Comparison of the effect of lipopolysaccharide on tumor necrosis factor α (TNF-α) secretion and TNF and TNFR1 mRNA levels in feline endometrium throughout the estrous cycle during pyometra and after medroxyprogesterone acetate treatment

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Abstract. Endotoxins released by Gram-negative bacteria are potent stimulators of tumor necrosis factor α (TNF-α) production. The objectives of this study were to evaluate plasma levels of TNF-α, TNF-α secretion, and mRNA levels of TNF and TNF-α receptor type 1 (TNFR1) following exposure to lipopolysaccharide (LPS). For this, we used cultured endometrial cells or organ cultures, throughout the estrous cycle, after hormone treatment with medroxyprogesterone acetate (MPA), and during pyometra. Plasma TNF-α concentrations were increased in animals at estrus (P < 0.05) compared to other groups. In the LPS-challenged endometrium, secretion of TNF-α by tissues collected during estrus increased (P < 0.001) compared to that of other groups. LPS, alone or combined with TNF-α, upregulated TNF gene expression in the feline endometrium at diestrus (P < 0.001 for both treatments), in queens treated short-term with MPA (P < 0.01 and P < 0.05, respectively) and in queens treated long-term with MPA (P < 0.01 and P < 0.001, respectively). During pyometra, TNF and TNFR1 mRNA were increased only after tissues were challenged with TNF-α and LPS (P < 0.001 and P < 0.01, respectively). When cultured endometrial cells were challenged with LPS, the concentration of TNF-α increased only in epithelial cells after 4 h and 12 h (P < 0.05 and P < 0.01, respectively). Since LPS did not affect stromal cells, but TNF-α increased its own transcript after 2 h (P < 0.01), 4 h (P < 0.05) and 12 h (P < 0.001), we assume that stromal cells are not directly involved in pathogen recognition, as was the case for epithelial cells.

Key words: Feline endometrium, Lipopolysaccharide (LPS), Medroxyprogesterone acetate (MPA) treatment, Pyometra, Tumor necrosis factor α (TNF-α)

The endometrium, consisting of epithelial, stromal, endothelial, smooth muscle, and immune cells, is the first line of defense against invading pathogens. A single layer of columnar epithelium is in constant contact with the uterine lumen, which may be intermittently contaminated by opportunistic bacteria or pathogens. Epithelial cells are able to recognize pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), which include the Toll-Like Receptor family (TLR) [1]. Among nine types of feline TLRs (TLR 1-9) [2, 3], TLR2/4 are the best characterized, particularly with regard to their role in pathogen recognition. We previously confirmed the presence of both receptor types at the gene and protein level in feline endometrial epithelial and stromal cells [4]. In dogs, engagement of TLRs initiates a signaling cascade, resulting in altered expression of genes such as prostaglandin-endoperoxide synthase 2 (PTGS2), prostaglandin-endoperoxide synthase 2 (PTGS2), PGE2-synthase (PGES), interleukin (IL)-1β, and tumor necrosis factor alpha (TNF) [5]. In cats, lipopolysaccharide (LPS), which is the main ligand for TLR4, is responsible for upregulation of PTGS2, PGES, and prostaglandin F2α synthase (PGFS) mRNA in the cultured endometrial cells, and also for increasing prostaglandin secretion [6]. In an earlier study, we showed that treatment of cultured endometrial cells with LPS results in abundant TNF-α secretion; however, this effect was specific to epithelial cells (and not stromal cells) [7]. TNF-α is a multifunctional proinflammatory cytokine that plays a role in the activation of immune cells such as macrophages, granulocytes, and cytotoxic T cells, and in the maturation of dendritic cells [8]. Besides its immunomodulatory function, TNF-α is involved in the control of cell differentiation, tissue renewal, and restructuring [9]. Although TNF-α is predominantly secreted by endotoxin-stimulated monocytes/macrophages [10], it can be synthesized by many different tissues and cell types, including feline endometrium, as recently shown [7]. Generally, concentrations of TNF-α correlate with the severity of illness and outcome [11]. Moreover, 10-fold higher plasma concentrations of TNF-α were observed in dogs with pyometra compared to healthy controls [12]. Pyometra, with the clinical manifestation of pus accumulation in the uterine lumen, is a common reproductive disorder in bitches but is observed less frequently in queens [13, 14]. Hormonal imbalances together with bacterial infections contribute to the development of pyometra. The development of this condition is observed during diestrus or the luteal-like phase and can be a result of treatment with megestrol or medroxyprogesterone acetate (used...
Materials and Method

Animals

All procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (No. 60/2010/DTN). Adult domestic queens (total n = 45, ranging in age from 7 months to 11 years, with an average age of 2.67 years) were used in this study. Based on circulating concentrations of progesterone in peripheral blood and macroscopic observations of ovaries [16], 30 animals were assigned to appropriate estrous cycle stage groups (anestrus, estrus, mid and late diestrus). Animals with a smooth ovarian surface and a basal progesterone (P4) level > 1 ng/ml were classified as being in anestrus phase. Uterine samples from queens in the anestrus phase were collected after breeding season. Estrus phase (day 1–7) was characterized by the presence of ovarian follicles ≥ 2 mm in diameter. Queens in estrus phase were housed individually or in pairs, with or without contact with an intact male. No pharmacological treatment was performed to provoke ovulation in the animals. Queens with the presence of a reddish corpus luteum (CL) on the ovaries and a mean P4 concentration above 20 ng/ml were classified as being in mid diestrus phase (day 15–20), whereas animals with a pale CL and a level of progesterone < 5 ng/ml were assigned to late diestrus (day 30–35). After interviewing owners, 10 additional cats were assigned equally to the following groups: (i) acute MPA-treated group, in which animals had previously been orally treated with MPA (Promon Vet®, Pfizer Animal Health, Louvain-la-Neuve, France; 5 mg/animal/week) for one month to prevent estrus behavior, or (ii) long-term MPA treated group (5 mg/animal/week), in which animals were treated for four to twelve months. The mean serum level of P4 in the MPA-treated group was > 10 ng/ml. No pharmacological treatment was performed to provoke ovulation in the animals. The last five queens were classified in the pyometra group. These animals were not treated with MPA and had a developing corpora lutea (CL), damage to the uterine tissue, and a fluid-filled uterus. The mean plasma concentration of P4 in the pyometra group ranged from 8 to 14 ng/ml. All the animals were ovariolyhysterectomized at the owners’ request and consent, and cats were anesthetized with ketamine (20 mg/kg) and acepromazine (0.5 mg/kg).

Tissue and blood collection

From all queens, 1–1.5 ml of blood was collected into EDTA-containing tubes for TNF-α analyses, immediately before surgery. Plasma was separated by centrifugation of whole blood at 1500 × g for 10 min, and stored at −20°C. Immediately after ovariolyhysterectomy (OVH), uteri were rinsed with sterile saline to remove blood contamination, placed into fresh saline at 4°C, and transported on ice to the laboratory. Both horns of each uterus (n = 35) were slit longitudinally and the endometrium was separated from the myometrium using a binocular microscope (Olympus SZX7, Tokyo, Japan).

Ex vivo organ cultures of endometrium

To evaluate the effect of LPS and TNF-α on the endometrium and TNF and TNFR1 gene expression, organ cultures of endometrium tissue were generated from each uterus (sixteen explants from each uterus, n = 35). Endometrial fragments were prepared as previously described [4, 6]. Using doses established in that study, cultured endometrial tissues were stimulated with 1 ng/ml human TNF-α (Sigma Aldrich, St. Louis, MO, USA), 50 ng/ml LPS purified from E. coli O55:B5 (Sigma Aldrich), and TNF-α with LPS (at the same concentrations). As a control, non-stimulated explants (n = 5 for each tested group) were treated with the same conditions as the treated cultures. After stimulation for 4 h, explants were placed into cryotubes filled with 1 ml of RNAlater (Ambion Biotechnologie, Wiesbaden, Germany). The samples were stored overnight at 4°C; RNAlater was then removed, and the tissues were stored at −80°C until RNA isolation. The supernatants recovered from the endometrial organ cultures were collected and used for measurement of TNF-α concentrations.

Isolation and culture of feline endometrial cells

Epithelial cells from cats at estrus (n = 5), and stromal cells from cats at diestrus (n = 5) were isolated from the endometrium according to a published protocol [6, 7]. After reaching 85% confluency, cells were stimulated at doses reported in a previous study [4] as follows: (i) 10 μl of sterile PBS served as a control, (ii) TNF-α (Sigma Aldrich) at 1 ng/ml, (iii) LPS purified from E. coli OS5:B5 (Sigma Aldrich) at 50 ng/ml, and (iv) TNF-α together with LPS at the same concentrations. After 2, 4, or 12 h of incubation, the supernatant was removed and the cells were suspended in 1 ml of TRIzol Reagent (Gibco-BRL Life Technologies, Karlsruhe, Germany) and stored at −80°C until RNA isolation.

TNF-α ELISA

Concentrations of TNF-α in plasma samples and culture media
were determined after incubating with LPS for 4 h, using an enzyme immunoassay TNF-α EIA kit (feline TNF-α DuoSet® ELISA DEVELOPMENT SYSTEM, R&D Systems, Minneapolis, USA; DY1814) according to the manufacturer’s instructions. The standard curve ranged between 15.62 pg/ml and 1000 pg/ml. The intra- and inter-assay coefficients of variation were 7.8% and 12.5%, respectively. Each sample was tested in duplicate. The cross-species reactivity of TNF-α was 34.1% for canine TNF-α, 1.4% for equine TNF-α, 7.5% human TNF-α, and 11.2% for rhesus macaque.

RNA isolation and reverse transcription

TRI-reagent® (Sigma Aldrich) was used to isolate total RNA according to the method of total RNA isolation, using a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture, described by Chomczynski and Sacchi [17]. RNA concentrations ranged from 200 to 300 ng/µl and were quantitated using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA); A260/A280 ratios ranged from 1.95 to 2.04. DNase treatment was performed using Amplification Grade DNase I (Sigma Aldrich) according to the manufacturer’s protocol. Complementary DNA (cDNA) synthesis was performed using the ImProm-II™ Reverse Transcription System (Promega, Dübendorf, Switzerland) according to the manufacturer’s instructions.

Real-Time PCR

All real-time PCR reactions were carried out in duplicate on a Via7 machine (Applied Biosystems, Carlsbad, California, USA) as previously described [16]. The reaction mix was set up as follows: 5 µl GoTaq Green Master Mix (Promega), 1 µl (0.5 µM) of each primer, and 3 µl cDNA (3.33 ng/µl). The primers are listed in Table 1; RPS7 and RPL17 were chosen as housekeeping genes [18]. All primers were purchased from Genomed S.A. (Warszawa, Poland) (Table 1). Each run included autoclaved water instead of cDNA as a negative control. The amplification was carried out as follows: denaturation for 20 sec at 95°C, followed by 40 cycles of 95°C for 1 sec and 60°C for 20 sec. The presence of product was confirmed by electrophoresis on a 2% agarose gel. Relative mRNA quantification data were then analyzed with the Real-time PCR miner algorithm [19]. According to the instructions supplied by the miner algorithm (http://www.miner.ewindup.info/), after determination of average cyclic threshold (Ct) and primer efficiency for each sample, the Ct levels were related to the primer efficiency level (E) using the equation [1/(1+E)]Ct.

Table 1. Primers used for real-time PCR

| Gene   | Gene name          | Forward       | Primer                      |
|--------|--------------------|---------------|-----------------------------|
| RPL17  | 60S ribosomal protein L17 | Forward | CTCTGGTGCATTGAGCACATCC |
|        |                    | Reverse       | TCAATGTGGCAGGGAGAC |
| RPS7   | 40S ribosomal protein S7 | Forward | GTCCCAGAAGGCAGCATTGAC |
|        |                    | Reverse       | CTCTTGCCCAACAATCTCGCTG |
| TNF    | Tumor necrosis factor α | Forward | TGGCCTTGCAACTAAATCAACC |
|        |                    | Reverse       | GTGTTGAGAGACATCCTTG |
| TNFR1  | TNF-α receptor type 1 | Forward | TCTCCTTGACAGTGTACCG |
|        |                    | Reverse       | AGGCAGAGGTGCAGTTTAG |

Thereafter, expression of the target genes was normalized against that of the reference genes and mRNA expression is presented in relation to different conditions in the endometria or to treatments.

Statistics

Data regarding the secretion of TNF-α by the feline endometrium in response to LPS and gene expression of TNF and TNFR1 after stimulation with TNF-α and LPS in endometrial explants were analyzed using a Kruskal-Wallis nonparametric one-way ANOVA followed by the Dunn’s Test (GraphPad PRISM v 6.0; GraphPad Software, San Diego, CA, USA). Expression is presented as an n-fold increase or decrease in mRNA level. Statistical significance was defined as P < 0.05. The differences in TNF-α in plasma samples, gene expression of TNF/TNFR1, and TNF-α secretion by epithelial and stromal cells were analyzed using a Kruskal-Wallis nonparametric one-way ANOVA followed by the Dunn’s Test (GraphPad PRISM v 6.0; GraphPad Software) and are presented as the mean (± SEM). Statistical significance was defined as P < 0.05.

Results

Plasma concentration of TNF-α

Mean TNF-α concentrations were higher in queens at estrus than those determined to be at anestru or diestrus, or those that were MPA-treated or those that had pyometra (P < 0.05) (Fig. 1).

Endometrial response to LPS via TNF-α secretion

LPS-stimulated explants from animals at estrus produced higher amounts of TNF-α than control explants (P < 0.001). No changes were found in TNF-α production, after LPS treatment, in endometrial explants from anestru, mid and late diestrus, or from queens treated short- or long-term with MPA. Differences were also not observed in explants from inflamed uteri (Fig. 2).

Transcription levels of TNF and TNFR1 genes in the feline endometrium

TNF-α (P < 0.05) and LPS (P < 0.001) treatment increased TNF gene expression in endometrial explants during estrus. A higher transcription level of TNF mRNA was observed in mid and late diestru after stimulation with LPS alone or in combination with TNF-α (P < 0.01). Expression of TNF was higher in explants derived from queens treated short-term with MPA after stimulation with LPS.
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Both TNF and TNFR1 gene expression were upregulated by TNF-α upon LPS challenge in inflamed endometrium (P < 0.001) (Fig. 3A).

Expression of TNFR1 was greater in the endometrium during estrus after stimulation with TNF-α (P < 0.01) and LPS (P < 0.001). LPS decreased TNFR1 gene expression in endometrial explants from queens at mid diestrus (P < 0.01). A higher transcription level of TNFR1 mRNA was observed in endometria from cats suffering from pyometra (P < 0.01) (Fig. 3B).

**TNF-α production by endometrial epithelial and stromal cells after LPS stimulation**

LPS stimulated TNF-α production in epithelial cells after 4 and 12 h (P < 0.01), compared to control treated cells (Fig. 4A). However, no changes were found in TNF-α production in stromal cells treated with LPS (Fig. 4B).

**Transcription levels of TNF and TNFR1 genes in cultured cells**

Expression of TNF was upregulated in epithelial cells after 2 (P < 0.01), 4 (P < 0.05), and 12 h (P < 0.01) of stimulation by LPS compared to control levels (Fig. 4C). Expression of TNFR1 was increased by LPS treatment in epithelial cells after 2 (P < 0.05) and 12 h (P < 0.01), but decreased after 4 h (P < 0.05), compared to control levels (Fig. 4D). In addition, TNF-α increased TNFR1 expression in epithelial cells after 4 (P < 0.05) and 12 h (P < 0.01) of incubation compared to control levels (Fig. 4F).

The expression of TNFR1 was lower in LPS-treated stromal cells after 2, 4 (P < 0.01), and 12 h (P < 0.001), compared to control levels (Fig. 4D), whereas expression of TNF was increased by TNF-α treatment in stromal cells after 2 (P < 0.01), 4 (P < 0.05), and 12 h (P < 0.001) of incubation (Fig. 4E). No significant changes in TNFR1 mRNA expression were seen in stromal cells after TNF-α treatment (Fig. 4F).

**Discussion**

This study found that LPS-challenge increased TNF-α secretion by endometrial tissue solely in estrous queens. However, an elevation of TNF mRNA was observed in the endometria of almost every estrous cycle stage (with the exception of anestrus) as well as in the endometria of MPA-treated cats and those suffering from pyometra. Increased cytokine production during estrus could be related to elevated levels of estradiol, enhancing the action of LPS. Treatment of ovariectomized rats with 17β-estradiol previously resulted in an increase in endometrial TNF-α mRNA and protein expression [20]. Furthermore, in human endometrial cells, TNF-α enhanced the intracellular estrogenic milieu by coordinating the expression of enzymes involved in local estrogen biosynthesis and metabolism [21]. A positive feedback loop between estradiol and TNF-α could be crucial for the enhanced proliferation of endometrial cells during the estrous cycle. Estradiol promotes endometrial proliferation of luminal and glandular epithelial cells and regulates the endometrial expression of cytokines as well as growth factors [22]. In turn, TNF-α,
via its two receptors, mediates different cellular responses. TNFR1 is related to cell death and apoptosis, whereas TNFR2 is associated with growth and proliferation [23]. Increased growth of glands, located in the endometrium, and under the influence of estradiol and TNF-α/TNFR2, could be a starting point for the development of cystic endometrial hyperplasia (CEH).

In this study, we confirmed the fundamental role of feline endometrial epithelial cells in pathogen recognition and the subsequent secretion of TNF-α. When activated by exposure to an infectious agent, macrophages and neutrophils recognize pathogen-associated molecular patterns via TLRs, resulting in intracellular signaling, activation of transcription factors, and production of pro-inflammatory cytokines [TNF-α, interleukin 1 (IL-1), and interleukin 6 (IL-6)] [24, 25] as well as secondary inflammatory mediators (prostaglandins, nitric oxide, and oxygen free radicals). Previously it was shown that an increased number of immune cells, including monocytes and neutrophils, in the blood of cats with pyometra, were able to produce TNF-α [26]. However, in our study no changes were observed in plasma TNF-α concentrations. A possible explanation for the lack of increased TNF-α plasma concentration in cats suffering from pyometra may be its short half-life [27]. Moreover, TNF-α has been considered as an early marker of acute endotoxin exposure in experimental animal sepsis [28]. Another study showed that TNF-α tends to precede the acute-phase response, and its detection was impossible as soon as 6 to 24 h after the induction of inflammation [29]. However, a study of 53 bitches with pyometra found plasma TNF-α concentrations to be significantly different between dogs with pyometra and healthy dogs [12]. The dogs showed a mean onset of pyometra approximately 1 week before veterinary examination; hence, these results were puzzling in the context of the experimental studies [12]. One possible explanation for this observation may be the continuous or intermittent systemic release of inflammatory mediators or bacterial compounds, including LPS. We assume that similar levels of TNF-α found in queues with diagnosed uterine inflammation compared to that of all other groups examined (with the exception of cats at estrus), may be due to the inherent characteristics of pyometra in cats, which is a severe disease. In this regard, our data are in accordance with earlier experimental studies [27–29].

We conclude this part of our study with the assumption that plasma TNF-α concentrations, as opposed to endometrial content, is not a reliable marker of pyometra.

Pyometra is associated with the upregulation of proinflammatory cytokines and chemokines in the inflamed uterus [30]. In a previous study, we clearly demonstrated that TNF-α is expressed mostly in epithelial cells of the feline endometrium. In the inflamed uterus, strong TNF-α immunolabeling was observed in the endometrial...
glands, whereas moderate to weak labeling was shown in the surface epithelium and weak or negative staining was found in the stroma [4]. However, in bitches with pyometra, the endometrial TNF-transcript level was not significantly altered [30]. Moreover, in the present study, LPS alone did not affect endometrial TNF gene expression or the secretion of TNF-α by feline endometrial explants from inflamed uteri. Only LPS enhanced by TNF-α led to an increase in the endometrial expression of TNF and TNFR1. A possible explanation for this result may be the severity of endometrial damage during the course of pyometra. In pyometra, serious endometrial epithelium impairment can be observed. However, LPS, alone or in combination with TNF-α, increased TNF transcription in the endometria of cats during diestrus and after hormone treatment with MPA. Nevertheless, in the study presented here, LPS had no effect on TNF-α production by endometrial explants, either from diestrus or from MPA-treated endometria. An elevation in TNF mRNAs, without a subsequent increase in protein levels may however suggest that the period of incubation (4 h) was not enough to observe changes in secreted TNF-α. This might be supported by an earlier observation that LPS-challenge led to TNF-α secretion in the endometrial epithelium, after 24 h of incubation [7]. Importantly, by taking advantage of endometrial epithelial or stromal cell cultures (as opposed to studying whole tissues containing other cell types such as immune cells), we could clearly show how these specific cells respond to LPS. The observed increases in TNF mRNA levels and TNF-α production by LPS-treated epithelial cells confirms our preliminary data using ELISpot. Therein we clearly demonstrated that feline epithelial cells, in response to LPS, release markedly greater amounts of TNF-α compared to stromal cells [7]. Endometrial stromal cells seem to play only a secondary role in the endotoxin challenge response, as LPS stimulation did not affect TNF-α concentration in these cells when levels were compared to those of untreated control cells. In fact, LPS negatively influenced TNFR1 expression in stromal cells and did not affect TNF mRNA when compared to levels of untreated cells. Since the only effect observed in stromal cells was the TNF-α-induced upregulation of TNF and TNFR1, one may speculate that stromal cells play a supporting role in the endometrial response to LPS.

Fig. 4. A, B) Time course of TNFα production by endometrial epithelial (n = 5) and stromal (n = 5) cells after LPS stimulation. C–F) Time course of TNF and TNFR1 mRNA expression in feline epithelial (n = 5) and stromal (n = 5) cells after LPS and TNFα treatment. Values were calculated in relation to controls (CTR) (separately for stromal and epithelial cells taking into account stimulation time) and presented as fold change in gene expression (C–F). Asterisks indicate statistical differences between expression levels depending on the treatment (* P < 0.05, ** P < 0.01, *** P < 0.001).
role in the production of proinflammatory cytokines and chemokines; however, they are not involved in an acute pro-inflammatory response. To conclude, we provided further support for a fundamental role of endometrial epithelial cells in pathogen recognition and subsequent upregulation of TNF-α production also evident in endometrial tissues obtained at estrus. Endometrial stromal cells seem to play only a secondary role in tissue responses to endotoxin-challenge.

**Conflict of interest:** The authors declare that they have no competing interests.

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