1%gated P < 0.03), Th17 (Placebo: 26 ± 1 vs. HAF: 47 ± 2%gated P < 0.05), and $CD4^+CD28^{null}$ T cells (Placebo: 2 ± 1 vs. HAF: 4 ± 0.1%gated, P < 0.04) were significantly increased in HAF rats compared to placebo, parallel to increased renal T lymphocyte subsets (Fig. 2A); $CD4^+$ (Placebo: 10 ± 1 vs. HAF: 22 ± 1%gated P < 0.007), Th17 (Placebo: 19 ± 1 vs. HAF: 23 ± 1%gated P < 0.002), and $CD4^+CD28^{null}$ (Placebo: 6 ± 1 vs. HAF: 15 ± 2%gated P < 0.001). Interestingly, despite the lower frequency of the splenic $CD4^+$ (Placebo: 11 ± 2 vs. HAF: 6 ± 1%gated P < 0.05) in the HAF rats, the number of Th17 (Placebo: 2 ± 1 vs. HAF: 3 ± 2%gated) and $CD4^+CD28^{null}$ (Placebo: 4 ± 1 vs. HAF: 3 ± 1%gated) T cells were not significantly changed compared to the placebo (Fig. 3A). Moreover, circulating anti-inflammatory T regulatory (Treg) cells (Placebo: 19 ± 5 vs. HAF: 39 ± 2%gated P < 0.001; Fig. 1A) were significantly higher in HAF rats, in contrast to significant suppressed percentage in the spleen and kidney (Placebo: 51 ± 4 vs. HAF: 38 ± 1%gated P < 0.01; Fig. 2A) and (Placebo: 3 ± 1 vs. HAF: 2 ± 0.7%gated P < 0.03; Fig. 3A) compared to placebo rats, respectively.

In the present study despite the lower percentage of circulating natural killer (NK) cells (Placebo: 5 ± 1 vs. HAF: 1 ± 0.1%gated P < 0.00001; Fig. 1B), the splenic and renal NK cells (Placebo: 17 ± 2 vs. HAF: 28 ± 3%gated P < 0.008; Fig. 2B) and (Placebo: 11 ± 1 vs. HAF: 15 ± 1%gated P < 0.01; 3B) were significantly higher in HAF rats, respectively. Furthermore, data show that expression of CD45 marker was significantly lower on the PB (Placebo: 2 ± 0.4 vs. HAF: 0.3 ± 0.2%gated P < 0.001; Fig. 1B) and renal leukocytes (Placebo: 16 ± 2 vs. HAF: 5 ± 2%gated P < 0.001; Fig. 3B), in contrast to the significant higher CD45 expression on splenic leukocytes (Placebo: 32 ± 2 vs. HAF: 52 ± 4%gated P < 0.002; Fig. 2B) of HAF rats.

Remarkably, HAF rats had higher percentage of renal mast cells (MCs) (Placebo: 6 ± 1 vs. HAF: 20 ± 1%gated P < 0.00009; Fig. 2C) in contrast to the significant lower splenic MCs (Placebo: 10 ± 1 vs. HAF: 4 ± 1%gated P < 0.002; Fig. 2B). Nonetheless, there was no change in the circulating MCs percentage (Placebo: 10 ± 1 vs. HAF: 9 ± 2%gated; Fig. 1B). Finally, there was no significant change in the number of PB, splenic, and renal $CD8^+$ T cells and IgM^+-B cells in HAF compared to placebo control rats.

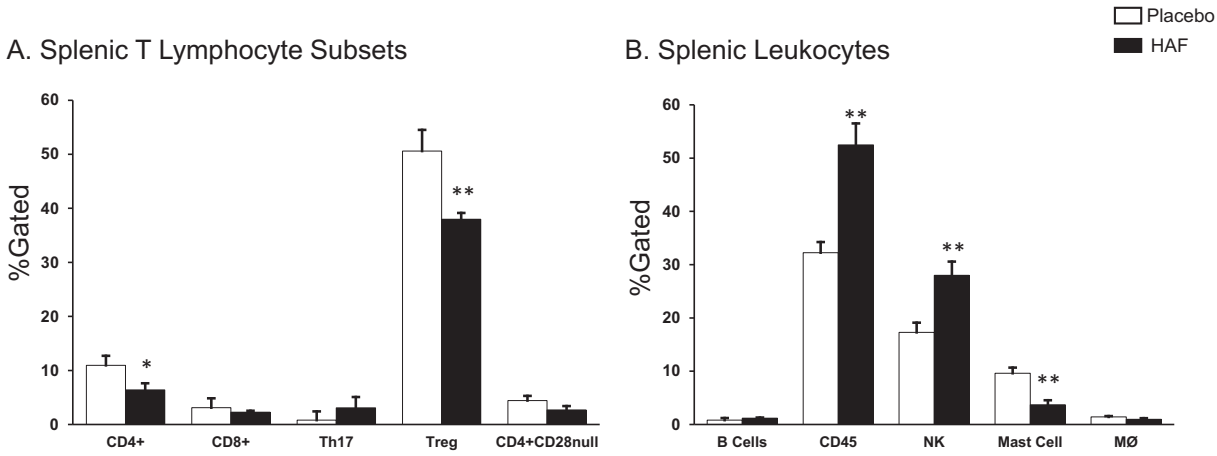

Fig. 2. Splenic T lymphocyte subsets (A) and other splenic leukocytes (B) of placebo and HAF rats. The percentage of splenic CD4+, CD8+, Th17, Treg, CD4+CD28null, IgM+, CD45+, CD161+, mast cell, and macrophage were measured using flow cytometry in Placebo and HAF rats (n = 5–7/group). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, compared to Placebo.

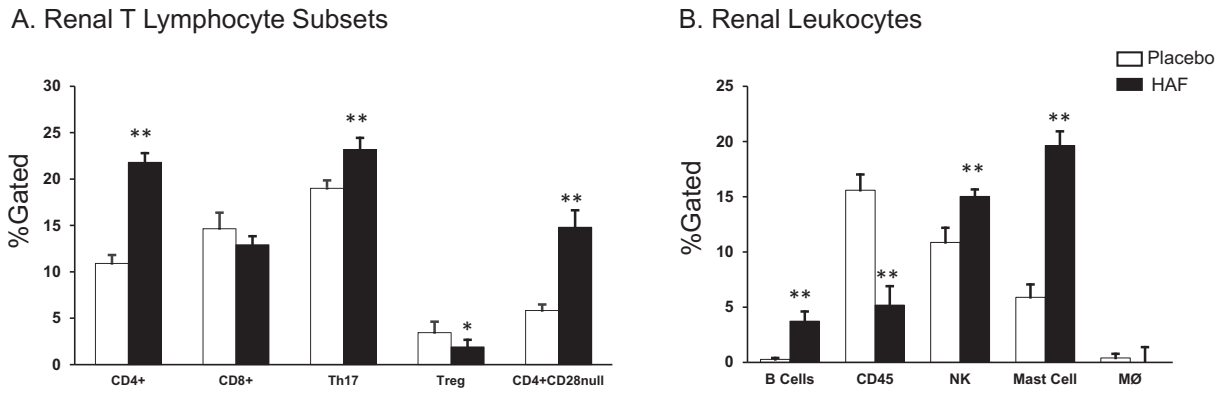

Fig. 3. Renal T lymphocyte subsets (A) and other renal leukocytes (B) of placebo and HAF rats. The percentage of renal CD4+, CD8+, Th17, Treg, CD4+CD28null, IgM+, CD45+, CD161+, mast cell, and macrophage were measured using flow cytometry in Placebo and HAF rats (n = 5–7/group). Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, compared to Placebo.

4. Discussion

The present study characterized, for the first time, the immune cell populations profile in the hyperandrogenemic female rat (HAF), an animal model of PCOS. Result from the current study demonstrated that modest androgen excess in HAF rats modulates cellular immunity by quantitative changes in circulating, splenic, and renal leukocyte populations. Body of evidence support the association of elevated leukocytes as a marker of inflammation with the later development of T2D [30,31], decline in insulin sensitivity [31], correlation between diabetic complication [32], and a higher risk of glucose metabolism disorder [33]. T lymphocytes play a crucial role in mediating inflammation and insulin resistance by secreting pro-inflammatory cytokines in various metabolic organs. To date there are findings that reveal the imbalance of T cell subsets population in patient with T2D; elevated T lymphocytes and pro-inflammatory cytokines in the kidneys [34,35], increased Th17 [36], and decreased Treg cell [37,38].

While, adequate information about Th17 T lymphocytes in animal models of PCOS [23–27] is lacking, the result of this study provides the evidence of expansion of pro-inflammatory Th17 T lymphocyte subset not only in the PB which is comparable to women with PCOS [18] but also in the kidney. Functional significance of Th17 cells in renal inflammation has been provided in a murine model of crescentic glomerulonephritis [39]. Of note, that Th17 T cells mediate the initiation and progression of renal injury by secretion of inflammatory IL-17 [40]. IL-17 recruits leukocytes to the site of inflammation and promotes inflammation by directly causing tissue injury and enhancing secretion of pro-inflammatory cytokines and chemokines by resident cells [41].

Increased $CD4^+CD28^{null}$ T cells frequency in blood and kidney in HAF rats suggest a high risk of immune-mediated cardiovascular disease and renal injury in PCOS as seen in patients with acute coronary syndrome [42–44], chronic kidney disease, atherosclerosis, and CVD [45]. The result of this study is comparable to the report of a significant increase in PB $CD4^+CD28^{null}$ in PCOS when compared with controls [46]. Notable, that $CD4^+CD28^{null}$ T cells show high pro-inflammatory and tissue-damaging properties. They induce renal injury by secretion of soluble mediators and/or by direct cytotoxicity [47]. However, the mechanisms of infiltration of renal T cell and its recruitment processes are still not well characterized and are being investigated by many laboratories.

The next significant T cell subset, Treg cells CD4(+) CD25(+) FOXP3(+), are critical for maintaining self-tolerance [48,49], preventing autoimmunity [50], and reproductive function [51]. Any shortfall in anti-inflammatory Treg number and/or function may impair the immune responses leading to increased inflammation, autoimmune disorders [50], tumor [51], and maternal allo-rejection of fetus [52]. The existing data regarding frequency of circulating anti-inflammatory Treg cells in PCOS patients is controversial. Previously, Wei and colleagues [18] have reported that there was no change in the PB Treg cells in PCOS patients, however Krishna and colleagues observed reduction in PB Treg of PCOS patients [53], while Walecki and colleagues [54] demonstrated that women Treg cells differentiate and proliferate in response to androgen treatment and women Treg cells are more responsive to androgen treatment compared to male T cells, suggesting gender-specific androgen signaling. Despite Treg cell vital role in a number of immune responses and reproduction, data on tissue distribution and frequency of Treg cell in PCOS does not exist due to the limited availability of human tissue and animal models. The result of this study, for the first time, provides 1) novel evidence for effect of androgen on Treg cell frequency in female rat model of PCOS, 2) the imbalance of Treg/Th17 in HAF rats in the PB, spleen, and kidney suggesting alteration of the immunological niche of tissues in HA state, since HAF rats exhibited significantly elevated Treg cells in the blood in contrast to the significant reduction in the spleen and kidney. The result of the present study is in good agreement with Walecki and colleague [54] and the report that shown a higher level of Treg cells in the circulation under inflammatory condition [55].

In this study HAF rats exhibited lower circulating NK cells contrary to their expansion in the kidney. Therefore, further studies are required to evaluate the role of renal NK cells in mediating the inflammation and CVD because it is significantly enhanced in PCOS women with pronounced cardiovascular risk [56]. The role of splenic NK cells requires more investigation as well, since they were significantly elevated in HAF rats. Remarkably, MCs as regulator of inflammatory interactions were lower in the spleen, in contrast to high infiltration in the kidney. This result supports the idea of tissue specific distribution of MCs in PCOS as they are increased in the soft palate in a rat model of PCOS [25] and decreased in ovaries of PCOS and postmenopausal women [57]. These studies collectively suggest that MCs which reside within a variety of tissues/organs may participate in the pathogenesis of PCOS by releasing cytokines/chemokines and selectively inducing T cell recruitment and activation.

Noteworthy, that CD45 is an important regulator factor of B and T cells activation and maturation [58–60] and has been shown that its expression is less prominent on ovarian leukocytes in PCOS women [61]. In the current study, CD45 expression was significantly reduced on leukocytes in PB and kidney, opposite to the elevated expression on the splenic leukocytes of HAF rats. Therefore, suggesting its expression is tissue specific in HA state and PCOS. However, precise role of CD45 in HA remains to be investigated, given the finding in the present study.

Although, macrophage does not appear to play a role in mediating the pro-inflammatory condition in HAF rats, since it was not altered, but this remains to be determined since Lima and colleagues [27] reported that macrophage frequency was altered in SD rat ovaries after

DHT treatment. Taken together these data, the result suggests that macrophage alteration might be tissue specific as well.

4.1. Limitation of the study

The major limitation of the study was lack of prior research studies on the leukocytes profile in PCOS animal models. However, the current study will positively, lay a foundation for understanding the mechanisms in HA and immune dysfunction in PCOS animal model and PCOS patients. In addition, the findings in this study are subject to limitation, additional research is required to understand the underlying risk factors of the observed leukocytes alterations in HAF rats and more studies required to clarify the contribution of insulin resistance in immune dysfunction in PCOS. In spite of a large body of evidence of leukocyte populations' alteration in the T2D and metabolic syndrome, the mechanisms by which leukocytes contribute to the development of insulin resistance and/or T2D remain unidentified. There are additional mechanisms associated with insulin resistance in POCS such as; sex hormones and insulin receptors on the surface of leukocytes [62] which suggest a possible link between insulin sensitivity and leukocytes. However, their investigation are beyond the scope of the current study. Therefore, conceivable roles of immune cells in insulin resistance/T2D will doubtless be topics for potential exploration.

5. Conclusion

In conclusion, this is the first study demonstrating inflammatory leukocytes alterations in the various tissues of HAF rat suggesting a systemic inflammatory phenomenon in PCOS, and confirms that this is not barely an ovarian-localized inflammation. In addition, the data raise the intriguing possibility of hormonal imbalance association with leukocyte expansion and tissue distribution, although a direct explanation for this observation is still to be resolved. Moreover, since T lymphocytes play a crucial role in orchestrating adaptive immune responses [63], they may contribute to increased long-term cardiovascular risk that PCOS women exhibit by producing large amount of pro-inflammatory cytokines [64,65]. Of note, the treatment options for women with PCOS are limited, mainly focusing on reducing insulin resistance. Lack of specific therapy directed at inflammatory mechanisms increases the necessity to study the immune cell activation and immune-mediated inflammatory diseases in PCOS to prevent associated CVD and other organ-specific impairment in the future.

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the author.

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