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Pathogenic *Escherichia coli* and LPS enhance the expression of IL-8, CXCL5 and CXCL10 in canine endometrial stromal cells

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SUMMARY

Chemokines play a central role in cellular communication in response to bacterial infection. However, the knowledge of the chemokine responses to bacterial infections in dogs remains limited. Uterine bacterial infection (pyometra) is one of the most common bacterial diseases in dogs and causes sepsis in most of the cases. We have shown previously that dogs with pyometra have higher mRNA levels of chemokines in uterus. To assess whether the stromal part of endometrium express chemokines in response to bacterial infection, we cultured endometrial stromal cells isolated from healthy dogs and exposed them to either live pathogenic *Escherichia coli* (*E. coli*) isolated from uterus of a dog with pyometra, or to lipopolysaccharide (LPS). Changes in the mRNA expression of ELR^+CXC chemokines IL-8, CXCL5, CXCL7, and ELR^- CXC chemokine CXCL10 were measured after 24 h using quantitative real-time PCR. Levels of IL-8, CXCL5 and CXCL10 were upregulated in endometrial stromal cells exposed to *E. coli* and to LPS whereas the level of CXCL7 was decreased or unaffected. In addition, levels of IL-8 and CXCL5, but not CXCL7 or CXCL10, were significantly higher in dogs with pyometra as compared to healthy dogs. Our findings show that pathogenic uterine-derived *E. coli* induces a CXC chemokine response both in cultured endometrial stromal cells within 24 h and in pyometra-affected uteri from dogs. Stromal cells could therefore play an important role in early neutrophil and T cell recruitment to the site of inflammation during Gram negative bacterial infection of the uterus. Further study is needed to clarify the role of chemokines in host response to bacterial infection in dogs and the possibility of using chemokines as diagnostic parameters for bacterial infection in this species.

38 Keywords: infection, chemokines, endometrium, dog/canine, pyometra.

1. INTRODUCTION

Chemokines belong to an ancient system of cellular communication [1] and [2] and play a central role in virtually every aspect of host-pathogen interaction [3]. Based on the arrangement of the first two cysteins, the chemokines are divided into C, CC, CXC and CX_3C families [4]. Members of the CXC family, such as IL-8 (CXCL8) and CXCL10, are important in recruiting leukocytes to the site of inflammation and they are expressed in different cell types in response to bacterial infection in humans [5], [6], [7], [8] and [9], mice [10] and [11], pigs [12], and [13] and cattle [14]. However, the CXC chemokine response in bacterial infections in dogs remains largely unexplored.

Uterine infection (pyometra) is one of the most common bacterial diseases in dogs, resulting in life-threatening conditions such as systemic inflammatory response syndrome in most of the cases [15] and [16]. Pyometra is caused mainly by Gram negative bacteria, particularly *Escherichia coli (E. coli)* strains [17] and [18] that originate from the normal vaginal flora of the bitch [19]. Lipopolysaccharide (LPS), the main component of bacterial cell wall, acts as endotoxin once released and elicits a strong inflammatory response in animals as well as in humans. It has been shown that bovine, human and murine endometria respond to LPS and *E. coli* by expressing higher levels of chemokines [20], [14] and [21]. In canine uteri affected by pyometra the expression of CXC chemokines such as IL-8 and CXCL10 is upregulated [22], but the role of the endometrium in chemokine response to pathogenic bacteria in the canine uteri is unknown.

IL-8, CXCL5 and CXCL7 are ELR^+ CXC chemokines because their N-terminal sequences contain glutamic acid–leucine–arginine (ELR) motif before the first cysteine, and are potent neutrophil chemoattractants [23] and [24]. Despite their similar sequences and identical monomeric 3-D structures [25], these chemokines may exert different functions during an inflammatory response [26]. IL-8 is one of the most clinically evaluated chemokines of the CXC chemokine family [27]. Although IL-8 is known as a potent neutrophil chemoattractant, blocking with IL-8 antibodies does not abrogate the neutrophil migration towards LPS-treated bovine endometrial cells [14], suggesting that other chemokines are involved in neutrophil recruitment to the uterus during a bacterial infection.

CXCL5, also known as epithelial cell-derived neutrophil-activating peptide-78 (ENA 78) in humans, is preformed and stored in platelet granules. Under homeostatic conditions platelets are thought to be the main source of this chemokine [28]. During inflammation, however, tissue-resident cells have been shown to produce increased levels of CXCL5 *in vivo*, as shown in epithelium samples from cows with endometritis [29] and in cultured human stromal endometrial cells stimulated with LPS [30].

CXCL7, known also as neutrophil-activating protein (NAP-2) or platelet basic protein (PBP), is stored and secreted by platelets both in homeostatic conditions and during inflammation in humans [31] and [32]. CXCL7 was shown to bind neutrophils with higher affinity than CXCL5 and to be more suitable for infection imaging in rabbit muscle infected with *E. coli* [33]. CXCL7 was recently shown to improve innate

immunity to bacterial infection in *K. pneumoniae* pneumonia in mice [34], but its role in canine bacterial infection remains unknown.

CXCL10, or IFN-γ-inducible protein (IP-10), is an ELR^- CXC chemokine that does not appear to have chemotactic activity for neutrophils [35], and is one of the few chemokines of this group studied in dogs [36]. CXCL10 has been suggested to have antimicrobial effect both for Gram positive and Gram negative bacteria [37], [38] and [39], and we have previously shown that CXCL10 was upregulated in uteri from dogs with pyometra [22].

Bacterial infection of the uterus causes disruption of the endometrial epithelium, leaving the stroma exposed to live bacteria and bacterial products [40]. Our hypothesis is that endometrial stromal cells may contribute substantially to the production of CXC chemokines in response to bacterial infection of the canine uterus. In this study we investigated the expression levels of CXC chemokines IL-8, CXCL7, CXCL10 and a canine homolog to CXCL5 in canine endometrial cells exposed to pathogenic *E. coli* and to the main proinflammatory factor in Gram negative bacterial infection, LPS. Knowledge of the endometrial cell chemokine response to bacterial infection can provide a better understanding of the pathology that leads to organ-damaging inflammatory conditions in dogs. This knowledge may contribute to the development of novel means of detecting and tempering severe inflammatory conditions in dogs and possibly also other animal species and humans.

2. MATERIALS AND METHODS

2.1 Isolation and culture of stromal endometrial cells

For isolation of endometrial cells, uteri were obtained from five healthy bitches of different breeds (two Beagles, one German shepherd and two Rottweilers) between 1.8 and 3 years old presented for routine spay (ovariohysterectomy) at the University Animal Hospital, Swedish University of Agricultural Sciences or at the Rembackens Animal Clinic, Uppsala, Sweden. Dogs were clinically healthy as examined by the veterinary surgeon in charge prior to surgery, and showed no signs of systemic or localized inflammation at the time of surgery. The study was approved by Uppsala local ethical committee, permission number C242/7, and performed with the permission of the dog owners.

All the following procedures were performed using sterile technique, equipment and solutions. Immediately after surgical removal, the uterus was placed in ice-cold phosphate buffered saline (PBS) containing 2 μg/mL gentamicin, 6 U/mL nystatin (Sigma-Aldrich, St. Louis, MO, USA), 6 mg/mL penicillin, 5 mg/mL streptomycin (VWR, Stockholm, Sweden), and used within 1 h. Adhering fat and ligaments were removed from the uterus with a scalpel, thereafter the uterine surface was rinsed with PBS solution containing antibiotics as described above to remove blood cells and tissue debris. Both of the uterine horns were cut open, and the endometrial layer was separated from the myometrium with a pair of scissors and/or a scalpel. The endometrium was mashed using a scalpel on a dry petri dish and transferred into a 50-mL Falcon tube containing 250 U/mL collagenase from *Clostridium histolyticum* and 250 U/mL bovine

hyaluronidase (C5138 and H3506, respectively; Sigma-Aldrich, St. Louis, MO, USA) in 25 mL PBS with antibiotics. The resuspended tissue was homogenized with a 10-mL syringe with an 18G×2″ needle and incubated with vigorous shaking for 2.5 h at 39°C [41]. The homogenisation step was repeated every 10 min during the enzymatic digestion.

The solution with digested endometrium was centrifuged at 200×g for 6 min and the resulting pellet was resuspended in PBS with antibiotics. The suspension was filtered first through a 40 mesh screen (CD1, Sigma-Aldrich) and then through a 40-μm nylon sieve (Falcon, Becton Dickinson & Company, Franklin Lakes, NJ) to remove mucus and undigested tissue. The filtrate was subsequently centrifuged at 200×g for 6 min and the cell pellet resuspended in Dulbecco's Modified Eagle's Medium/Nutrient Mixture (DMEM) F12 (D6434; Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2.4 mM L-glutamine, insulin-, transferrin- and sodium selenite (ITS) liquid media supplement (0.5×; I3146, Sigma-Aldrich), and antibiotics as described above. Cells were counted using Bürker chamber, plated in 25-cm^2 tissue culture flasks (Sarstedt, Wiener Neudorf, Austria) at a concentration of 1 to 2×10^6 cells/mL, and cultured at 37°C, in 5% CO_2. The medium was renewed within the first 24 h to remove nonattached cells and changed every fourth day thereafter. Passages were performed at 80 to 100% cell confluence using trypsin-EDTA (VWR, Stockholm, Sweden). One independent experiment was performed with each of the five endometrial cell cultures of passage two or three, and cell cultures used for the experiment were > 99% morphologically homogenous as determined using light microscopy.

2.2 Immunocytochemistry

Endometrial stromal cells of passage two or three were allowed to attach in a chamber slide (cat. 177429; NuncInc, Naperville, IL, USA) at a density of 0.3 to 0.5×10^6 cells/mL in DMEM F12 medium for 48 h at 37°C with 5% CO_2, washed twice with PBS and fixed using ice-cold methanol for 10 min. Cells were then washed with Tris-buffered saline (TBS), dried at room temperature for 15 to 30 min and kept at -20°C prior to staining. Cells were subjected to peroxidase blocking (Dako, Glostrup, Denmark) for 5 min, washed with TBS and then blocked with goat serum (diluted 1:50) for 1 h at room temperature prior to addition of primary antibodies. The primary antibodies (0.4 mg/L mouse cytokeratin clones AE1/AE3, 0.3 mg/L mouse anti-vimentin clone V9, 0.3 mg/L mouse smooth muscle actin clone 1A4, or 0.7 mg/L mouse IgG1 antibody as control; Dako, Glostrup, Denmark) were incubated with cells overnight at 4°C in a sealed humid container, thereafter washed 3 times with 0.05% Tween-20 in TBS. The Envision polymer system (Dako) together with 3,3′-diaminobenzidine (DAB; Dako) was used for detection, and hematoxylin staining was used for nuclei visualisation. Paraffin cross-sections (4 μm thin) of dog uteri obtained after OHE of one healthy dog were deparaffinised, subjected to heat-induced antigen retrieval with PT Module buffer (Labvision [Thermo Fisher Scientific], Fremont, CA) in a Pascal decloaking chamber (DakoCytomation, Glostrup, Denmark), washed with TBS and stained as described above.

2.3 *Escherichia coli* bacterial strain

The bacterial strain, *E. coli* P170 was earlier isolated from a female dog with severe uterine infection (pyometra) [42] and serotyped at Statens Serum Institut (Division of Diagnostics, Danish Ministry of Health, Copenhagen, Denmark) as O6:K14:H31 with P- and/or S-fimbria and α hemolysin. The strain was stored in PBS containing 15% glycerol at -70°C. Prior to use in experiments, the bacterial cells were first plated onto an LB-agar plate and incubated at 37°C overnight, then they were inoculated in liquid LB broth and incubated for 12 to 16 h at 37°C with shaking. The culture was washed twice with PBS. To determine bacterial concentration, a series of dilutions were plated on LB-agar plates and incubated overnight at 37°C. The number of colonies was counted and the colony forming units (cfu) were calculated.

2.4 *In vitro* exposure of stromal endometrial cells to *Escherichia coli* and LPS

Endometrial cells were detached from the culture flask using trypsin-EDTA, and then pelleted and resuspended in antibiotic-free DMEM F12 medium to a density of 0.3 to 0.5 x 10^6 cells/mL in 24-well flat bottom tissue plates (Sarstedt Inc., Newton, NC 28658, USA). The cells were then allowed to attach for 24 h prior to addition of bacteria *(E. coli P170)* or LPS from *E. coli* 055:B5 (L2880; Sigma-Aldrich, St. Louis, MO, USA). The *E.* coli P170 bacteria were added at the multiplicity of infection (MOI) of 3 to 5 (Bact Low) or 30 to 50 (Bact Hi). After 1 h incubation, 120 μg/mL penicillin and 100 μg/mL streptomycin were added to the cell culture to kill the bacteria. Alternatively, endometrial cells were incubated with 100 ng/mL (LPS Low) or 1 μg/mL (LPS Hi) of LPS [46, 53]. Nontreated endometrial cells were used as controls. After 24 h [14], [46] and [53] the cells were detached by trypsination, pelleted by centrifugation and stored at −20°C. All

199 treatments were performed in triplicates, and data shown are mean values from five
200 independent experiments.
201
202 **2.5 Dogs with pyometra**
203 Cross-sections of frozen uterine tissue samples obtained from four female dogs with
204 pyometra and four healthy female dogs were obtained as described previously [22].
205 Briefly, the dogs diagnosed with pyometra according to criteria and method described
206 [43] and [44] were subjected to surgical treatment (ovariohysterectomy, OHE) of
207 pyometra, and the removed uterus was sectioned and snap-frozen in liquid nitrogen
208 whereby stored at −80°C. The control samples from healthy female dogs admitted for
209 elective spay (OHE) were obtained and treated identically to samples from dogs with
210 pyometra.
211
212 **RNA preparation and cDNA synthesis**
213 Total RNA of cultured endometrial cells was isolated using NucleoSpin kit (Macherey-
214 Nagel, Düren, Germany) according to the manufacturer's instructions. First-strand cDNA
215 was synthesized with Super-Script II reverse transcriptase (Invitrogen) according to the
216 manufacturer's instructions with 10 μL of RNA and a final concentration of 7.5 ng/μL
217 random hexameres (Invitrogen) in a total volume of 20 μl. cDNA from cross-sections of
218 frozen uterine tissue samples obtained from four female dogs with pyometra and four
219 healthy dogs was prepared as previously described [22].
220
221 **Quantitative real-time RT-PCR**

Quantitative real-time RT-PCR was performed on an ABI Prism 7900 HT using iQ SYBR Green Supermix (Bio-Rad) in a final volume of 10 μL as described previously [45]. All samples were assayed in duplicate and non-template wells were used as negative control. The target transcript levels were calculated by the comparative CT method (user bulletin no. 2 for ABI Prism 770 sequence detection system; P7N 4303859; Applied Biosystems) and normalized to the corresponding levels of a housekeeping gene glucuronidase beta (GusB). Primer efficiency was validated by performing qPCR with cDNA dilutions of 1:1, 1:10, and 1:100. The primer sequences are summarized in Table 1.

Statistical analyses

Data were analyzed for normality using Kolmogorov-Smirnov, D'Agostino and Pearson omnibus, and Shapiro-Wilk tests and were found to be nonnormally distributed which is why Mann-Whitney's *u*-test for two-tailed distributions was used to analyze the differences between the groups. All statistical analyses were performed using Graph Pad Prism 4.0c, and a P-value < 0.05 was considered significant.

238 3. RESULTS

239 **3.1 Canine endometrial stromal cells**

240 Evaluation of uterine tissue sections after the isolation of stromal endometrial cells
241 confirmed that the endometrium was removed from the uterus leaving the muscle layer
242 (myometrium) intact (Fig 1A and B). Freshly isolated endometrial cells contained a
243 mixed population of cells including high numbers of red blood cells. The number of cells
244 decreased dramatically at the day of first passage, followed by the expansion of a > 99%
245 morphologically homologous cell population with every further passage (Figure 1C).
246 The isolated stromal endometrial cells were positively stained with antibodies to smooth
247 muscle α–actin (Fig. 2A and B) and to the mesenchymal cell marker vimentin (Fig. 2B
248 and D), but were negatively stained with antibodies to the epithelial cell marker
249 cytokeratin (Figure 2E and F) or with the isotype control (Figure 2G and H). This
250 confirms that the cell population surviving the culture conditions were endometrial
251 stromal cells.

252

253 **3.2 Elevated expression levels of IL-8, CXCL5 and CXCL10 in canine endometrial**
254 **stromal cells in response to LPS or *Escherichia coli* P170.**

255 Our earlier study has shown that mRNA levels of IL-8 and CXCL10 were upregulated in
256 uterine tissue from dogs with severe bacterial infection [22]. To investigate the effect of a
257 clinically relevant pathogenic bacterial strain on chemokine expression in canine
258 endometrium, we incubated cultured canine endometrial cells with high and low doses of
259 *E. coli* P170 and measured mRNA levels of IL-8, CXCL5, CXCL7 and CXCL10. Both
260 low and high doses of *E. coli* P170 caused an increased expression of IL-8 ($P < 0.001$),

CXCL5 ($P < 0.001$) and CXCL10 ($P < 0.001$ and $P < 0.01$, respectively) in endometrial cells from different dogs (Figure 3A, B and C). The levels of CXCL7 were, however, unaffected (Figure 3D).

To compare the difference between cell response to live bacteria and purified bacterial endotoxin, we incubated cultured endometrial cells with different doses of LPS for 24 h. Endometrial cells incubated with a high dose of LPS (1 μg/mL, LPS Hi) had higher expression of IL-8 ($P < 0.001$), CXCL5 ($P < 0.001$) and CXCL10 ($P < 0.001$) compared to untreated control cells (Figure 3A, B and C). The cells incubated with a lower dose of LPS (100 ng/mL, LPS Low) had a lower expression of CXCL7 ($P < 0.001$; Figure 3D) when compared to control.

3.3 Enhanced expression of IL-8 and CXCL5 in endometrial tissue from dogs with pyometra

To compare the *in vitro* data with a natural bacterial infection *in vivo*, uterine mRNAs from dogs with pyometra and healthy dogs were used to measure the expression levels of IL-8, CXCL5, CXCL7 and CXCL10. Levels of IL-8 and CXCL5 were upregulated 28-fold (range 1.5 to 205; $P < 0.05$) and 176-fold (range 4 to 740; $P < 0.05$) respectively in the uterus from dogs with pyometra compared to those of healthy dogs (Figure 4A and B). In contrast, the CXCL10 and CXCL7 expression levels were not different in uteri from dogs with pyometra and healthy controls (Figure 4C and D).

4. DISCUSSION

This study shows that canine endometrial stromal cells express significantly increased elevated levels of the CXC chemokines IL-8, CXCL5 and CXCL10, but not CXCL7, in response to stimulation with *E. coli* or LPS *in vitro*. These findings are in agreement with previous reports showing that human, bovine and canine endometrial stromal cells express Toll-like receptor 4 (TLR4) [46], [47], [48] and [49], a receptor which is utilized by LPS [50]. Our results are also in agreement with those of Silva and collaborators [49] showing an upregulation of TLR4 expression in uteri from dogs with pyometra.

We showed that canine endometrial stromal cells increase IL-8 mRNA synthesis by nearly 30-fold in response to LPS, and by nearly 60-fold in response to a pathogenic *E. coli* strain within 24 h. We showed also that in uteri from dogs with pyometra the expression of IL-8 is highly upregulated, which in line with our earlier study [22]. LPS and *E. coli* were reported to upregulate IL-8 production in murine, bovine and human epithelial and stromal endometrial cells [21], [51], [52] and [53]. IL-8 is known as an early marker of bacterial infection in humans because the expression of IL-8 mRNA is detected within minutes after LPS exposure in peripheral blood mononuclear cells [54]. Increased serum concentration of IL-8 preceded the onset of fever and increased blood concentrations of C-reactive protein in humans with sepsis [54]. Serum concentrations of IL-8 were higher in dogs with sepsis secondary to pyometra compared to dogs without sepsis [55]. Our data suggest that stromal cells may be an important early source of IL-8 for neutrophil recruitment during a bacterial infection of the uterus when the epithelial

304 layer of the endometrium is disrupted. Moreover, IL-8 can be a useful early biomarker of
305 uterine infection and possibly also sepsis in dogs.
306
307 We showed that the mRNA expression of CXCL5, but not CXCL7, is significantly
308 potentiated in canine pyometra and in cultured canine stromal endometrial cells exposed
309 to LPS and *E. coli*. This is in agreement with another report showing that human stromal
310 endometrial cells, but not epithelial endometrial cells, produce and secrete increased
311 amounts of CXCL5 (ENA-78) in a dose-dependent response to LPS stimulation *in vitro*
312 [30]. Human endometrial stromal cells, in contrast to epithelial endometrial cells, do not
313 express CXCL5 constitutively, but the CXCL5 mRNA production is increased when
314 stimulated with a potent inflammatory trigger IL-1β [56]. It has been shown that tissue-
315 resident cells, such as lung epithelial cells, are the main source of both local and systemic
316 CXCL5 during a bacterial infection, as shown in *E. coli*-induced lung inflammation and
317 in tuberculosis model in mice, and that CXCL5 expression by these cells is harmful for
318 bacterial clearance and inflammation regulation [57] and [58]. In bovine endometrial
319 epithelium samples, CXCL5 mRNA levels were higher in cows with subclinical and
320 clinical endometritis compared to healthy cows [29], supporting a role for CXCL5 in
321 endometrial response to bacterial infection. Several other cell types have been shown to
322 produce CXCL5 during inflammation, such as murine enterocytes [59], human
323 eosinophils [60] and monocytes [61], suggesting that CXCL5 may have an important role
324 in inflammation. To our knowledge, this study is the first to show that the CXCL5 gene is
325 expressed at the mRNA level in canine endometrial stromal cells. Further studies are
326 needed to clarify the specific roles of CXCL5 in canine bacterial infection.

In the current study, we show that canine endometrial cells express CXCL7, and that the level of expression is not affected by co-culture with bacteria. Similarly, in uterus from dogs with pyometra the CXCL7 mRNAs are at the same levels as those of healthy dogs. However, the mRNA expression of CXCL7 was downregulated in cells stimulated with a lower dose of LPS. Our results indicate that the role of CXCL7 differs from that of IL-8 and CXCL5 in response to Gram negative bacterial infection in canine uterus, suggesting that CXCL7 may be not a part of early chemokine response to Gram negative bacterial infection in endometrial stromal cells in dogs.

In our study both LPS and *E. coli* caused a significant upregulation of CXCL10 in stromal endometrial cells. CXCL10 is known to facilitate T effector cell generation and trafficking in response to LPS stimulation both in mice and in humans [62] and [63]. Human connective tissue fibroblasts were reported to release high concentrations of CXCL10 in response to LPS stimulation *in vitro* [64]. The present data show that endometrial stromal cells produced up to 27-fold more CXCL10 mRNA in response to LPS, suggesting that the stromal cells may have an important role in T cell recruitment during Gram negative bacterial infection. In uteri from dogs with pyometra the CXCL10 mRNA levels were similar to those of healthy dogs, in contrast to an earlier observation that CXCL10 expression was increased in uterus from dogs with pyometra, obtained by using a less quantitative method and involved a larger, and more diverse group of animals with pyometra [22]. Collectively, our data suggests that the expression of CXCL10 is highly variable between individuals with uterine infection. Because the phase of the

estrus cycle and other cofactors of uterine infection, e.g. closed or opened cervix, affect the degree of leukocyte proliferation and migration to the infected uterus [65], [66] and [67], the expression of CXCL10 may also be influenced by these factors.

5. CONCLUSIONS

In this study, we investigated the expression of both ELR^+ and ELR^- CXC chemokines in canine bacterial infection both *in vivo* and *in vitro* and showed that pathogenic uterine-derived *E. coli* induces a CXC chemokine response both in pyometra-affected uteri and in cultured endometrial stromal cells from dogs. These findings suggest that stromal cells are important players in the early neutrophil and T cell recruitment to the site of inflammation during bacterial infection of the uterus. Further study is needed to clarify the role of chemokines in host response to bacterial infection in dogs and the possibility of using chemokines as diagnostic parameters for bacterial infection in this species.

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374
375 DISCLOSURES
376 All authors have materially participated in the research and/or article preparation and
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587 bitches and those with pyometra. Theriogenology 2011;75:979-87.

588 **Figure legends**

589 **Figure 1**. Endometrial cell isolation and culture. (A, B) Tissue sections after removal of
590 the endometrium stained with hematoxylin and eosin, magnification ×40. The
591 endometrium is partially (A) or completely (B) separated from muscle tissue. (C) Cell
592 survival dynamics in culture. Viable cells were staining with trypan blue and counted
593 using a Bürker chamber on the day of isolation and during each passage. The graph is
594 showing the representative data for five independent experiments.
595
596 **Figure 2**. Characterization of cultured canine endometrial cells. Chromogenic
597 immunostaining with smooth muscle actin (SMA) (A, B), vimentin (C, D), cytokeratin
598 (E, F) and negative control IgG1 (G, H) antibodies was performed to identify the type of
599 isolated cells. The tissue sections of healthy canine uterus (B, D, F, H; magnification
600 ×40) were used as staining control. The positive staining of the cytoskeleton with anti-
601 SMA and anti-vimentin in combination with a negative staining with anti-cytokeratin
602 indicated stromal origin of the isolated canine endometrial cells (magnification ×200).
603
604 **Figure 3**. *E. coli*- or LPS-induced CXC chemokine expression in cultured canine
605 endometrial stromal cells. Levels of mRNA expression of IL-8 (A), CXCL5 (B),
606 CXCL10 (C) and CXCL7 (D) in cultured canine endometrial stromal cells in response to
607 treatment with 100 ng/mL PLS (LPS Low), 1 μg/mL LPS (LPS Hi), co-culture with MOI
608 3 to 5 or 30 to 50 of *E. coli* P170 (Bact Low and Bact Hi, respectively). Canine
609 endometrial stromal cells incubated with saline were used as control (Control). All
610 treatments were performed in triplicates. Data shown are mean values ± SEM from five

611 independent experiments normalized to the LPS Lo treatment for each experiment. ** P
612 < 0.01; *** P < 0.001; compared to Control.
613
614 **Figure 4**. CXC chemokine expression in uterine tissue from dogs with pyometra and
615 healthy dogs. Levels of mRNA expression of IL-8 (A), CXCL5 (B), CXCL10 (C) and
616 CXCL7 (D) in uterine tissue from healthy dogs (Control, n = 4) and dogs diagnosed with
617 uterine bacterial infection pyometra (Pyometra, n = 4). Data shown are median values
618 with 25 and 75% percentile (box) and range of values (whiskers). * P < 0.05, compared
619 to Control.

620 **Table 1.** Primers used for quantitative real-time RT-PCR

621

Target	Sequence
canine	
CXCL5*, fw[a]	5'-CCG CGG GTG GAA GTC ATA GCC-3'
CXCL5*, rev[b]	5'-CCG CGG GTG GAA GTC ATA GCC-3'
CXCL7, fw	5'-ACC TCA GAC CTA AGG CCA CCT CC-3'
CXCL7, rev	5'-CAG CAG CAG CCC CCG TAG GA-3'
IL-8, fv	5'-TCT GTG AAG CTG CAG TTC TGT CAA G-3'
IL-8, rev	5'-TTG GGG TCC AGG CAC ACC TC-3'
CXCL10, fw	5'-CTT GAA TGA ACC AAA GTG CTG TTC-3'
CXCL10, rev	5'-ACA GCG TAT AGT TCT AGA GAG AGG-3'
GusB, fw	5'-GCC CCT GTC CTC CTG CCG TA-3'
GusB, rev	5'-GGG CCA CGA CCT TGC CTT CC-3'

622 [a] fw, forward primer

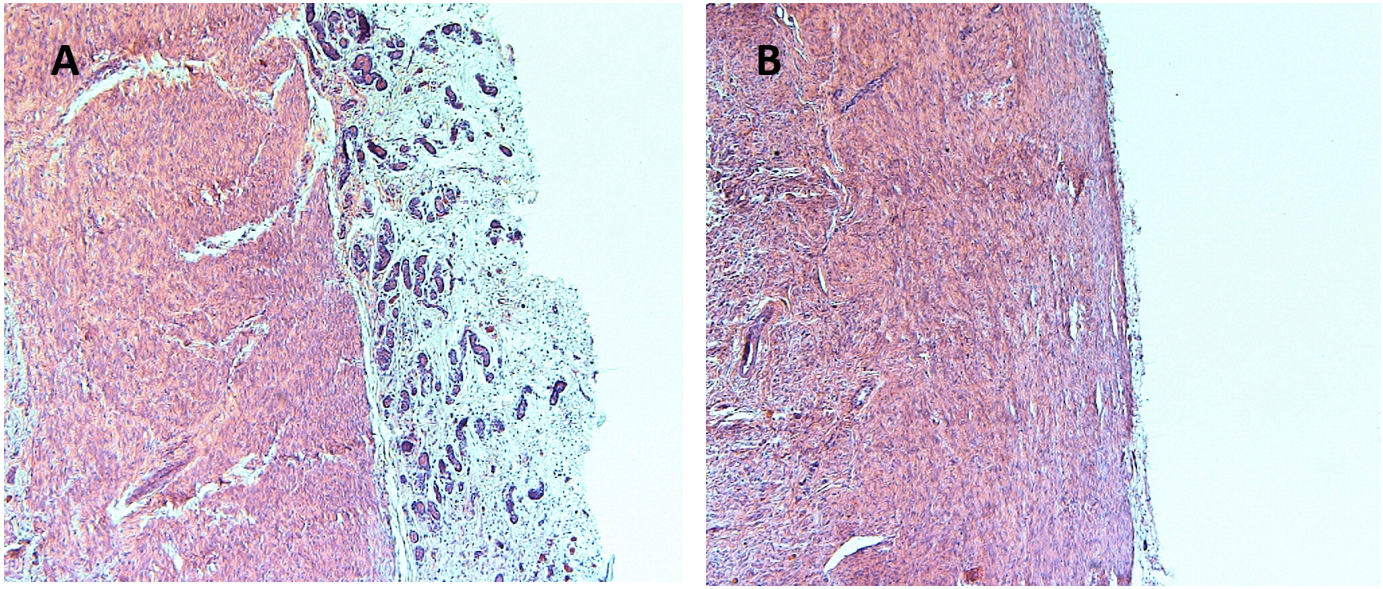
623 [b] rev, reverse primer

624 * The primers for canine CXCL5 were designed using predicted mRNA sequence of the

625 gene (ID 611927) in NCBI database and available on March 15, 2012: XM_849650,

626 length 375 bp.

Figure 1



C

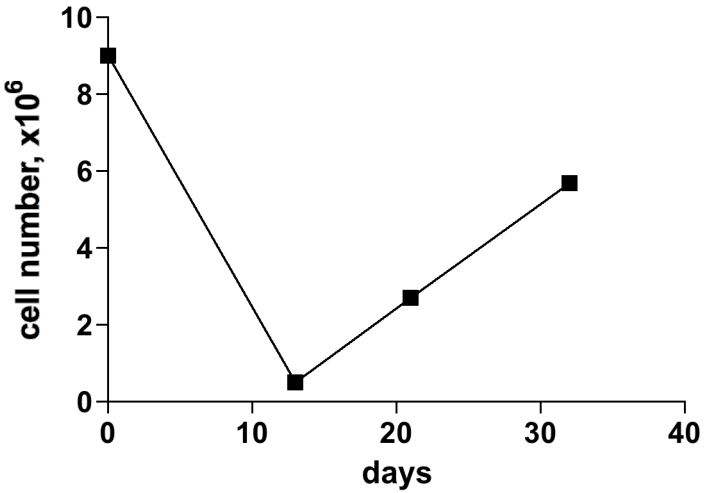


Figure 2

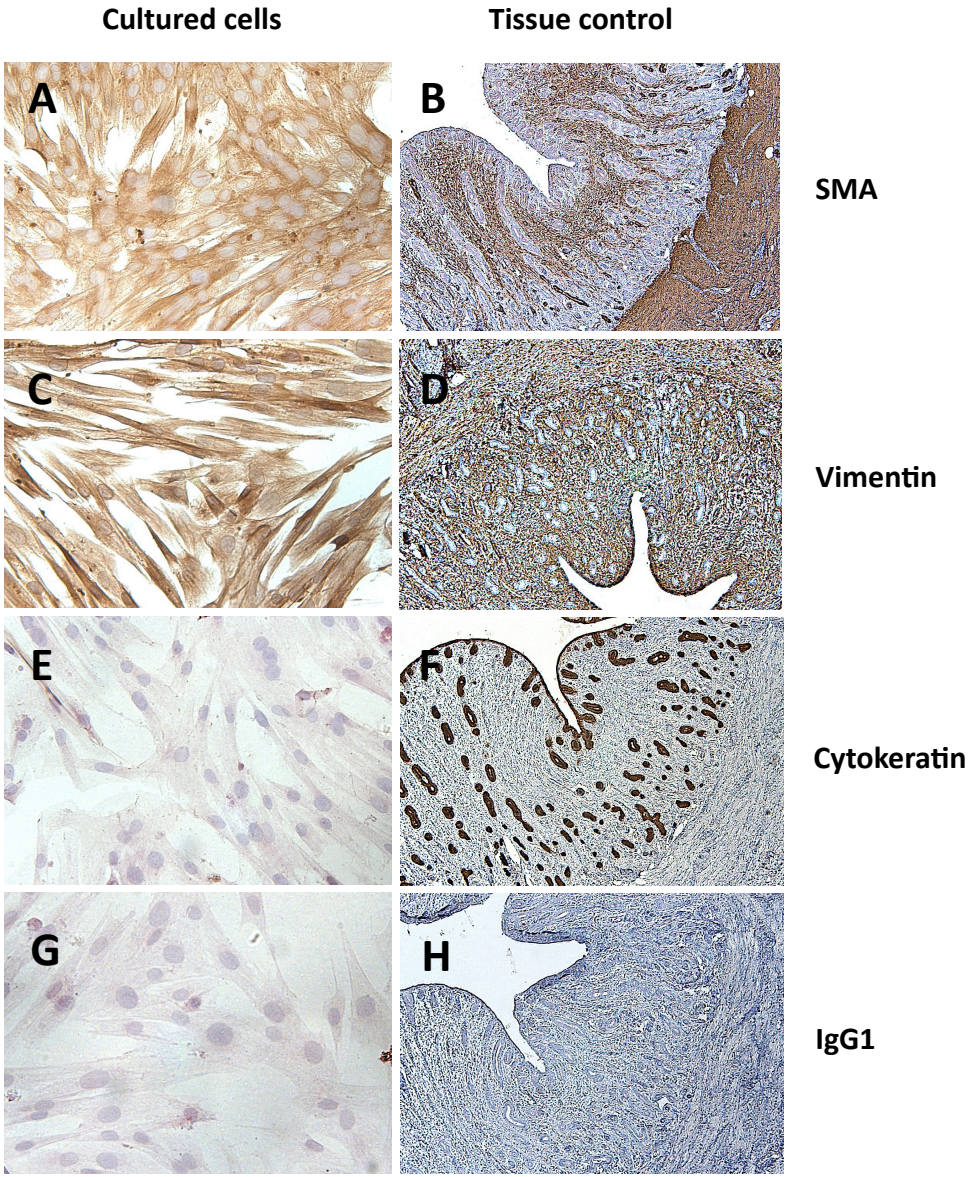

Cultured cells
Tissue control
A
B
SMA
C
D
Vimentin
E
F
Cytokeratin
G
H
IgG1

Figure 3

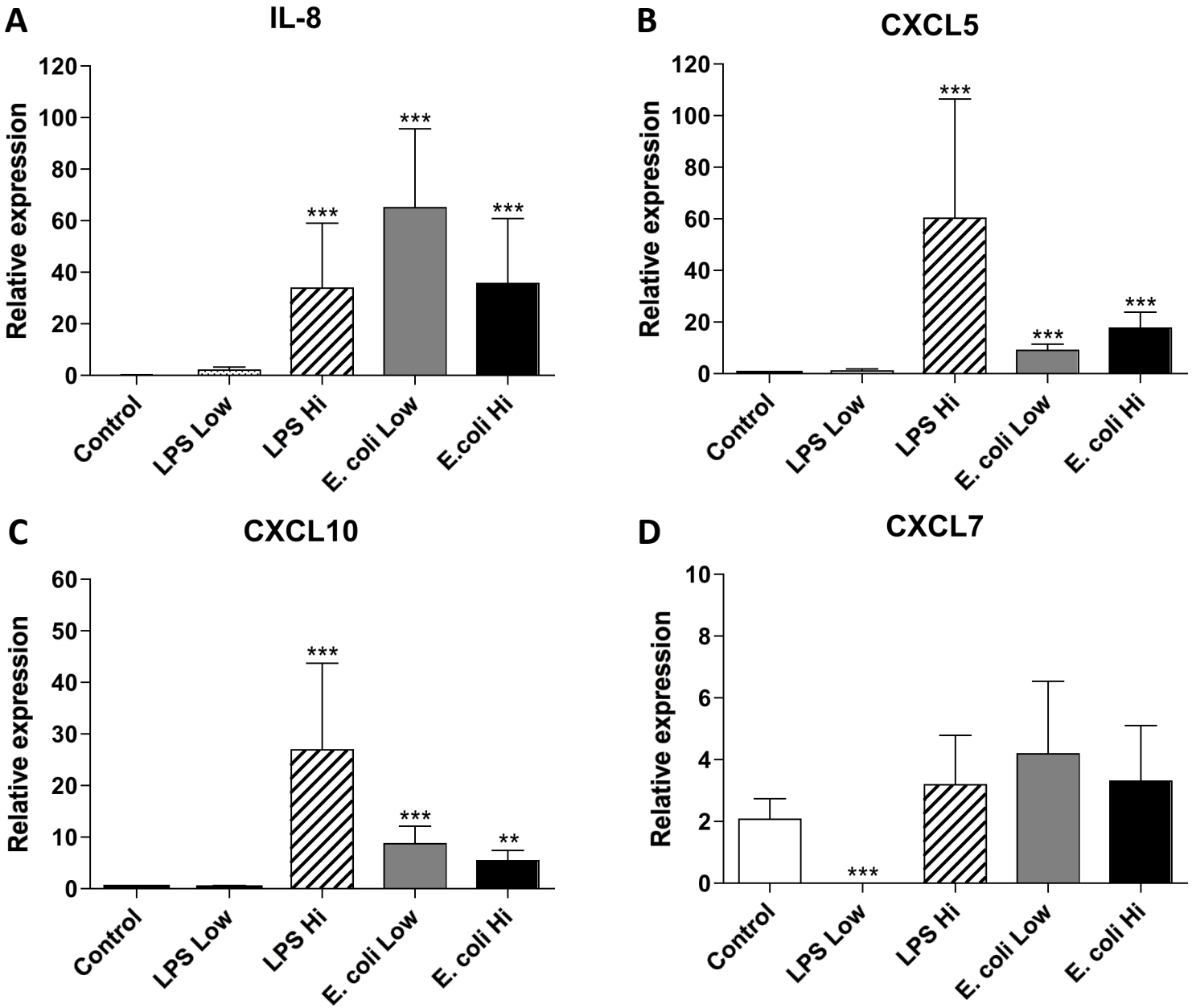
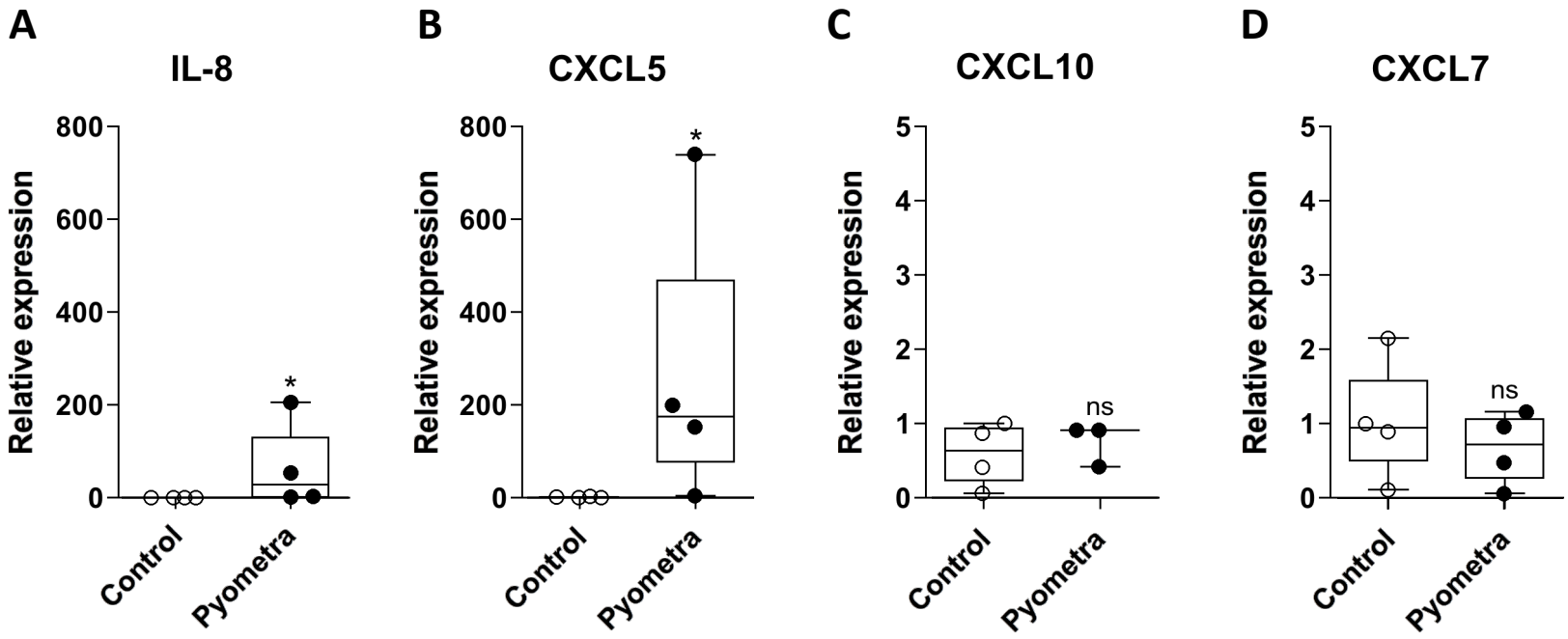

A
IL-8
Relative expression
120
100
80
60
40
20
0
***
***
***
Control
LPS Low
LPS Hi
E. coli Low
E.coli Hi
B
CXCL5
Relative expression
120
100
80
60
40
20
0
***
***
***
Control
LPS Low
LPS Hi
E. coli Low
E. coli Hi
C
CXCL10
Relative expression
60
50
40
30
20
10
0
***
***
**
Control
LPS Low
LPS Hi
E. coli Low
E. coli Hi
D
CXCL7
Relative expression
10
8
6
4
2
0
***
Control
LPS Low
LPS Hi
E. coli Low
E. coli Hi

Figure 4


A
IL-8
Relative expression
800
600
400
200
0
*
Control
Pyometra
B
CXCL5
Relative expression
800
600
400
200
0
*
Control
Pyometra
C
CXCL10
Relative expression
5
4
3
2
1
0
ns
Control
Pyometra
D
CXCL7
Relative expression
5
4
3
2
1
0
ns
Control
Pyometra