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FOXP3+ T cells and immune dysregulation in canine pyometra

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ABSTRACT

Canine pyometra is a suppurative uterine infection associated with immune dysregulation. This study investigated the role of regulatory T cells (Tregs) and associated factors in the pus accumulation within the canine uterus. Sixteen client-owned intact bitches, eight diagnosed with pyometra and the other eight healthy animals undergoing elective ovariohysterectomy, were enrolled. Blood samples were collected into ethylenediaminetetraacetic acid (EDTA)-coated tubes for flow cytometry. Tissue samples were obtained after ovariohysterectomy and used to examine localization of interleukin (IL)-2 and IL-2Rα as key regulators of Treg functions. Gene expression was analyzed by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Results of flow cytometry analysis revealed a significant increase in the population of Tregs in the uterine tissue and their corresponding decrease in the peripheral blood. This shift is likely reflective of the recruitment of Tregs from the peripheral blood to the decidua in pyometra. There was a marked upregulation in the expression of IL-2 in the uterine tissue. There was dysregulation in the expression of anti-inflammatory cytokines produced by Tregs (such as IL-10) and pro-inflammatory factors secreted by effector T cells (such as RORγt and IL-17A), which gave a deeper insight into the mechanism underlying the immune dysfunction in canine pyometra. Taken together, these observations elucidate the dynamic changes in Tregs and related factors during canine uterine pyometra, thus providing a new perspective on the equilibrium of the uterine immune microenvironment.

1. Introduction

Canine pyometra is an acute or chronic suppurative uterine infection associated with immune dysregulation in response to pathogenic invasion. The underlying pathogenesis is intricately linked to dysfunction in the immune regulatory network [1–3], as evident from changes in blood parameters. One such effect involves the modulation of lymphocyte activity, which plays critical functions in the development of a systemic inflammatory response [1,4]. The dynamic equilibrium in the subsets of T lymphocytes in the human uterine microenvironment has become a focus of scientific investigations [5,6]. T cells play a role in maintaining the immune/peripheral tolerance, thereby limiting the chances of chronic inflammatory diseases [7] and mediate several local immune functions [8]. While this tolerance function can be directly mediated by regulatory T cells (Tregs) via inhibition of the overactivation of effector immune cells such as $CD4^+$ T cells [9,10], the role of cytokines such as interleukin (IL)-10 and transforming growth factor-beta (TGF-β) in this phenomenon is significant [7]. In the uterus, in particular, the distribution and function of Tregs is very disease-specific. For instance, under the influence of endometrial hyperplasia, there is an abnormal increase in the population of Tregs in the lesion area. This immunosuppressive function may weaken the activity of effector immune cells, consequently restraining the body's inherent ability to fight and eliminate pathogens. This phenomenon may indirectly promote the immune evasion of pathogens [11,12]. The effects of uterine pathologies are also reflected through changes in the peripheral blood Tregs [13,14].

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The transcription factor FOXP3 is specific to Tregs and regulates the expression of CTLA-4, CD25, and other surface molecules, thus forming the molecular basis of Treg immunosuppressive functions [15]. Notably, IL-2 was found to not only play a crucial role in the activation of effector T cells but also promoted the development and functional maintenance of Tregs via the signal transducer and activator of transcription 5 (STAT5) signaling pathway [16]. This bidirectional regulatory feature of the FOXP3/IL-2 signaling axis is considered a hallmark of the immune mechanism underlying the pathogenesis of pyometra in canines.

Canine pyometra is characterized by an immunosuppressive state induced by progesterone along with changes in the characteristics of cytokines in the blood, including upregulation in IL-4 and IL-10 expression [17]. This immunosuppressive state may contribute to changes in the population of Tregs. This observation is supported by the typical immunosuppressive functions of Tregs in autoimmune diseases and tumor microenvironments. To this end, we hypothesized that Tregs in the uterine tissue and peripheral blood undergo change due to pyometra, which is accompanied by changes in molecular transcription factors and cytokines. Therefore, herein we aimed to investigate Treg activity in the uterine tissue and blood of dogs with pyometra and analyze the related expression of *FOXP3*, RAR-related orphan receptor gamma (*RORγt), IL-17A, IL-10*, and TGF-β and the localization of IL-2, IL-2Rα, FOXP3, and IL-17A in the uterine tissue. The knowledge of their behavioral changes will help further characterize the status of the uterine immune microenvironment.

2. Materials and methods

The animal experiment protocol was approved by the Animal Administration and Ethics Committee of Chengdu Normal University and Zhi Pet Animal Hospital (Approval number: CDNU of Canine Pyometra: 03-2023-017C and 20230612 ZP). All experimental procedures were conducted following strict adherence to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (https://arriveguidelines.org).

2.1. Animals and sampling

A total of 16 intact female dogs (mean age 7.21 ± 1.18 years) were enrolled, 8 diagnosed with pyometra and 8 healthy controls in the diestrus period of their cycle, that underwent elective ovariohysterectomy. The diestrus period was confirmed by medical history and vaginal cytology, as described in a previous study [18]. Diagnosis of pyometra was conducted based on medical history, abdominal ultrasonography, and vaginal discharge [19]. Blood samples for flow cytometry analysis were collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Animals were surgically treated by ventral midline ovariohysterectomy, as previously described [4]. Uterine tissue samples were collected from cranial, medial, and caudal portions of uterine horns from healthy controls and canines with pyometra [4]. Collected entire uterine wall samples were subjected to flow cytometry, gene expression, and Western blot analyses. The samples were washed with sterile saline solution, placed into tubes filled with RNAlater solution, and frozen in liquid nitrogen before being stored at −80 °C. The remaining uterine tissue samples were fixed in 10 % buffered formalin at room temperature for 24–48 h and employed for immunohistochemical and immunofluorescence analyses.

2.2. Flow cytometry

Peripheral venous blood samples (excluding hemolysis, coagulation, and chylous samples) were obtained and peripheral blood mononuclear cells isolated using Ficoll-Paque PLUS (GE Healthcare Life Sciences) [20]. The blood was mixed thoroughly with an anticoagulant (EDTA) prior to centrifugation. Once centrifuged, the plasma layer from the sample was carefully harvested by avoiding any contamination of red blood cells. The uterine tissue sample was mechanically minced and filtered through a 40 μm mesh filter to generate a single-cell suspension. Throughout the process, cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 2 % fetal bovine serum (FBS). Red blood cells were lysed using a lysis buffer for 1–2 min to eliminate erythrocytes while minimizing any cell damage caused due to prolonged exposure. Surface staining of cells was conducted by incubation of approximately 10^6 cells in 50 μL of a fluorescence-activated cell sorting (FACS) buffer containing surface marker antibodies such as CD25 (1:200, #39475, Cell Signaling Technology, USA) at 4 °C for 30 min. Cells were then rinsed twice with 500 μL of cold FACS buffer for complete removal of unbound antibodies. Cells were then fixed and permeabilized using a fixation/permeabilization buffer set for 30 min. Cells were subsequently washed and stained with PE (Phycoerythrin) or other labeled anti-FOXP3 (1:200, #12653, Cell Signaling Technology, USA) antibodies in permeabilization buffer at 4 °C for 1 h. Finally, cells were counted and analyzed by flow cytometry. In total, 10,000 white blood cells were counted per sample, and the data obtained were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

2.3. Immunohistochemistry

Tissue samples incubated in a fixative for 24–48 h were washed, embedded in paraffin, and sectioned into 4–5 μm thick slices. These paraffin sections were sequentially immersed in three eco-friendly dewaxing solutions for 10 min each, and then dehydrated by treatment with ethanol for 5 min per step. The samples were finally rinsed with distilled water and subjected to antigen retrieval. In the next step, the sections were washed thrice with phosphate-buffered saline (PBS) for 5 min each and incubated with a 3 % hydrogen peroxide solution in the dark for 25 min. Later, the samples were blocked with 3 % bovine serum albumin (BSA) for 30 min and incubated overnight at 4 °C with primary antibodies specific to IL-2 (1:200, GB11114, Servicebio, China) and IL-2Rα (1:200, GB11612, Servicebio, China). Following incubation, the samples were probed with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:200, GB23303, Servicebio, China) at room temperature for 50 min. Color development was performed by treatment with 3,3′-diaminobenzidine (DAB), and the reaction was terminated by rinsing the samples under tap water. Nuclear staining was performed with hematoxylin. The samples were finally dehydrated with alcohol and xylene, and mounted for observation under a white light microscopy.

Immunohistochemical staining was semi-quantitively evaluated on the basis of staining score and intensity scores [21–23] as follows: Cells with <10 % staining were deemed as negative (−, 1); cells with 10–49 % staining were scored as (+, 2); cells with 50–74 % staining were scored as (++, 3); and cells with 75–100 % staining were scored as (+++, 4). Staining intensity was assessed as follows: 0, negative; 1, weak staining; 2, moderate staining; 3, strong staining.

2.4. Immunofluorescence

After paraffin removal, uterine sections were rehydrated and incubated with a blocking buffer containing 10 % normal rabbit serum at room temperature for 2 h. This step was followed by an blocking step using 1 % BSA in PBS [16]. The sections were eventually incubated overnight at 4 °C with primary antibodies specific for FOXP3 (1:200, GB112325, Servicebio, China) and IL-17A (1:200, GB11110, Servicebio, China). After incubation, the slides were washed thrice with PBS (Servicebio, China, G0002; pH 7.4) on a shaker (Servicebio, China, SYC-Z100), with each wash lasting for 5 min. Then the sections were probed with a goat anti-rabbit IgG conjugated with HRP (1:200, GB23303, Servicebio, China) at room temperature for 50 min. The samples were dried and placed in a dark humidified chamber (Servicebio, China, SIB-20F) and subjected to 4′,6-diamidino-2-phenylindole (DAPI) staining (Servicebio, China, G1012) at designated areas for 10

Table 1
Forward and reverse primer sequences of the genes.

Name	Primer sequence	Product size (bp)
FOXP3	F:5′-GCTGGGAAGATGACCCTGACC-3′ R:5′-TGGAAGAACTCTGGGAATGTGCT-3′	**197**
TGF-β1	F: 5′-ACCAACTACTGCTTCAGCTCCAC-3′ R: 5′-GTGTCCAGGCTCCAAATGTAGG-3′	**158**
IL-17A	F: 5′-AATGAGGACCCTGAGAGATACCC-3′ R:5′-GACTCCCTTCGCAGAACCAG-3′	**143**
GAPDH [26]	F: 5′-GAGATCCCGCCAACATCAAATGG-3′ R: 5′-TACTTCTCATGGTTCACGCCCAT-3′	**181**

min at room temperature in the dark. Following incubation, tissue sections were analyzed under a confocal microscope, and immunofluorescence signal was quantified using ImageJ software. Each slide was analyzed in at least three distinct regions: the luminal epithelium (LE), endometrial glands/cysts (GE/CE), and endometrial stroma (SC).

2.5. Gene expression analysis

In brief, approximately 50 mg tissue samples were treated with 1 mL TRIzol Reagent (15596018, Thermo Fisher Scientific, USA) for total RNA isolation. Isolated RNA was dissolved in 20–40 μL of nuclease-free water according to the pellet size obtained. The purity (A260/A280 > 1.8) and concentration of RNA was measured using Merinton SMA-1000 Spectrophotometer, and the integrity of the samples was determined by 1 % agarose gel electrophoresis. The samples were treated with DNase I (EN0521, Thermo Fisher Scientific, USA), and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (4368814; ThermoFisher Scientific, USA). As per the manufacturer's instructions, 1 μg of RNA was used and the final volume of the reaction was maintained to 200 μL using nuclease-free water. After treatment, samples were stored at −80 °C.

The expression of *FOXP3*, *TGFβ1*, and *IL-17a* genes was determined by amplification of samples using quantitative polymerase chain reaction (qPCR; Rotor-Gene Q, MDx 5Plex HRM, Qiagen, USA) with a SYBR Green I dye-containing kit (4367659, Power SYBR Green PCR Master Mix, Thermo Fisher Scientific, USA). All samples were examined in duplicates, with one of the most stable reference gene in uterine tissue, *GAPDH* serving as a reference gene [24,25]. The qPCR cycle comprised an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 60 s. Melting curve analysis was performed, and the reaction was subjected to a temperature gradient from 62 °C to 99 °C at an increment of 0.5 °C per step. The primers used for gene amplification were designed by Primer Premier 5 and then also checked by Primer BLAST (NCBI). The primer sequences are presented in Table 1.

2.6. Western blot analysis

The inflammatory and immunomodulatory status of the canine uterine tissue was determined by analysis of the expression of proteins such as FOXP3 (1:1000, ab215206, Abcam, UK), RORγt (1:800, ab232516, Abcam, UK), IL-17A (1:1000, ab218013, Abcam, UK), IL-10 (1:1000, #12163, Cell Signaling Technology, USA), and TGF-β (1:1500, ab215715, Abcam, UK) using β-actin (1:1000, #4967, Cell Signaling Technology, USA) as an endogenous protein control. In brief, 60 mg of uterine tissue sample was homogenized in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, China) in the presence of a protease/phosphatase inhibitor cocktail (Beyotime, China) using a high-speed tissue disruptor. Total protein concentrations were determined using a bicinchoninic acid (BCA) assay (Servicebio, China). Later, 30 μg of protein per sample was loaded onto a 10 % sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and the proteins were separated. The separated protein bands were transferred onto polyvinylidene fluoride (PVDF) membranes initially at 80 V for 30 min to partially adhere proteins to the membrane and then at 120 V for 40 min for their complete transfer. Membranes were then blocked with 5 % BSA in TBST for 2 h at 25 °C and incubated with species-specific primary antibodies (16 h, 4 °C). Following incubation, the membranes were washed four times (8-min each) with TBST and then probed with HRP-conjugated secondary antibodies (1:1000) for 1.5 h at room temperature. Protein bands were visualized using a SuperSignal™ West Pico ECL substrate (Thermo Scientific, USA) and quantified by densitometry analysis in ImageJ following normalization to β-actin expression. All experimental procedures were conducted in triplicate biological replicates to ensure statistical robustness.

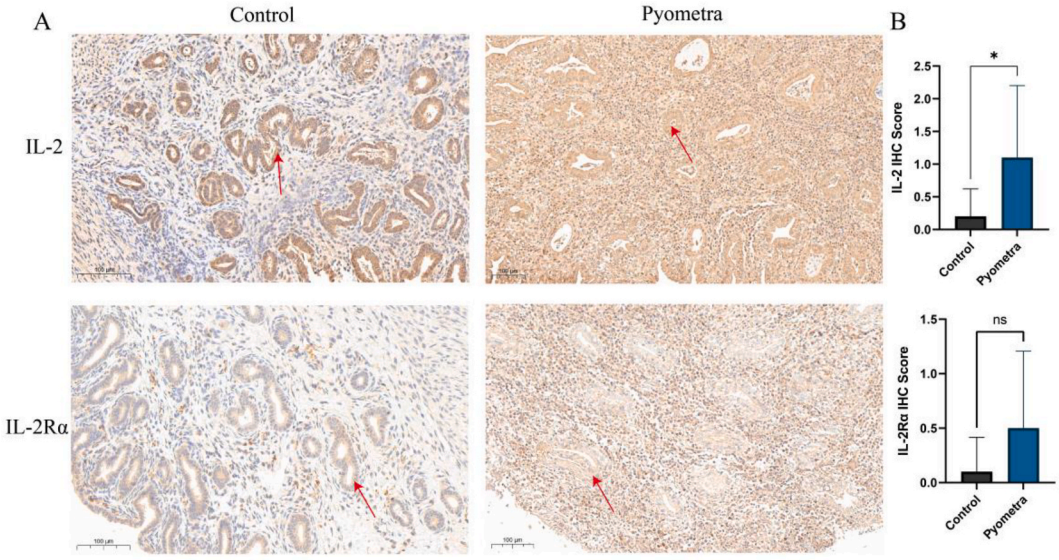
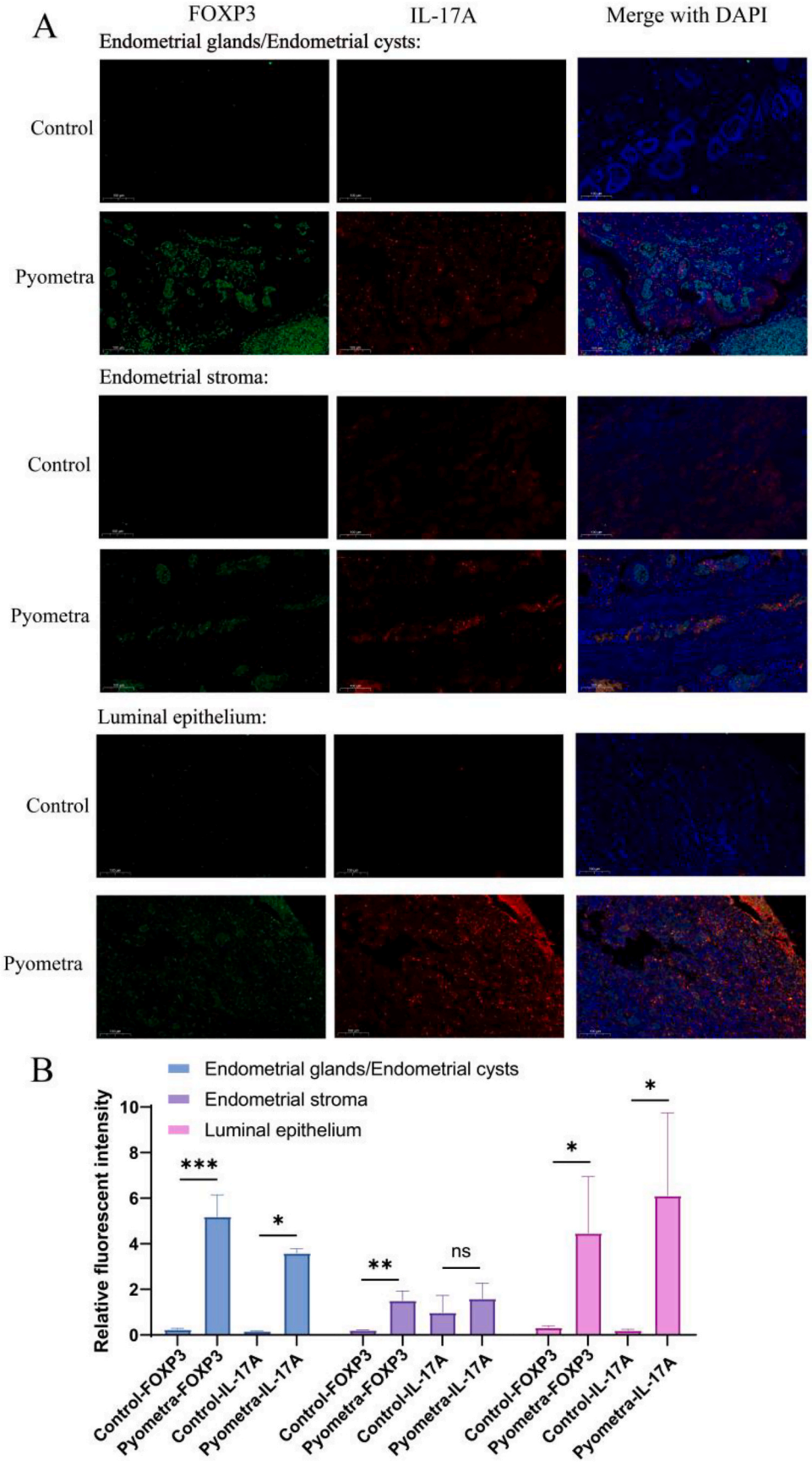


Fig. 1. Immunohistochemical staining analysis of uterine tissue. (A) The expression of IL-2 and IL-2Rα in healthy controls and pyometra affected uterine tissue samples was examined using immunohistochemistry (IHC) at x20 (100 μm) magnification with Aperio ScanScopev12.4.6 digital scanning analysis of slides. The red arrow indicates the uterine glands; (B) IHC Score (ns = no significant; *$p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



(caption on next page)

Fig. 2. Uterine immunofluorescence. (A) Immunofluorescent expression of FOXP3 and IL-17A proteins in the uterine tissue of healthy dogs and dogs with pyometra, with representative images showing FOXP3 (green) and IL-17A (red) immunoreactivity, while nuclei were stained with DAPI (blue) (Scale bars = 100 μm); (B) Relative fluorescence intensity. (ns = no significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

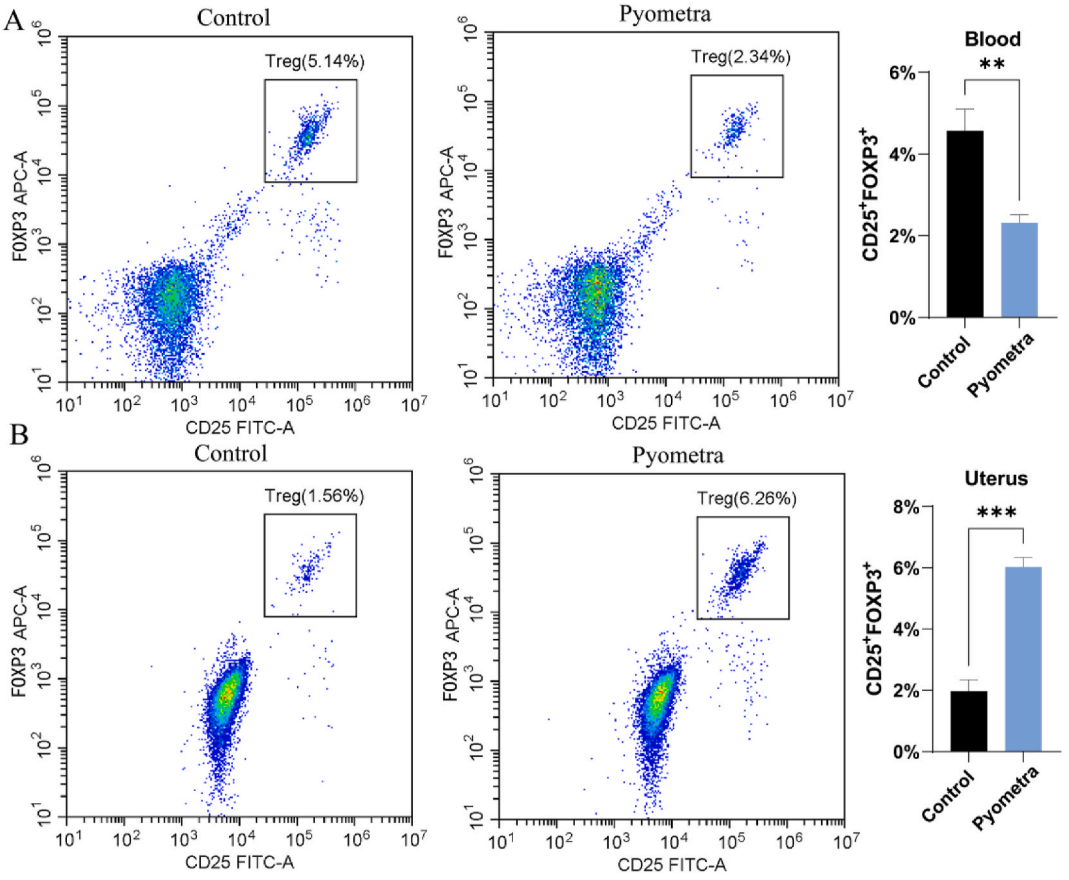


Fig. 3. Flow cytometry analysis. (A) Proportion of Treg in canine peripheral blood; (B) Treg ratio in canine uterine tissue (**$p < 0.01$; ***$p < 0.001$). Among those, cells that expressed both CD25 and FOXP3 were defined as Treg suppressor cells.

2.7. Statistical analysis

Experimental data were independently replicated at least thrice and are expressed as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism 9.5.0 software. All data were initially tested for normal distribution using the Shapiro-Wilk test, and homogeneity of variances was assessed using the Brown-Forsythe test. Between group comparisons were performed using a two-sample *t*-test where a value of $p < 0.05$ indicated significant difference.

3. Results

3.1. Immunohistochemistry for IL-2 and IL-2Rα expression in the canine uterus

In comparison with control animals, animals with pyometra showed elevated expression of both IL-2 and IL-2Rα in their uterine tissue. In particular, a strong positive staining for IL-2 was detected in the epithelial and stromal cells of the uterine glands ($p < 0.05$) (Fig. 1).

3.2. Immunofluorescence for FOXP3 and IL-17A expression in the canine uterus

The expression of FOXP3 and IL-17A in the canine uterus was determined by double-labeling immunofluorescence. There was a significant upregulation in the expression of both FOXP3 and IL-17A in the uterine tissue of the dogs affected with pyometra as compared to the uterine tissue of healthy controls ($p < 0.05$). Further analysis revealed a marked increase in fluorescence signals for FOXP3 and IL-17A in the endometrial glands/endometrial cysts and luminal epithelium ($p < 0.05$; Fig. 2).

3.3. Quantification of Tregs in the canine peripheral blood and uterus

Flow cytometry analysis was employed to detect the number of Tregs in the peripheral blood and uterine tissue of dogs (Fig. 3). In comparison with healthy canines, those with pyometra showed a decrease in Treg population in the peripheral blood ($p < 0.01$) with a simultaneous increase in their cell count in the uterine tissue ($p < 0.001$).

3.4. Immune response of the canine uterus

Western blotting results confirmed the significant increase in the expression levels of RORγt, IL-17A, and IL-10 ($p < 0.05$; Fig. 4) in the uterine tissue of the canines with pyometra. The expression of FOXP3 and TGF-β was consistent between the two groups. RT - qPCR analysis revealed that *IL-17A* mRNA expression was significantly upregulated in dogs with pyometra ($p < 0.001$; Fig. 4).

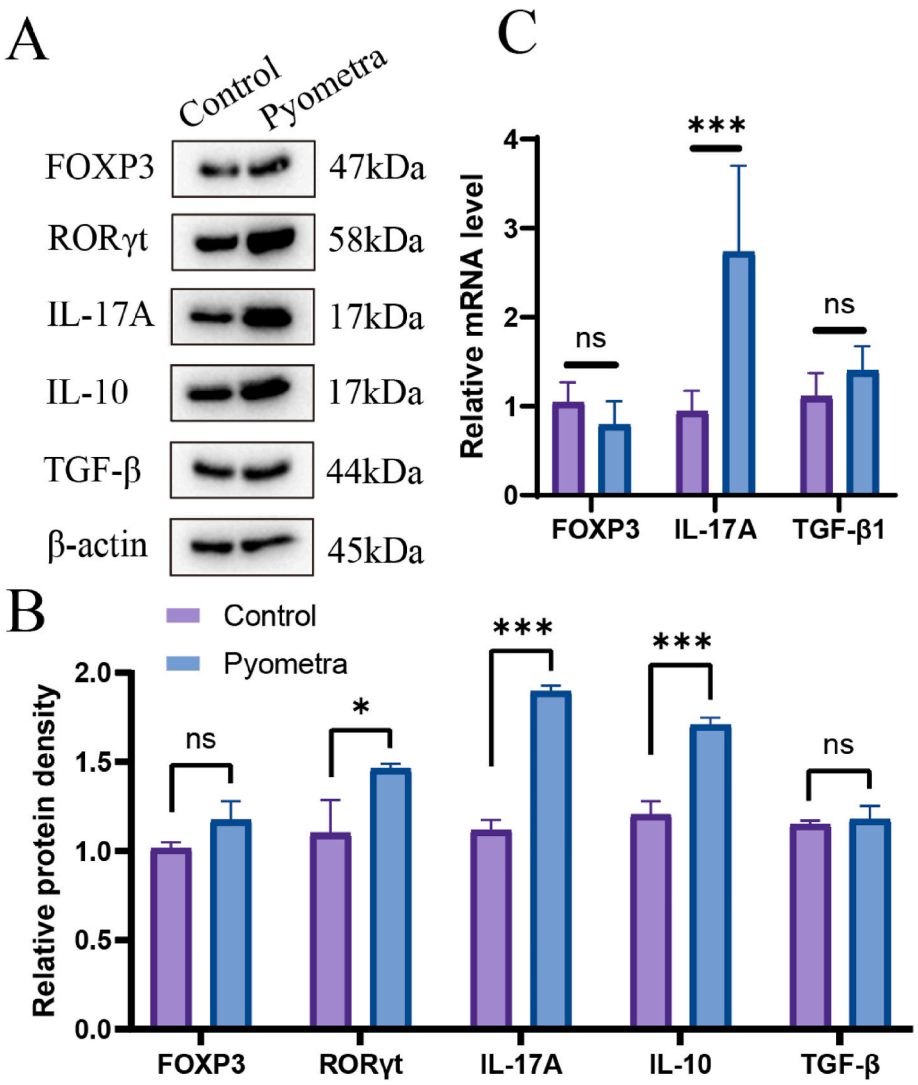


Fig. 4. Uterine tissue expressions of FOXP3, RORγt, IL-17A, IL-10, and TGF-β immune response. (A) Western blot detection of FOXP3, RORγt, IL-17A, IL-10, TGF-β protein expression; (B) FOXP3, RORγt, IL-17A, IL-10, TGF-β relative protein density. (C) RT- qPCR analysis. (ns = no significant; *$p < 0.05$; ***$p < 0.001$).

4. Discussion

While the critical functions of Tregs in immune regulation are well established, there is only limited evidence on the mechanism underlying the immune dysregulation and disease progression specific to canine pyometra. Our findings highlight the significant enhancement in the population of Tregs within the uterine tissue along with a decrease in their cell count in the peripheral blood of canines with pyometra. Previous studies have shown that Tregs typically accumulate in the decidua during the reproductive cycle or under disease conditions, which may potentially explain the observed reduction in the peripheral blood Tregs and their increased presence in the uterine tissue in our study [27].

As a pivotal cytokine, IL-2 regulates Treg homeostasis [28]. As Tregs are highly sensitive to low concentrations of IL-2, these cells can effectively detect and respond to IL-2 signals within the immune microenvironment and maintain their immunosuppressive functions [29]. IL-2 binds to IL-2Rα expressed on the surface of Tregs to activate downstream signaling pathways such as STAT5, which eventually promotes the survival and functional stability of Tregs [29]. Our observations show that the expression of IL-2 was significantly upregulated in the uterine tissue of canines with pyogenic conditions and is plausibly reflective of the dysregulation in the uterine immune system under these conditions.

Tregs exert critical immunomodulatory functions in the physiological and pathological processes of the uterus via various mechanisms. For instance, Tregs maintain the homeostasis of the uterine immune microenvironment by secreting anti-inflammatory cytokines such as IL-10 and TGF-β and modulating the behavior of effector T cells, including Th17 cells [30]. In the present study, the characterization of Th17 cells ($CD4^+$ IL-17+ RORγt+) was not conducted. However, it will be interesting to assess the expression profile of T cell-related pro-inflammatory factors, given that the γδT cell population is increased in canines with pyometra [8]. The expression of IL-17A, a Th17-produced key pro-inflammatory cytokine, is markedly increased in patients with endometriosis [31]. Eosinophils are thought to enhance the proliferation and expansion of Tregs via direct contact in a TGF-β–dependent manner [32]. Our observations confirmed the significantly increase in levels of IL-17A, IL-10, and RORγt in the uterine tissue, which is indicative of the disruption in the immune balance during the pathological state of pyometra.

5. Conclusion

This study investigated the alterations in the expression and functions of Tregs and associated factors in canines with pyometra to elucidate the potential mechanism underlying immune dysregulation. There was a significant increase in Treg population within the uterine

tissue of canines with pyometra, which was consistent with a corresponding decrease in their cell population in the peripheral blood. The expression of key cytokines and transcription factors involved in immune responses, including IL-2, IL-17A, IL-10, and RORγt, was upregulated in animals with pyometra. These observations are suggestive of the disruption in the Th17/Treg equilibrium, which contributed to the shift in the local immune microenvironment toward a pro-inflammatory state.

CRediT authorship contribution statement

Zhiqiang Li: Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Wei Zhao:** Software, Methodology. **Xin Deng:** Writing – original draft, Software, Methodology. **Murat Onur Yazlık:** Writing – review & editing. **Hüseyin Özkan:** Writing – review & editing, Methodology. **Shiyi Liu:** Methodology. **Ling Mei:** Methodology. **Shangfeng Li:** Resources. **Jiasui Zhan:** Writing – review & editing. **Binhong Hu:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Formal analysis, Conceptualization.

Ethics approval and consent to participate

The animal study was approved by the Animal Administration and Ethics Committee of Chengdu Normal University and Zhi Pet Animal Hospital (Approval number: CDNU of Canine Pyometra: 03-2023-017C and 20230612 ZP). The collection of canine uterine samples is conducted with the written consent of the dog owners, and all experiments adhere strictly to the Animal Ethical Procedures and Guidelines of the People's Republic of China to ensure humane treatment throughout the process. All procedures in this study were conducted in strict adherence to the ARRIVE guidelines (https://arriveguidelines.org).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. Datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2025.117445.

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