Title: Bovine lactoferrin suppresses inflammatory cytokine expression in endometrial stromal cells in chronic endometritis

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Highlights:

・Lactoferrin suppresses the expression of inflammatory cytokines in endometrial stromal cells with chronic endometritis.

・Lactoferrin suppresses the inflammation that induced TNF-α in endometrial stromal cells.

・AKT and MAPK are involved in the anti-inflammatory effect of lactoferrin on endometrial stromal cells.

Keywords: Chronic endometritis, Endometrial stromal cells, Lactoferrin

Authors' contributions: Conception and design: AN, FK; acquisition of data: AN, ST, TH, ATakebayashi, ATakahashi, JK, AM; analyzed the data: AN, FK, TH, ATakebayashi, ATakahashi, JK, AM, RK; drafting the manuscript: AN, FK; substantively revised it: ST, TA, RK, TM; final approval of the version: TM. All authors read and approved the final manuscript.

Ethics statement:

The authors confirm the ethical policies of the journal, as noted on the journal's author guidelines page. This research was approved by the Ethics Committee of Shiga University of Medical Science (R2014-090). The information on conducting the study was made public, and the opportunity for refusal was guaranteed as much as possible by optout as the Ethics Committee suggested.

Abstract

Chronic endometritis (CE) is a type of chronic inflammation in the endometrium that is associated with infertility, which is primarily due to implantation failure. Antibiotics are the most common treatment for CE. However, some patients with CE are resistant to antibiotic treatment, while others refuse this treatment. Therefore, we focused on lactoferrin (Lf), which exhibits antimicrobial and anti-inflammatory properties, and studied its effect on inflammation in endometrial stromal cells (ESCs) from patients with CE.

Endometrial tissue was collected from patients with CE, and ESCs were isolated and cultured. When ESCs were cultured with bovine lactoferrin (bLf: 1 mg/mL), the mRNA expression of TNF-α ($p < 0.05$) and IL-1β ($p < 0.01$) was significantly decreased compared with that in cells cultured without bLf. The level of TNF-α protein in the culture medium was significantly decreased ($p < 0.01$), while that of IL-1β was also decreased, but not significantly ($p < 0.10$), when 1 mg/mL of bLf was added to the culture medium. When more inflammation was induced artificially by adding 0.1 ng/mL of TNF-αto ESCs, the addition of bLf (1 mg/mL) to ESCs decreased IL-6 and IL-1β mRNA expression to levels similar to those in ESCs without TNF-α treatment. Furthermore, it was revealed that the actions of bLf are mediated by the AKT and MAPK intracellular signaling pathways, which are mechanisms by which the increase in TNF-α-induced cytokine expression is suppressed in ESCs. bLf suppresses the expression of inflammatory cytokines in human ESCs and may be a new therapeutic candidate for CE.

Main text

1. Introduction

The endometrium is essential for the implantation and maintenance of pregnancy. Since inflammation of the endometrium contributes to implantation failure, miscarriage, and preterm birth, the endometrium has various defense mechanisms against inflammation. In the endometrium, innate immunity resulting from natural antimicrobial components functions as well as defense through immunocompetent cells (King *et al.*, 2003).

Lactoferrin (Lf) is a glycoprotein responsible for the innate immunity of the endometrium and is expressed in the human endometrial glandular epithelium (Masson *et al.*, 1968). Lf is contained within mammalian exocrine secretions, such as breast milk, tears, saliva, bile, pancreatic juice, semen, and cervical mucus, as well as in the secondary granules of neutrophils (Levy, 2004, Vogel, 2012). Lf exerts various effects, such as iron binding ability and antimicrobial, anti-inflammatory, and antitumor activity. It has also been reported that Lf induces cell proliferation of endometrial stromal cells (ESCs) in human endometrium (Yanaihara *et al.*, 2000). Lf expression is estrogen-responsive, and in endometrial carcinoma and hyperplasia, Lf is overexpressed in endometrial glandular epithelial cells (Walmer *et al.*, 1995, Kelver *et al.*, 1996, Teng *et al.*, 2002), but any other effects are unknown.

Chronic endometritis (CE) is a disease characterized by continuous and subtle inflammation of the endometrium. CE is usually asymptomatic, but sometimes it presents only with non-specific symptoms, such as abnormal uterine bleeding, pelvic pain, dyspareunia, and leucorrhea (Rotterdam, 1978, Yörükoğlu and Kuyucouğlu, 1998). CE is pathologically diagnosed by plasma cell infiltration in the endometrial stromal region, where they are not typically present except just before and during menstruation (Crum *et al.*, 1983). Plasma cell expression suggests the presence of continuous immune response to some component of the endometrium (Kimura *et al.*, 2019).

CE has been suggested to be involved in infertility and implantation failure (Johnston-

MacAnanny *et al.*, 2010, Smith *et al.*, 2010, Hirata *et al.*, 2021). We have previously reported that CE decreases term birth and live birth rates per pregnancy and increases miscarriage rates (Morimune *et al.*, 2021). Antibiotics, such as doxycycline, ofloxacin, and metronidazole, currently comprise the most common treatments for CE, as administration of antibiotics to women with CE improves both clinical and pathological findings (McQueen *et al.*, 2014, Kitaya *et al.*, 2017). However, in some cases, CE is not cured even after several administrations of antibiotics. Moreover, some patients do not want to take antibiotics because their frequent use can lead to disruption of microbial flora in the body, including in the intestines. The development of a method to regulate inflammation in the endometrium of patients with CE without the use of antibiotics is expected. Therefore, in this study, we investigated whether Lf exerts an anti-inflammatory effect on the human endometrium.

2. Materials and methods

2.1. Ethics

The study protocol was reviewed and approved by the Ethics Committee/Institutional Review Board of the Shiga University of Medical Science (No. R2014-090), and the study was conducted according to the guidelines described in the Declaration of Helsinki. Written informed consent was provided by all subjects before study inclusion.

2.2. Patients and samples

The participants were women undergoing infertility treatment by *in vitro* fertilization-embryo transfer (IVF-ET) at our institute from January 2020 to March 2021. None of the patients was diagnosed with immune diseases, cancer, or diseases that increase susceptibility to infections. We collected patient data from the medical records.

Endometrial tissue was collected during the mid-secretory phase. To collect endometrial tissue during the implantation period, ovulation was predicted by urinary luteinizing hormone

measurements. Patients planned to undergo hysteroscopy and curettage 5–9 days after a positive urine test. Endometrial tissue obtained from each patient during the first curettage was used to diagnose CE. The endometrial tissues were fixed in 10% formaldehyde, embedded in paraffin, and sectioned at a 4 μm thickness. The sections were immunostained with an anti-CD138 antibody (product No. B-A38, Nichirei Corp. Tokyo, Japan), as previously reported (Wu *et al.*, 2017). One pathologist examined these specimens. When one or more CD138-positive plasma cells in 10 high-power fields (HPFs) (a field magnified 400 times under a microscope) were observed, the patient was diagnosed with CE. In this study, only the endometrium of patients diagnosed with CE was cultured with bLf and used for the experiment. The endometrium of patients without CE was cultured for comparison with CE.

To obtain endometrial tissue for cell culture experiments, mild curettage was performed several times. On the day of hysteroscopy and curettage, blood samples were obtained to measure serum levels of estradiol (E2) and progesterone (P4). Patients with E2 levels less than 50 pg/mL and P4 levels less than 6 ng/mL were excluded.

2.3. Isolation and culture of ESCs

Endometrial tissues were used to derive primary cells, as previously reported (Yamanaka *et al.*, 2014, Wu *et al.*, 2017). Briefly, minced endometrial tissue mixed with 0.2% collagenase (Sigma-Aldrich, St. Louis, MO, USA) and 0.005% deoxyribonuclease I (Worthington Biochemical Co., Lakewood, NJ, USA) was incubated for 1 h at 37°C in a humidified atmosphere of 5% CO_2 in the air with gentle pipetting every 15 min. After digestion, the same volume of DMEM/F12 (1:1) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% charcoal-treated FBS (HyClone, GE Healthcare Life Sciences, Pittsburgh, PA) was added to inactivate the collagenase, after which the cell suspension was placed in an upright position for 10 min to allow large glandular structures to sink to the bottom of the tube. Then, the supernatant containing a rich fraction of stromal cells was filtered through a 70-μm cell

strainer (Falcon Life Sciences, Corning, NY, USA) followed by centrifugation for 3 min at room temperature. After the pellet was resuspended, 2.2×10^6 viable cells per dish were transferred to a 10-cm dish (Thermo Fisher Scientific). The ESCs were cultured in 10 mL of DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. After 1 h, the culture medium was exchanged for a fresh medium. The culture medium was changed every two or three days. By immunocytochemistry for vimentin, cytokeratin, vWF, and α-smooth muscle actin, we confirmed that the purity of ESCs was more than 95%. When the ESCs reached confluence, they were seeded into a 24-well plate (Thermo Fisher Scientific) at a density of 10^5 cells per well and were used for the experiments described below.

2.4. ELISA of cell culture supernatant

The ESCs were cultured with or without bLf (0 ng/mL, 1 ng/mL, 1 µg/mL, or 1 mg/mL, Tatura Milk Industries Ltd., Tatura, Australia) at 37°C. The culture medium was changed 24 h before the collection of culture media and cell harvest. The culture medium was collected, centrifuged at 15,000 rpm at 4°C for 10 min after which the supernatant was collected and stored at −80°C for further analysis. The numbers of ESCs in each well were counted using an inverted microscope with a cell counter after exposure to 0.3 mL of trypsin for several minutes followed by deactivation after the addition of 0.3 mL of DMEM/F12 with 10% FBS.

TNF-α, IL-1β, and IL-6 levels in the cell culture supernatant were measured using ELISA kits (Human TNF-α Quantikine ELISA Kit, Product No. DTA00D, Human IL-1β/IL-1F2 Quantikine ELISA Kit, Product No. DLB50, Human IL-6 Quantikine ELISA Kit, Product No. D6050, R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturers' instructions.

2.5. RNA extraction from cultured cells and real-time polymerase chain reaction

ESCs were cultured with or without bLf (0 ng/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL, 1 µg/mL,

10 μg/mL, 100 μg/mL, or 1 mg/mL) at 37°C for 24 h. In subsequent experiments, ESCs were cultured with bLf, TNF-α (0.1 or 1 ng/mL, R&D Systems Inc.), and anti-Toll-like receptor 4 (TLR4) antibody (10 μg/mL, Hycult Biotech, Uden, The Netherlands). Regarding the PI3K/Akt and MAPK pathway, we cultured ESCs added LY294002 (10 μM, Sigma-Aldrich) as a PI3K/Akt inhibitor and U0126 (10 μM, Sigma-Aldrich) as a MAPK inhibitor with bLf and TNF-α.

Total RNA was extracted from ESCs using an RNeasy Micro kit (QIAGEN, K.K., Tokyo, Japan) according to the manufacturer's instructions. Each sample (500 ng) was reverse transcribed using Prime Script RT Master Mix (Takara Biotechnology, Shiga, Japan). Real-time PCR was performed using the LightCycler 480 SYBR Green I Master (Roche, Castle Hill, Australia) with specific primers for TNF-α, IL-1β, IL-6, and GAPDH, as follows: TNF-α, F:5′-CTGCCTGCTGCACTTTGGAG-3′ and R:5′-ACATGGGCTACAGGCTTGTCACT-3′ (Takara); IL-1β, F:5′-CCAGGGACAGGATATGGAGCA-3′ and R:5′-TTCAACACGCAGGACAGGTACAG-3′ (Takara); IL-6, F:5′-AAGCCAGAGCTGTGCAGATGAGTA-3′ and R:5′-TGTCCTGCAGCCACTGGTTC-3′ (Takara); GAPDH, F:5′-AAATCCCATCACCATCTTCCA-3′ and R:5′-AATGAGCCCCAGCCTTCTC-3′ (Sigma-Aldrich). The mixture of reaction reagents was incubated at 95°C for 5 min and cycled according to the following parameters: 95°C for 10 s, 59°C for 15 s, and 72°C for 10 s for a total of 45 cycles, followed by cooling for 10 s at 40°C in the LightCycler 480TL system II (Roche). Melting curve analysis was also performed.

2.6. Western blot analysis

ESCs were cultured with or without bLf (1 mg/mL) at 37°C for 24 h. Then, TNF-α (0.1 ng/mL) was added to the medium for the indicated times for time course experiments. Incubation was terminated by aspiration of the medium, two washes in ice-cold PBS, and the addition of 50 μL RIPA buffer (Nacalai Tesque Inc., Kyoto, Japan). After scraping the ESCs, 200 μL of RIPA

233 buffer was added to collect the lysate, which was centrifuged at 10,000 × g at 4°C for 10 min to
234 obtain supernatant. The supernatant was collected and stored at −80°C until Western blot was
235 performed.
236 Equal quantities of proteins (30 μg) were heated in 2 × Laemmli buffer (Bio-Rad Laboratories
237 Inc., Richmond, CA) under reducing condition at 95°C for 5 min and were separated by
238 electrophoresis on 4%–20% SDS-polyacrylamide gels after which the proteins were transferred
239 to polyvinylidene difluoride membranes (Bio-Rad Laboratories Inc.). The membranes were then
240 incubated with 3% BSA at room temperature for 1 h to block non-specific protein binding sites,
241 followed by incubation with primary antibodies at 4°C overnight. The following antibodies were
242 used: p-AKT (9271; 1:1000; Cell Signaling Technology, Danvers, MA), AKT antibody (9272;
243 1:1000; Cell Signaling Technology), p-p38 (4511; 1:1000; Cell Signaling Technology), p38
244 (9212; 1:1000; Cell Signaling Technology), p-NFκB (3033; 1:1000; Cell Signaling
245 Technology), NFκB (8242; 1:1000; Cell Signaling Technology) and βactin (A5441; 1:10000;
246 Sigma-Aldrich) diluted in Can Get Signal (Toyobo Co Ltd., Osaka, Japan). After washing with
247 TBST 3 times for 10 min each time, the membranes were reacted with a peroxidase-labeled
248 anti-rabbit or anti-mouse IgG (7074 or 7076; 1:5000; Cell Signaling Technology) for 1 h at
249 room temperature. Peroxidase labeling was detected by chemiluminescence using the Chemi-
250 Lumi One Super (Nacalai Tesque Inc.). Image J (National Institutes of Health, USA) was used
251 to quantify the ratios of AKT to βactin, p-AKT to βactin, p-AKT to AKT, p38 to βactin, p-p38 to
252 βactin, p-p38 to p38, NFκB to βactin, p-NFκB to βactin and p-NFκB to NFκB.
253
254 **2.7. Statistical analysis**
255 All data are expressed as means ± standard error of the mean. A *t*-test or Mann–Whitney test
256 was performed for comparisons between two groups based on whether they followed a Gaussian
257 distribution, and one-way ANOVA was performed for comparisons between three or more
258 groups using GraphPad Prism ver.7.00. Both unpaired and paired analyses were performed. A

value of $P < 0.05$ was considered significant.

3. Results

ESCs from 15 CE and seven non-CE patients were cultured and compared for mRNA expression of inflammatory cytokine (Table 1). At the mRNA level, expression of TNF-α and IL-6 was significantly higher in CE patients than in non-CE patients (Fig. 1a, c). No significant difference in IL-1β expression was observed between CE and non-CE (Fig. 1b). Endometrial tissues for cell culture added bLf were obtained from eight patients with CE (Table 2). At the mRNA level, significant differences were observed in TNF-α and IL-1β according to one-way ANOVA. Expression of TNF-α and IL-1β was significantly decreased in cells cultured with bLf (1 mg/mL) compared with those cultured without bLf (Fig. 1d, e). No significant difference in IL-6 expression was observed in cells treated with and without bLf (Fig. 1f).

In cell culture supernatants, tendency for differences was found in the concentrations of TNF-α and IL-1β proteins according to the one-way ANOVA. The concentration of TNF-α secreted from the cells was significantly decreased, and the concentration of IL-1β was decreased in cells cultured with bLf (1 mg/mL) compared with those without bLf, but the decrease was not significant (Fig. 2).

TNF-α with or without bLf was added to ESCs obtained from nine patients (Table 3). At the mRNA level, IL-6 expression was significantly increased in cells treated with 0.1 or 1 ng/mL TNF-α compared with the level in cells not treated with TNF-α (Fig. 3a, b). With the addition of 0.1 ng/mL TNF-α and 1 mg/mL bLf, IL-6 mRNA expression was decreased to the same level seen in cells without TNF-α and bLf treatment (Fig. 3a). When 1 ng/mL TNF-α and 1 mg/mL bLf were added, IL-6 mRNA expression tended to decrease compared with the level in cells not treated with bLf, but the IL-6 level did not decrease to the same level as the control (Fig. 3b). IL-1β mRNA expression was induced by 0.1 ng/mL TNF-α and suppressed by 1 mg/mL bLf in a manner similar to that of IL-6 (Fig. 3c). Moreover, 1 ng/mL TNF-α induced IL-1β mRNA

expression, but no significant change was observed with the addition of bLf (Fig. 3d). Addition of anti-TLR4 antibody to the culture medium did not reverse the decreased mRNA expression of IL-6 and IL-1β induced by bLf (Fig. 3e, f).

Western blot analysis showed maximum expression of AKT and phosphorylated AKT when TNF-α was added to ESCs for 5 min without bLf. The addition of 1 mg/mL bLf suppressed TNF-α-induced AKT expression (Fig. 4a). Compared with βactin, protein expression of AKT and p-AKT induced by the addition of TNF-α for 5 min was most significantly suppressed by 1 mg/mL bLf (Fig. 4b, c). Similarly, the addition of TNF-α to ESCs for 5 min without bLf showed maximum expression of MAPK p38 and phosphorylated p38 (Fig. 4a). Compared with βactin, TNF-α-induced p38 protein expression was significantly suppressed by 1 mg/mL bLf, and p-p38 expression tended to be suppressed (Fig. 4e, f). There was no significant change in the ratios of p-AKT to AKT and p-p38 to p38 with or without bLf (Fig. 4d, g). TNF-α-induced protein expression of NFκB and p-NFκB did not differ significantly with or without bLf (Fig 4h, i, j, k).

Regarding the PI3K/Akt pathway and MAPK pathway, LY294002 as a PI3K/Akt inhibitor and U0126 as a MAPK inhibitor were added with bLf and TNF-α to culture ESCs and analyzed mRNA expression of IL-6 and IL-1β. Examination of five samples did not confirm that a PI3K/Akt inhibitor or a MAPK inhibitor offset the anti-inflammatory effects of bLf on ESCs (Supplemental fig. 1).

4. Discussion

In this study, we found that the addition of bLf to cultured ESCs from patients with CE decreased the expression of inflammatory cytokines. In addition, bLf administration to ESCs in which inflammation was artificially induced with TNF-α markedly suppressed that inflammation. This effect was not mediated by TLR4, as administration of an anti-TLR4 antibody did not alter this suppressive effect. Furthermore, AKT and MAPK induction, which

311 plays a central role in the promotion of cellular inflammation, in ESCs by TNF-α was
312 suppressed by bLf. These results show that bLf suppresses inflammation of the human
313 endometrial stroma. Regarding the anti-inflammatory effect of Lf, some studies have reported
314 sepsis (Lepanto *et al.*, 2019), necrotizing enterocolitis in newborns (Chatterton *et al.*, 2013,
315 Lepanto *et al.*, 2019), and endometritis in mares (Fedorka *et al.*, 2017), but this is the first report
316 to show the effect of Lf on inflammation in human endometrium.
317 Unfortunately, no information is available on bLf blood levels after oral administration of bLf,
318 although there has been an article showing that human Lf levels fluctuate after oral
319 administration of bLf (Iigo *et al.*, 2014). Moreover, bLf has been reported to accumulate in
320 inflammatory areas in mice (Yanagisawa *et al.*, 2022). In other words, the results of this study
321 infer that local concentrations of bLf are much higher where inflammation is present after oral
322 administration of bLf. On the other hand, the concentration of Lf in human such as cervical
323 mucus, blood, and breast milk (1-7 mg/mL), which is the highest concentration in the human
324 body have been reported. Lf has also been shown to be secreted by neutrophils, and it seems
325 that its concentration is considerably higher in the endometrium around Lf is secreted. In the
326 present study, the concentration of bLf was determined based on these factors.
327 Lf has been reported to exert anti-inflammatory effects in various tissues. It has been reported
328 that the function of Lf is mediated by the PI3K/Akt and NFκB pathways in oral squamous cell
329 carcinoma (Chea *et al.*, 2018), the PI3K/Akt and MAPK pathways in the human keratinocyte
330 cell line HaCat (Uchida *et al.*, 2017), TLR4 and NFκB in the monocytic leukemia cell line THP-
331 1 (Ando *et al.*, 2010), and the C-X-C-motif cytokine receptor 4 (CXCR4) in HaCat cells and
332 Caco-2 colon cancer cells (Takayama *et al.*, 2017). We found that the anti-inflammatory effect
333 of bLf on human ESCs is mediated by the intracellular signaling pathway AKT and MAPK.
334 Compared with βactin, protein expression of AKT, p-AKT, MAPK p38 and phosphorylated p38
335 induced by the addition of TNF-α was suppressed by bLf. Moreover, although a PI3K/Akt
336 inhibitor or a MAPK inhibitor was administered in combination with bLf to determine by what

mechanism mRNA expression of inflammatory cytokines is suppressed in the cultured ESCs, they did not modify the effect of bLf. From these results, bLf is thought to work via the PI3K/Akt and MAPK signaling. As this study showed that the action of Lf on ESCs is not mediated by TLR4, which is a representative Toll-like receptor that recognizes molecules characteristic of pathogens, it appears that bLf does not directly affect TLR4 signaling. In addition, bLf did not modify the NFκB protein level and its phosphorylation. In this study, although representative intracellular signals were examined, Lf has a variety of effects, and further studies on the additional effects of Lf on ESCs are warranted.

Microorganisms detected in the uterine cavity of patients with CE are usually common bacteria (*Streptococcus* species, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Staphylococcus* species, and *Corynebacterium*) and mycoplasma species (*Mycoplasma genitalium* and *Ureaplasma urealyticum*), which are also present in the uterine cavity of healthy women (Cicinelli *et al.*, 2008, Cicinelli *et al.*, 2009, Kitaya *et al.*, 2017). It is believed that the immunological response to these bacteria, rather than their presence, is involved in CE development (Kimura *et al.*, 2019). When antibiotics are administered to women diagnosed with CE, pathologically, the plasma cells present in the endometrial stromal region are reduced or eliminated, and clinically, the pregnancy rate improves and the miscarriage rate decreases (McQueen *et al.*, 2014, Kitaya *et al.*, 2017). This is thought to be due to changes in the bacterial flora in the uterus caused by antibiotics. On the contrary, although we have demonstrated that bLf directly suppresses inflammation of the endometrium in CE, clinically, bLf may regulate the causative microorganism of CE via its antimicrobial action. To answer these remaining questions, we await the results of future studies.

The strength of the present study is that it shows that bLf suppressed inflammation in both endometrial stromal cells of patients with CE and those further inflamed with TNF-α. bLf inhibited the TNF-α- induced cycle of inflammation amplification in the endometrium of patients with CE. We also show that bLf is an efficient inhibitor of inflammation.

The limitation of this study is that suppression of inflammatory cytokine expression in ESCs and the curing of CE are not equal. It is unclear whether Lf alters plasma cell infiltration into the endometrial stromal region. Inflammatory cytokine expression in the endometrium adversely affects implantation and continued pregnancy, and suppression of inflammatory cytokine expression may contribute to the improvement of infertility treatment and obstetric prognosis. Another limitation of this study is that the diagnostic criteria for CE have not yet been established, and cases with equal to less than 4 plasma cells/HPF that might be classified in the non-CE group according to other criteria are included in the CE group in this study. We have conducted a prospective study on how to establish diagnostic criteria for CE based on the results of infertility treatment at our hospital (Hirata et al., 2021). The results showed that the most valid criterion should be the presence of even one plasma cell found in 10 HPFs. We adopted this criterion in the present study. Because of this, in Figures 1-3, the results for patients with plasma cells of 5 or more cells in 10 HPFs are marked with white symbols, and those with 1-4 cells are marked with black symbols. Furthermore, the expression of each inflammatory cytokine was found to be significantly different between CE and non-CE groups. Including these results, this study uses the diagnostic criterion of at least one plasma cell in 10 HPFs to diagnose CE.

5. Conclusions

In conclusion, bLf suppresses the expression of inflammatory cytokines in human ESCs. Lf may be a new therapeutic candidate for CE cases that are resistant to antimicrobial treatment.

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Declaration of Competing Interest

None of the authors has any conflict of interest related to this manuscript.

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Figure Legends

Figure 1. The mRNA levels of TNF-α, IL-1β, IL-6 in endometrial stromal cells (ESCs) cultured in the absence (control) or presence of bovine lactoferrin (Lf).

a. The mRNA level of TNF-α in ESCs from patients with chronic endometritis (CE) and without CE (nCE) cultured without bovine Lf.

b. The mRNA level of IL-1β in ESCs from CE and nCE patients cultured without bovine Lf.

c. The mRNA level of IL-6 in ESCs from CE and nCE patients cultured without bovine Lf.

d. The mRNA level of TNF-α in ESCs from CE patients.

e. The mRNA level of IL-1β in ESCs from CE patients.

f. The mRNA level of IL-6 in ESCs from CE patients.

In this figure, the results for patients with plasma cells of 5 or more cells in 10 HPF are marked with white symbols, and those with 1-4 cells are marked with black symbols.

*$p<0.01$, **$p<0.05$

Figure 2. Secretion of TNF-α and IL-1β from endometrial stromal cells (ESCs) cultured in the absence (control) or presence of bovine lactoferrin (Lf).

a. The concentration of TNF-α in the medium of cultured ESCs evaluated by ELISA.

b. The concentration of IL-1β analyzed by ELISA.

In this figure, the results for patients with plasma cells of 5 or more cells in 10 HPF are marked with white symbols, and those with 1-4 cells are marked with black symbols.

*$p<0.01$, ***$p<0.10$

Figure 3. The mRNA levels of IL-6 and IL-1β induced by TNF-α in endometrial stromal cells (ESCs) cultured with or without bovine lactoferrin (bLf) and anti-Toll-like receptor 4 (TLR4) antibody.

a. The mRNA level of IL-6 induced by 0.1 ng/mL of TNF-α in ESCs.

b. The mRNA level of IL-6 induced by 1 ng/mL of TNF-α.

c. The mRNA level of IL-1β induced by 0.1 ng/mL of TNF-α.

d. The mRNA level of IL-1β induced by 1 ng/mL of TNF-α.

e. Effect of 1 mg/mL of bLf and 10 μg/mL of anti-TLR4 antibody on the IL-6 mRNA level induced by 0.1 ng/mL of TNF-α in ESCs.

f. Effect of 1 mg/mL of bLf and 10 μg/mL of anti-TLR4 antibody on the IL-1β mRNA level induced by 0.1 ng/mL of TNF-α.

In this figure, the results for patients with plasma cells of 5 or more cells in 10 HPF are marked with white symbols, and those with 1-4 cells are marked with black symbols.

*p<0.01, **p<0.05, ***p<0.10, †not significant

Figure 4. The time-course analysis of TNF-α-induced AKT and MAPK p38 protein expression in endometrial stromal cells (ESCs).

The ESCs cultured with or without 1 mg/mL of bovine lactoferrin (bLf) were exposed to a medium with 0.1 ng/mL of TNF-α for 0, 5, 10, 20 or 60 min.

a. Protein expression of p-AKT, AKT, p-p38, p38 and βactin using western blot.

b. The ratio of protein expression of AKT to βactin in ESCs cultured with or without bLf.

c. The ratio of p-AKT to βactin in ESCs cultured with or without bLf.

d. The ratio of p-AKT to AKT in ESCs cultured with or without bLf.

e. The ratio of protein expression of p38 to βactin in ESCs cultured with or without bLf.

f. The ratio of p-p38 to βactin in ESCs cultured with or without bLf.

g. The ratio of p-p38 to p38 in ESCs cultured with or without bLf.

h. Protein expression of p-NFκB, NFκB and βactin using western blot.

i. The ratio of protein expression of NFκB to βactin in ESCs cultured with or without bLf.

j. The ratio of p-NFκB to βactin in ESCs cultured with or without bLf.

k. The ratio of p-NFκB to NFκB in ESCs cultured with or without bLf.

*p<0.01, **p<0.05, ***p<0.10

Supplemental figure 1. The mRNA levels of IL-6 and IL-1β induced by TNF-α in endometrial stromal cells (ESCs) cultured with or without bovine lactoferrin (bLf) and PI3K/Akt inhibitor (LY294002) or MAPK inhibitor (U0126).

a. Effect of 1 mg/mL of bLf and 10 μM of PI3K/Akt inhibitor or MAPK inhibitor on the IL-6 mRNA level induced by 0.1 ng/mL of TNF-α in ESCs.

b. Effect of 1 mg/mL of bLf and 10 μM of PI3K/Akt inhibitor or MAPK inhibitor on the IL-1β mRNA level induced by 0.1 ng/mL of TNF-α.

**p<0.05

578 **Tables**

579 **Table 1. Patient characteristics in the cell culture experiment for comparison of mRNA**

580 **expression in ESCs from CE and non-CE patients**

	CE n = 15	non-CE n = 7	P value
Age, median (IQR)	37.00 (30.00-40.00)	33.00 (28.00-40.00)	n.s.
Gravidity, median (IQR)	0 (0-2)	0 (0-2)	n.s.
Parity, median (IQR)	0 (0-1)	0 (0-1)	n.s.
Serum level of estradiol (IQR) (pg/mL)	166.00 (86.90-325.80)	187.30 (90.10-360.4)	n.s.
Serum level of progesterone (IQR) (ng/dL)	17.96 (11.51-31.28)	22.37 (15.58-25.80)	n.s.
Body mass index (IQR) (kg/m^2)	23.70 (17.28-32.24)	20.60 (19.35-25.39)	n.s.
Number of plasma cells (IQR) (/10HPFs)	2.0 (1-10)	0	
Infertility cause			
Ovarian factor	0	0	
Tubal factor	2	0	
Endometriosis	5	1	
Male factor	2	2	
Fertilization failure	0	0	
Immune factor	0	0	
Unexplained	6	4	

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588 **Table 2. Patient characteristics in the cell culture experiment with lactoferrin**

	n = 8
Age, median (IQR)	34.50 (28.00-40.00)
Gravidity, median (IQR)	0.50 (0-2.00)
Parity, median (IQR)	0
Serum level of estradiol (IQR) (pg/mL)	154.80 (52.80-325.80)
Serum level of progesterone (IQR) (ng/dL)	21.47 (8.07-31.28)
Body mass index (IQR) (kg/m^2)	23.03 (17.97-26.80)
Number of plasma cells (IQR) (/10HPFs)	4.5 (1-26)
Infertility cause	
Ovarian factor	0
Tubal factor	2
Endometriosis	2
Male factor	0
Fertilization failure	0
Immune factor	0
Unexplained	4

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598 **Table 3. Patient characteristics in the cell culture experiment with lactoferrin and TNF-α**

	n = 9
Age, median (IQR)	36.00 (29.00-39.00)
Gravidity, median (IQR)	1.00 (0-2.00)
Parity, median (IQR)	1.00 (0-2.00)
Serum level of estradiol (IQR) (pg/mL)	135.00 (53.70-222.50)
Serum level of progesterone (IQR) (ng/dL)	15.05 (6.28-20.32)
Body mass index (IQR) (kg/m^2)	22.01 (16.01-29.55)
Number of plasma cells (IQR) (/10HPFs)	4.0 (1-15)
Infertility cause	
Ovarian factor	0
Tubal factor	0
Endometriosis	4
Male factor	1
Fertilization failure	0
Immune factor	0
Unexplained	4

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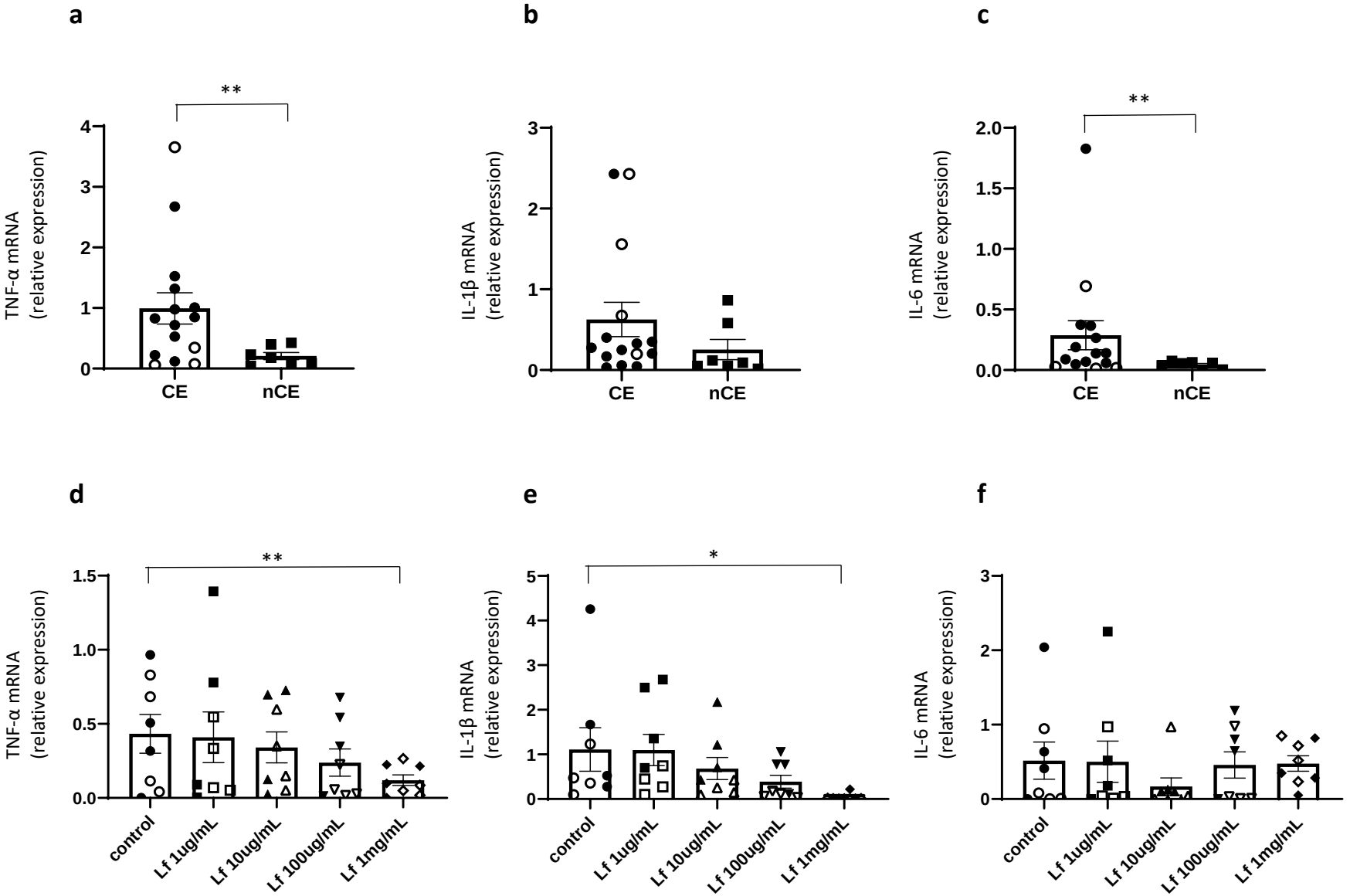
Figure 1


a
b
c
d
e
f
TNF-α mRNA (relative expression)
IL-1β mRNA (relative expression)
IL-6 mRNA (relative expression)
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*
CE
nCE
control
Lf 1ug/mL
Lf 10ug/mL
Lf 100ug/mL
Lf 1mg/mL

Figure 2

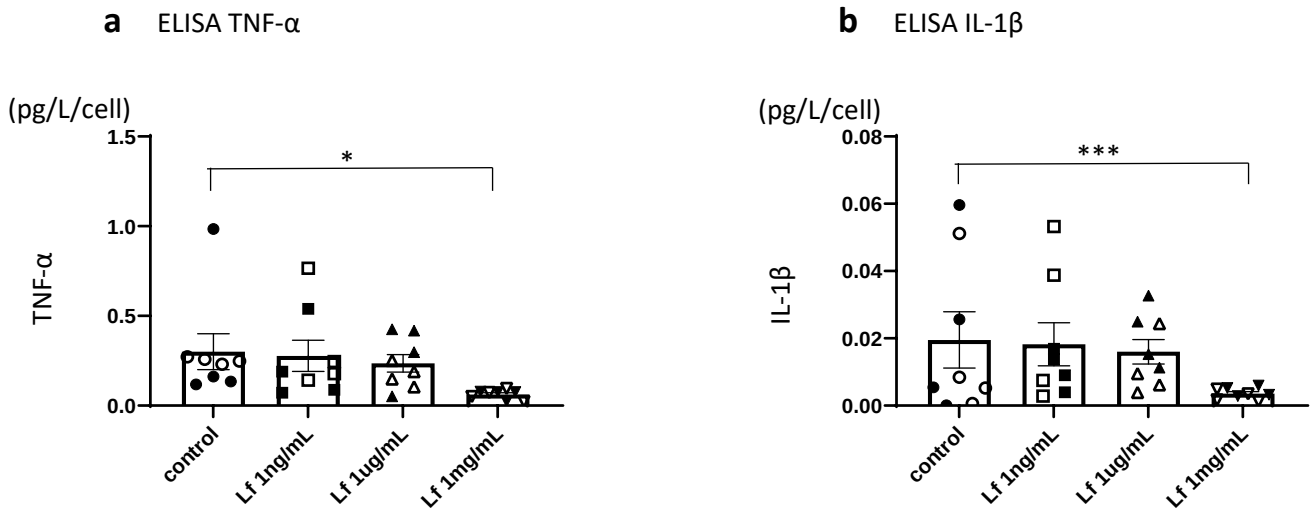

a
ELISA TNF-α
(pg/L/cell)
TNF-α
1.5
1.0
0.5
0.0
*
control
Lf 1ng/mL
Lf 1ug/mL
Lf 1mg/mL
b
ELISA IL-1β
(pg/L/cell)
IL-1β
0.08
0.06
0.04
0.02
0.00
***
control
Lf 1ng/mL
Lf 1ug/mL
Lf 1mg/mL

Figure 3

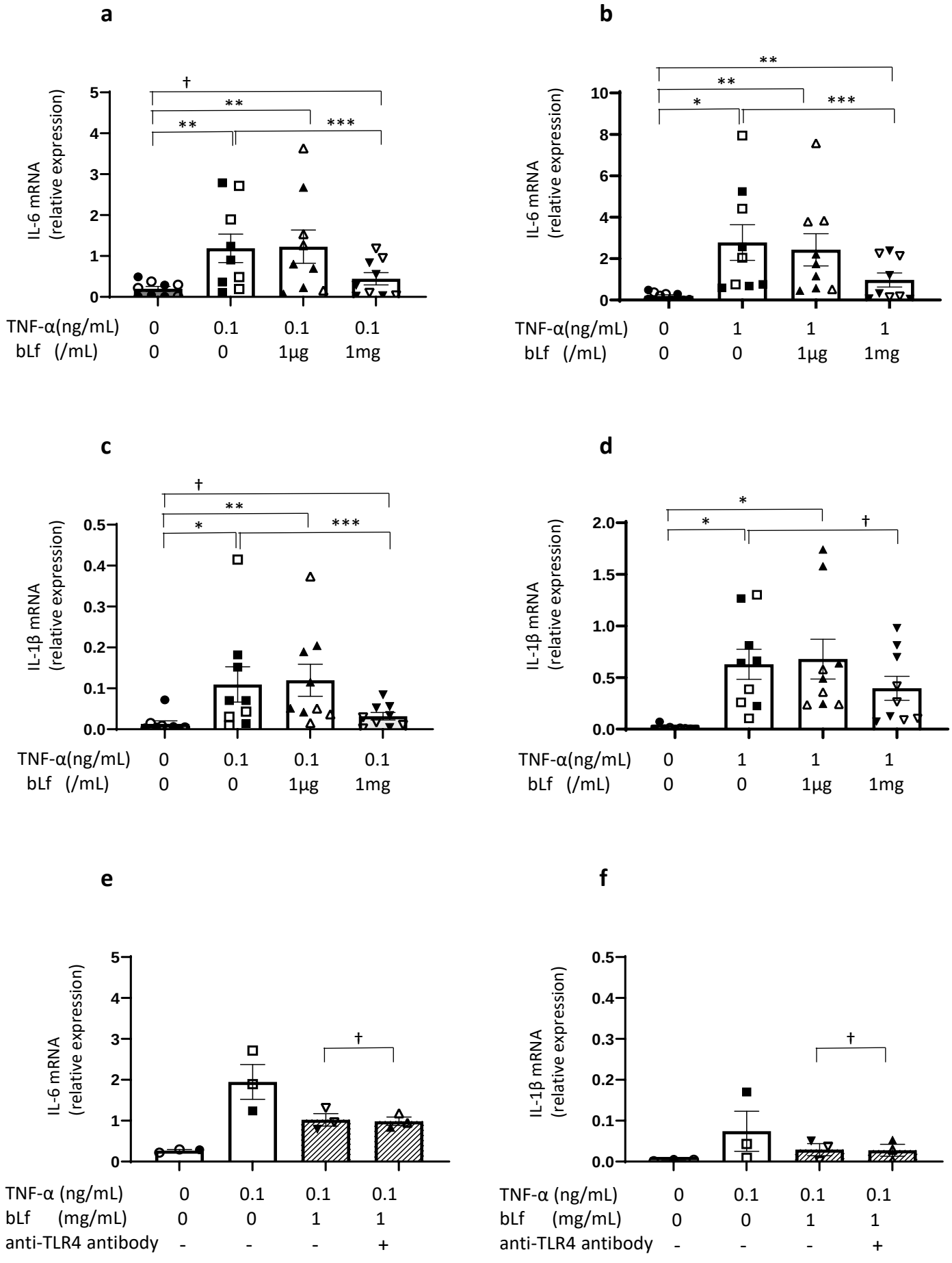
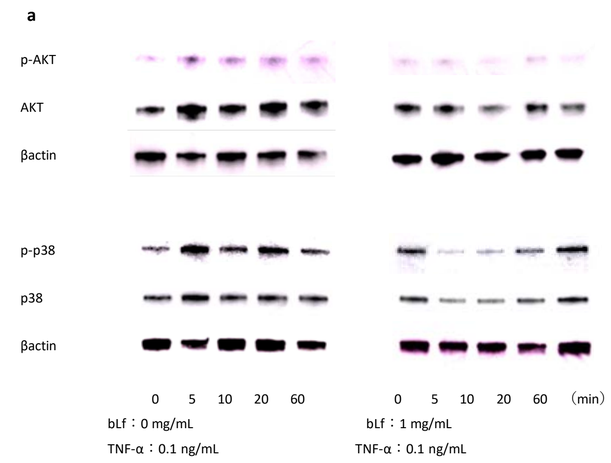
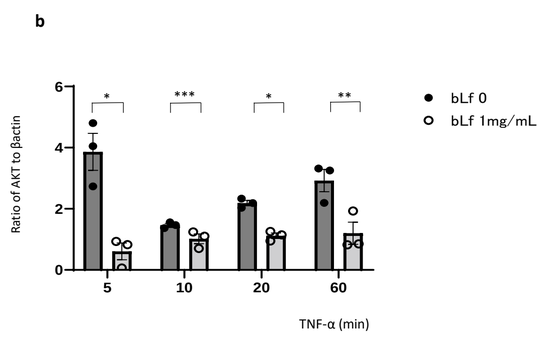
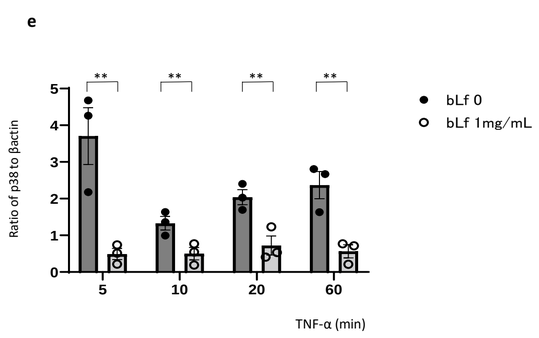
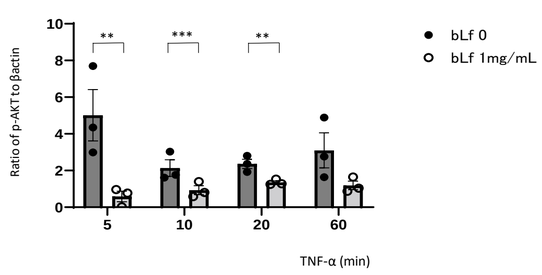

a
IL-6 mRNA (relative expression)
TNF-α(ng/mL) 0 0.1 0.1 0.1
bLf (/mL) 0 0 1μg 1mg
b
IL-6 mRNA (relative expression)
TNF-α(ng/mL) 0 1 1 1
bLf (/mL) 0 0 1μg 1mg
c
IL-1β mRNA (relative expression)
TNF-α(ng/mL) 0 0.1 0.1 0.1
bLf (/mL) 0 0 1μg 1mg
d
IL-1β mRNA (relative expression)
TNF-α(ng/mL) 0 1 1 1
bLf (/mL) 0 0 1μg 1mg
e
IL-6 mRNA (relative expression)
TNF-α (ng/mL) 0 0.1 0.1 0.1
bLf (mg/mL) 0 0 1 1
anti-TLR4 antibody - - - +
f
IL-1β mRNA (relative expression)
TNF-α (ng/mL) 0 0.1 0.1 0.1
bLf (mg/mL) 0 0 1 1
anti-TLR4 antibody - - - +

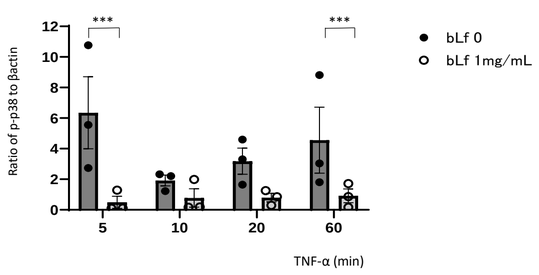
Figure 4

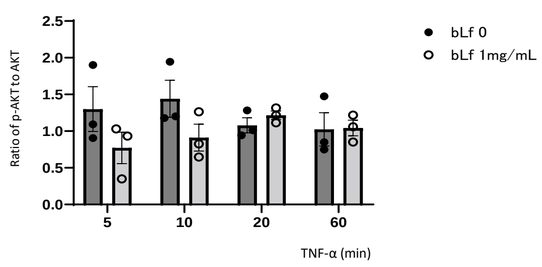

a
p-AKT
AKT
βactin
p-p38
p38
βactin
0 5 10 20 60
0 5 10 20 60 （min）
bLf：0 mg/mL
TNF-α：0.1 ng/mL
bLf：1 mg/mL
TNF-α：0.1 ng/mL

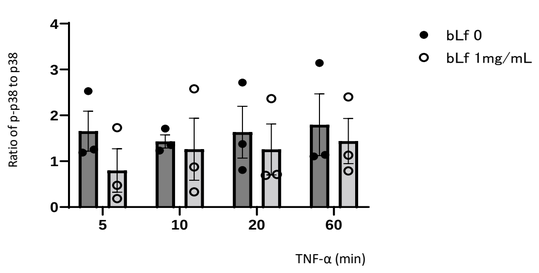

b
Ratio of AKT to βactin
TNF-α (min)
bLf 0
bLf 1mg/mL

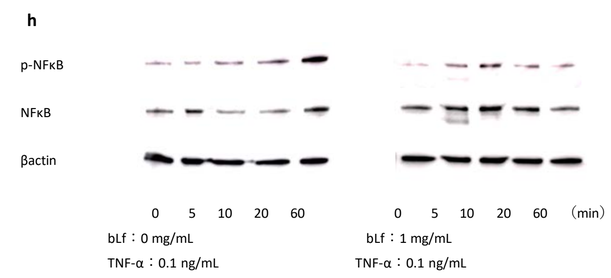

e
Ratio of p38 to βactin
TNF-α (min)
bLf 0
bLf 1mg/mL

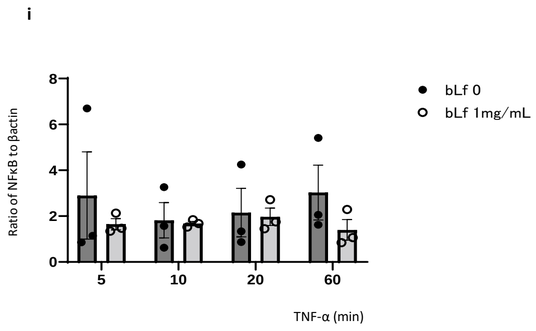

c
Ratio of p-AKT to βactin
TNF-α (min)
bLf 0
bLf 1mg/mL

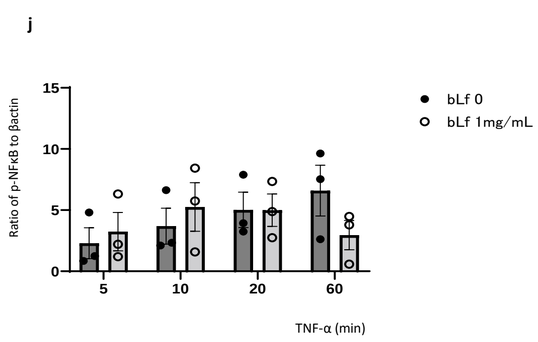

f
Ratio of p-p38 to βactin
TNF-α (min)
bLf 0
bLf 1mg/mL

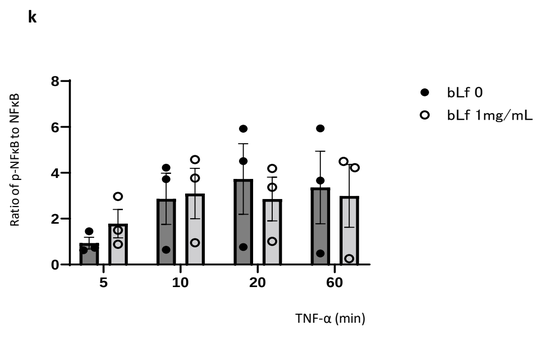

d
Ratio of p-AKT to AKT
TNF-α (min)
bLf 0
bLf 1mg/mL


g
Ratio of p-p38 to p38
TNF-α (min)
bLf 0
bLf 1mg/mL


h
p-NFκB
NFκB
βactin
0 5 10 20 60
0 5 10 20 60 （min）
bLf：0 mg/mL
TNF-α：0.1 ng/mL
bLf：1 mg/mL
TNF-α：0.1 ng/mL


i
Ratio of NFκB to βactin
TNF-α (min)
bLf 0
bLf 1mg/mL


j
Ratio of p-NFκB to βactin
TNF-α (min)
bLf 0
bLf 1mg/mL


k
Ratio of p-NFκB to NFκB
TNF-α (min)
bLf 0
bLf 1mg/mL

Supplemental figure 1