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γδ T lymphocytes are recruited into the inflamed uterus of bitches suffering from pyometra

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ABSTRACT

Very little is known about the occurrence of immune system cells in the canine uterus. The aim of this study was to generate information about lymphocyte subsets that are present in the healthy canine uterus and that are recruited under inflammatory conditions caused by pyometra. Using immunohistochemistry and flow cytometry, a significant influx of γδ T lymphocytes was found in pyometra samples mainly due to recruitment of $\gamma\delta^+/CD8^-$ T lymphocytes. The relative expression of genes encoding selected cytokines/chemokines was evaluated in samples from healthy and pyometra-affected uteri. Expression of pro-inflammatory cytokines (including IL-1β, TNF-α, IL-8, IL-17 and IFN-γ) and chemokines (including CXCL10, CCL4 and CCL5) was upregulated in pyometra samples confirming the presence of inflammation. In contrast, the expression of the homeostatic chemokine CCL25 and of the anti-inflammatory cytokine IL-10 was downregulated and unchanged, respectively.

Introduction

Pyometra is one of the most common and serious diseases in bitches that affects mainly older nulliparous bitches in the luteal stage of the oestrous cycle and may or may not be associated with neutrophilia. Even though the aetiopathology of pyometra has been studied for decades, it is still not yet fully understood (Dow, 1959; Fransson and Ragle, 2003; Kida et al., 2010) but impaired immune function, including reduced activity of lymphocytes, has been reported (Faldyna et al., 2001a). Suppression of lymphocyte activity returned to normal within 7 days after surgical removal of the uterus (Bartoskova et al., 2007).

Undoubtedly, local uterine immunity (or lack thereof) has a part in the development of pyometra. The local immune system of the uterus is very specific due to the fact that, besides the necessity to protect against various kinds of infections, it is under the influence of sex hormones and has to interact with allogeneic spermatozoa and the immunologically distinct fetus to enable successful reproduction. There is a large amount of information concerning both innate and adaptive immunity in the female genital tract (Wira et al., 2005). In contrast, there little is known about the prevalence of immune cells in the canine uterus compared to the situation in cows, sows, or mares (Cobb and Watson, 1995; Kaeoket et al., 2001; Arrighi et al., 2007; Noronha et al., 2012), although Groppetti et al. (2010) brought general insight into cytological changes taking place in canine uteri throughout the oestrous cycle and also under pathological conditions such as pyometra.

The aim of the current study was to generate information about the lymphocyte subsets that are present in healthy canine uteri and that are recruited into the uterus under inflammatory conditions caused by pyometra. We found that, as part of the incoming leukocytes, an increased percentage of γδ T lymphocytes can be detected in the uteri of bitches suffering from pyometra. More interestingly, the percentage of γδ T lymphocytes not expressing CD8 was the cause of the increased percentage of the total number of γδ T lymphocytes.

Materials and methods

Animals and sample collection

Samples of hysterectomized uteri were obtained from bitches of various breeds that were either surgically treated for pyometra ($n = 7$) or underwent elective ovariohysterectomy (referred to as 'healthy'; $n = 5$) as requested by their owners at the Department of Reproduction, Clinic of Dog and Cat Diseases of the University of Veterinary and Pharmaceutical Sciences, Brno. All samples were used with the expressed consent of the owners. The bitches with pyometra and the healthy animals were 8.14 ± 3.29 and 6.20 ± 3.96 years old, respectively.

All animals were in the luteal phase of the oestrous cycle, as confirmed by the presence of corpora lutea in the ovaries. The average time from the end of heat was 6.14 ± 2.73 weeks in bitches with pyometra and 9.20 ± 0.84 weeks in healthy animals. In pyometra cases, *Escherichia coli* was shown to be responsible of the bacterial contamination of the uterine lumen.

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Table 1
List of monoclonal antibodies used.

Antibody	Expressed on	Clone	Isotype	Manufacturer
CD3	T lymphocytes	CA17.2A12	IgG1	P.F. Moore[b]
CD4	T helper cells	CA13.1E4	IgG1	P.F. Moore
CD8	T cytotoxic cells	YCATE 55.9[a]	IgG1	Serotec[c]
CD21	B lymphocytes	CA2.1D6	IgG1	P.F. Moore
CD45	All leukocytes	CA12.10C12	IgG1	P.F. Moore
γδ-TCR	γδ T-lymphocytes	CA20.8H1	IgG2a	P.F. Moore
CD172α	Myeloid cells	DH59B	IgG1	VMRD[d]

[a] Directly conjugated with RPE.
[b] P.F. Moore, School of Veterinary Medicine, University of California, Davis, CA.
[c] AbD Serotec.
[d] Veterinary Medical Research & Development.

Table 2
List of primers used.

Gene	Primers
IL1-β	F: CCCTGGAAATGTGAAGTGCTGCTGCC R: TGCAACTGGATGCCCTCATCTACCAG
TNF-α	F: TTCTCCTTCCTCCTCGTCGCAGGG R: TGGGGGCCGATCACTCCAAAGTG
IFN-γ	F: TTGCGTGATTTTGTGTTCTTCTGGCTGT R: ACGAAAAGAGACCCACCGTCCGATACA
IL-17	F: TGACTCTGGTGACAACTTCATCCATGTTCC R: CCACCAGGCTCAGAAGCAGTAGCA
IL-8	F: CCAAGCTGGCTGTTGCTCTCTTGGC R: CAGCTTCACAGAGAGCTGCAGAAAGGACA
CXCL10	F: ACCTCTCTCTAGAACTATACGCTGTACCTGTATCA R: TGTGGCAATGATCTCAACATGTGGACACG
CCL3	F: TGTTGCCCAGCACCATGGAGGTC R: CAGCACCAAAAGTGGAAGAGCAGGTC
CCL4	F: GCGCTCTCAGCACCAATGGGTTCAG R: TACCACAGCTGGCTGGGAGCAGA
CCL5	F: GCCTCTGCCTCCCCATATGCCTCAG R: GACGACTGCTGGCATGGAGCACT
IL-10	F: TGCATGGCTCAGCACTGCTCTGTTG R: AGTGGGTGCAGTCGTCCTCAAGTAGG
CCL25	F: ACACTCAAGGTGTCTCTGAGGACTGCTG R: CTCACTTCCTGGCGCTGGTAGCC
GUSB[a]	F: AGACGCTTCCAA/GTACCCC R: AGGTGTGGTGTAGAGGAGCAC
HPRT	F: CGAAGTGTTGGCTATAAACCTGACTTTGTTGG R: TCAAGGGCATATCCTACAACAAACTTGTCTGGA
RPS19	F: CTCTCGCGAGCTCTCGCACCTCT R: TAACTCCAGGCATCGTGCGGCCTC

[a] Schlotter et al. (2009).

Immunostaining and flow cytometric analysis

Samples of uterine tissue were collected immediately after hysterectomy into RPMI 1640 medium (Sigma–Aldrich). Lymphocytes were isolated by incubating uterine pieces in Hanks' Balanced Salt solution containing collagenase type IV (50 U/mL, Sigma–Aldrich) for 90 min at 37 °C with stirring. Cells were then purified by gradient centrifugation (Histopaque 1.077, Sigma–Aldrich). Thereafter, isolated cells were washed twice in washing and staining buffer (WSB) (phosphate buffered saline [PBS] containing 0.2% gelatin from cold water fish skin, 0.1% sodium azide and 0.05 mM EDTA, all reagents from Sigma–Aldrich) and resuspended in WSB supplemented with 10% non-immune heat-inactivated goat serum to the density of 5×10^6/mL.

Cell suspensions were stained indirectly. A volume of 50 μL of cell suspension was incubated with a primary mouse monoclonal antibody (Table 1) at room temperature for 15 min. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin fraction (Southern Biotechnology Association) was added as the secondary antibody for 20 min at 4 °C. After another washing, the cells were resuspended in WSB. Samples stained with the secondary antibody only were used as controls. In the case of double staining of CD8 with γδ-T cell receptor (γδ-TCR), a modified protocol was used. Cell surfaces were indirectly stained with anti-γδ-TCR antibody and FITC-conjugated secondary antibody as described above. During the last washing step, 10% heat-inactivated mouse serum was added to WSB to block free binding sites on the secondary antibody. Then, R-phycoerythrin-conjugated antibody against CD8 was added to cell culture for 20 min (Faldyna et al., 2003). After another washing, propidium iodide was used to stain DNA in dead and damaged cells and to exclude such events from the analysis.

Data were acquired on a FACSCalibur flow cytometer (Becton–Dickinson) operated by the CELLQuest software (Becton–Dickinson). In each sample, 10,000–50,000 events were acquired. The PC-lysys software was used for data analysis. Gating of lymphocytes was based on forward and side scatter properties of cells. The common leukocyte antigen (CD45) and common myeloid antigen (CD172α) expression were used for the 'lymphogate' setup and lymphocyte purity determination (Faldyna et al., 2001b). Results obtained for the other surface markers were recalculated to 100% of $CD45^+$ and $CD172\alpha^-$ cells in the 'lymphogate'.

Immunohistochemistry

Uteri were cut into small pieces (1 cm^3) and rapidly frozen using N-heptane cooled with liquid nitrogen. Sections (5 μm) were cut using a routine cryo-microtome (Leica CM 1900) at −15 °C. Dry sections were fixed in ice-cold acetone for 3 min and microscopic slides were stored at −20 °C until used. Untreated cryo-sections were incubated for 5 min with Peroxidase-Blocking Reagent (Dako) for inhibition of endogenous peroxidase activity. After washing (3 × 5 min, TRIS), sections were incubated for 5 min with Protein Block reagent (Dako) to block non-specific background staining.

Immunohistochemical staining was performed using a two-step visualization high sensitivity peroxidase system. First, sections were incubated for 2 h with mouse monoclonal anti-dog antibodies (Table 1) in a humid chamber. Neutrophils were detected using rabbit polyclonal antibody (Dako) prepared against human lactoferrin (Sinkora et al., 2007). Antibodies were diluted in TRIS (dilutions 1:10 and 1:50). Second, after washing (3 × 5 min, TRIS), sections were incubated with the EnVision System Kit (Dako) for 1 h and washed again (3 × 5 min, TRIS). All previous steps were performed at room temperature. Sections were then incubated with the chromogenic substrate system diaminobenzidine (DAB) at 37 °C temperature for 5 min, counterstained with Harris haematoxylin and cover-slipped.

Quantitative real-time PCR detection of cytokines/chemokines

Samples of uteri from 12 bitches taken immediately after surgery were stabilized with RNAlater (Qiagen) and stored at −20 °C. Tissue samples were then homogenized on MagnaLyser (Roche) with 2.3 mm zirconia/silica beads (BioSpec Products) and lysed in 1 mL of TRI Reagent RT (Molecular Research Center). Total RNA obtained from the RNA phase after 4-Bromoanisole treatment was purified according to the manufacturer's instruction using the RNeasy Kit (Qiagen). RNA concentration was measured spectrophotometrically and RNA integrity was checked by agarose gel electrophoresis.

RNA was reversely transcribed using M-MLV reverse transcriptase (200 U) (Invitrogen) and oligo-dT primers at 37 °C for 1.5 h. cDNA was stored at – 20 °C until used. Real-time PCR was performed with the LightCycler 480 (Roche) using QuantiTect SYBR Green PCR Kit (Qiagen). Primers for 11 chemokines/cytokines and three candidate reference genes (*GUSB*, *beta-glucuronidase*; *HPRT*, *hypoxanthine–guanine phosphoribosyltransferase*; *RPS19*, *ribosomal protein S19*) (Table 2) were used. Primer design was performed using NCBI primer designing tool.[1] Each run included a no-template control to test the assay reagents for contamination. Using GeNorm software (Vandesompele et al., 2002), *GUSB* was selected as the most stable gene among the candidates for a reference gene in our experiment. The relative expression of a gene of interest was calculated as a ratio to *GUSB* using the following formula: $[1/(2^{CtGOI})]/[1/(2^{CtGUSB})]$ (Zelnickova et al., 2008).

Data analysis

For comparison of result from affected and healthy bitches, the Mann–Whitney *U* test was used. $P < 0.05$ was considered statistically significant. All calculations were performed with GraphPad Prism v.3.03 software.

Results

Flow cytometry

Results obtained by flow cytometry are shown in Fig. 1. The percentages of all T ($CD3^+$) and all B ($CD21^+$) lymphocytes did not differ significantly between healthy control and pyometra-affected uteri. Similarly, the percentages of Th (T helper, $CD4^+$) and Tc (T cytotoxic, $CD8^+$) lymphocytes did not show statistically significant

[1] See: http://www.ncbi.nlm.nih.gov/tools/primer-blast/.