

# Up-regulation of CMKLR1 in endometriosis and its relationship with inflammatory responses

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**Summary.** Inflammation plays a critical role in the pathogenesis of endometriosis. We aimed to study the proinflammatory effect of Chemerin chemokine-like receptor 1 (CMKLR1) in patients with endometriosis. Sixty patients with endometriosis and 50 healthy controls were recruited in this study for the collection of endometrial samples and peritoneal fluid. The expression levels of CMKLR1, IL-6, MCP-1, and TNF- $\alpha$  in peritoneal fluid and endometrial tissues were detected by ELISA, qRT-PCR, and immunohistochemical staining. Human endometrial stromal cells (HESCs) were used to measure the Chemerin-induced CMKLR1 activation and inflammatory responses. CMKLR1 level was significantly up-regulated in peritoneal fluid and endometrial tissues in patients with endometriosis. Interestingly, CMKLR1 overexpression positively correlated with pro-inflammatory cytokines and chemokine in both peritoneal fluid and ectopic endometrium. Chemerin treatment increased the expression of CMKLR1, and aggravated inflammatory responses in HESCs. CMKLR1 is up-regulated in peritoneal fluid and endometrial tissues, and promotes the inflammatory responses in of endometriosis.

**Key words:** CMKLR1, Endometriosis, Inflammatory responses, Chemerin

## Introduction

Endometriosis is a chronic, estrogen-dependent, inflammatory disease characterized by the presence of ectopic endometrium, which cause pain, lesion progression, and infertility (Giudice, 2010). Endometrial tissues (glands and stroma) appear, grow, infiltrate, and repeatedly bleed outside of the endometrium, and can form nodules and masses, therefore, endometriosis is a benign disease with characteristics of malignant tumor

disease (Greene et al., 2016). Endometriosis causes dysmenorrhea, pelvic pain, infertility, and so on (Fourquet et al., 2011). The incidence of endometriosis in women of childbearing age is about 15%, and about 20-50% of infertile population suffer from endometriosis, and the age of onset of endometriosis has tended to be younger recently (Koninckx et al., 2012). Moreover, endometriosis increases the risk of ovarian cancer by about 50% compared to the cases among normal population (Vercellini et al., 2014).

The inflammatory response is an extremely important factor in the pathogenesis of endometriosis, and several pro-inflammatory cytokines have been suggested to play a critical role in endometriosis-related inflammatory responses (Harada et al., 1997; Bulun, 2009; Suen et al., 2014). Systemic and local immune abnormalities have been demonstrated in endometriosis, with the activation of peripheral blood monocytes that produce high levels of pro-inflammatory cytokines, such as interleukin (IL)-1 $\beta$ , IL-6, and IL-12 (Carmona et al., 2012; Malutan et al., 2015). IL-6 has been reported to have important functions in the regulation of the survival and growth of cells in ectopic endometrium (Moini et al., 2013). Meanwhile, some chemokines are also somehow associated with the development of endometriosis and infertility, including monocyte chemoattractant protein-1 (MCP-1) and C-X-C motif chemokine ligand 12 (Borrelli et al., 2013).

Chemerin chemokine-like receptor 1 (CMKLR1) is a G protein-coupled receptor that binds chemerin and plays important roles in chronic inflammatory diseases (Zabel et al., 2005; Graham et al., 2014). CMKLR1 mainly exists in epithelial cells, vascular smooth muscle cells, endothelial cells, leukocytes, osteoclasts, and adipocytes (Rourke et al., 2015). The binding of Chemerin to CMKLR1 can promote the release of calcium ions in the cell, which will regulate nuclear transcription factors (NF- $\kappa$ B) and extracellular regulatory protein kinase 1 (ERK-1) and other signaling pathways and then play an important role in the pathogenesis of cardiovascular diseases, metabolic diseases, inflammatory response, osteoblastogenesis, and carcinogenesis (Muruganandan et al., 2010; Kostopoulos

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et al., 2014; De Henau et al., 2016; Tummler et al., 2017; Li et al., 2018). The interaction between CMKLR1 and IL-1 $\beta$  can result in a vicious circle of continuous amplification if the inflammatory cascade occurs (Xie et al., 2020). Recently, Jin et al. reported that Chemerin levels were higher in peritoneal fluid from patients with endometriosis, and its expression correlated with IL-6 and TNF- $\alpha$  in peritoneal fluid positively (Jin et al., 2015). However, the expression and role of CMKLR1 in endometriosis patients is still unclear. Here, we studied the expression of CMKLR1 in patients with endometriosis and disclosed the relationship between CMKLR1 and inflammatory responses in peritoneal fluid and endometrial tissues.

## Materials and methods

### Reagents

Human Chemerin recombinant protein (2324-CM-025) was ordered from R&D Systems (Minneapolis, MN, USA). Chemerin was provided in sterile phosphate-buffered saline (PBS) with the concentration at 100  $\mu$ g/ml.

### Patient recruitment

For patient inclusion and exclusion, all participants aged from 22 to 45 years-old, who had regular menstrual cycles for at least 3 months before surgery and without history of hormonal treatment, were included. The patients were excluded from the study if they suffered from infertility associated with factors of ovary, fallopian tube, uterus, and/or abnormal semen. In this work, a total of 60 patients' eutopic and ectopic endometrial tissues and 50 healthy controls were selected. All participants gave written informed consent before taking part in the study, and the study protocols have been approved by the Ethics Committee of Zibo Central Hospital (#2020.7jv1).

### Specimen collection

Peritoneal fluid samples from 60 patients and 50 healthy controls were collected from the utero-vesical or Douglas pouch using the laparoscopic cannula during surgery. The peritoneal fluid supernatant was frozen by liquid nitrogen and stored in -80°C freezer for subsequent analysis. The endometrial samples were collected by curettage during hysteroscopy. Ovarian endometriotic tissues (with 38 to 72 mm diameter) were collected from the inner cyst wall. For healthy control group, the endometrial specimens were collected from fertile women who were undergoing reversal of tubal sterilization or laparoscopic tubal ligation by hysteroscopy.

### Cell culture

Human endometrial stromal cells (HESCs) were

isolated from the endometrial biopsies and cultured in DMEM/F12 medium with L-glutamine, 15 mM HEPES, 10% fetal bovine serum (FBS), 1% Penicillin (10,000 U/mL) and Streptomycin (10,000  $\mu$ g/mL) (Pen Strep), and 1 $\times$ Na<sub>2</sub>HCO<sub>3</sub>, as described in the study of Michalski et al. (2018). In brief, tissues were minced into pieces (1 mm) and digested in PBS containing 2 mg/mL of type II collagenase (0.1%, Sigma-Aldrich, St. Louis, MO) at 37°C for 1h with constant agitation. Stromal cells were isolated from the epithelial cells and debris using 150 and 35  $\mu$ m nylon strainer. After overnight culture, the unattached blood cells and debris were removed by aspiration and the stromal cells were washed with PBS. The stromal cells were subsequently cultured in DMEM/F12. HESCs were identified by flow cytometry (vimentin<sup>+</sup> and E-cadherin<sup>-</sup>) as described previously (Palen et al., 2016; Liu et al., 2017). HESCs were exposed to different concentrations of recombinant human Chemerin (0, 1, 5, 10 nM) for 2h. After the treatment, cells were harvested for gene expression and Western blot analyses.

### Gene expression analysis

The total RNA of endometrial samples from patients and healthy controls was extracted by using the Trizol reagent (Invitrogen, Carlsbad, USA), following the protocol provided by the manufacturer. One  $\mu$ g RNA was reversely transcribed into cDNA by using the M-MLV reverse transcriptase and random primers (Promega, Madison, USA). The 7500 Real-Time PCR system was used to perform qRT-PCR reactions with the Bio-Rad qPCR Master Mix (Hercules, USA). GAPDH were used as internal control to normalize the expression level of target genes. The expression levels of targets were calculated by using 2<sup>- $\Delta\Delta$ CT</sup> method. Primers used in this study are listed below: *CMKLR1* F: 5'-GCC AAC CTG CAT GGG AAA ATA-3' and 5'-GTG AGG TAG CAA GCT GTG ATG-3'; *TNF- $\alpha$* : F: 5'-AGC CCC CAG TCT GTA TCC TT-3' and R: 5'-CTC CCT TTG CAG AAC TCA GG-3'; *IL-6*: F: 5'-GCC CAA ACA CCA AGTCAGT-3' and R: 5'-TATAGG AAA CAG CGGGTTGG-3'; *MCP-1*: F: 5'- CAG CCA GAT GCA ATCAATGCC-3' and R: 5'-TGG AAT CCT GAA CCC ACT CT-3'; *GAPDH*: F: 5'- TGT GGG ATCAA TGG ATTTGG-3' and R: 5'-ACA CCA TGT ATT CCG GGT AAT-3'.

### ELISA

The concentration of CMKLR1, IL-6, TNF- $\alpha$  and MCP-1 in peritoneal fluid, endometrial tissues, and the conditional medium of HESCs were determined by using the commercial ELISA kits: human CMKLR1 ELISA kit (LS-F49527, LSBio, Seattle, USA), human IL-6 quantikine ELISA kit (D6050, R&D Systems, Minneapolis, USA), TNF alpha human ELISA kit (KHC3011, Invitrogen, Waltham, USA), and MCP-1 human ELISA kit (BMS281, Invitrogen, Waltham, USA), following the protocols provided by the

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manufacturers. For tissue measurement, samples were lysed with cold radioimmunoprecipitation (RIPA) buffer (Bio-Rad, CA, USA). Then the homogenate was centrifuged at 10,000 rpm at 4°C for 20 min, after which the supernatant and membranes were separated. The concentrations of the proteins were determined by corresponding ELISA kits.

### Immunoblotting analysis

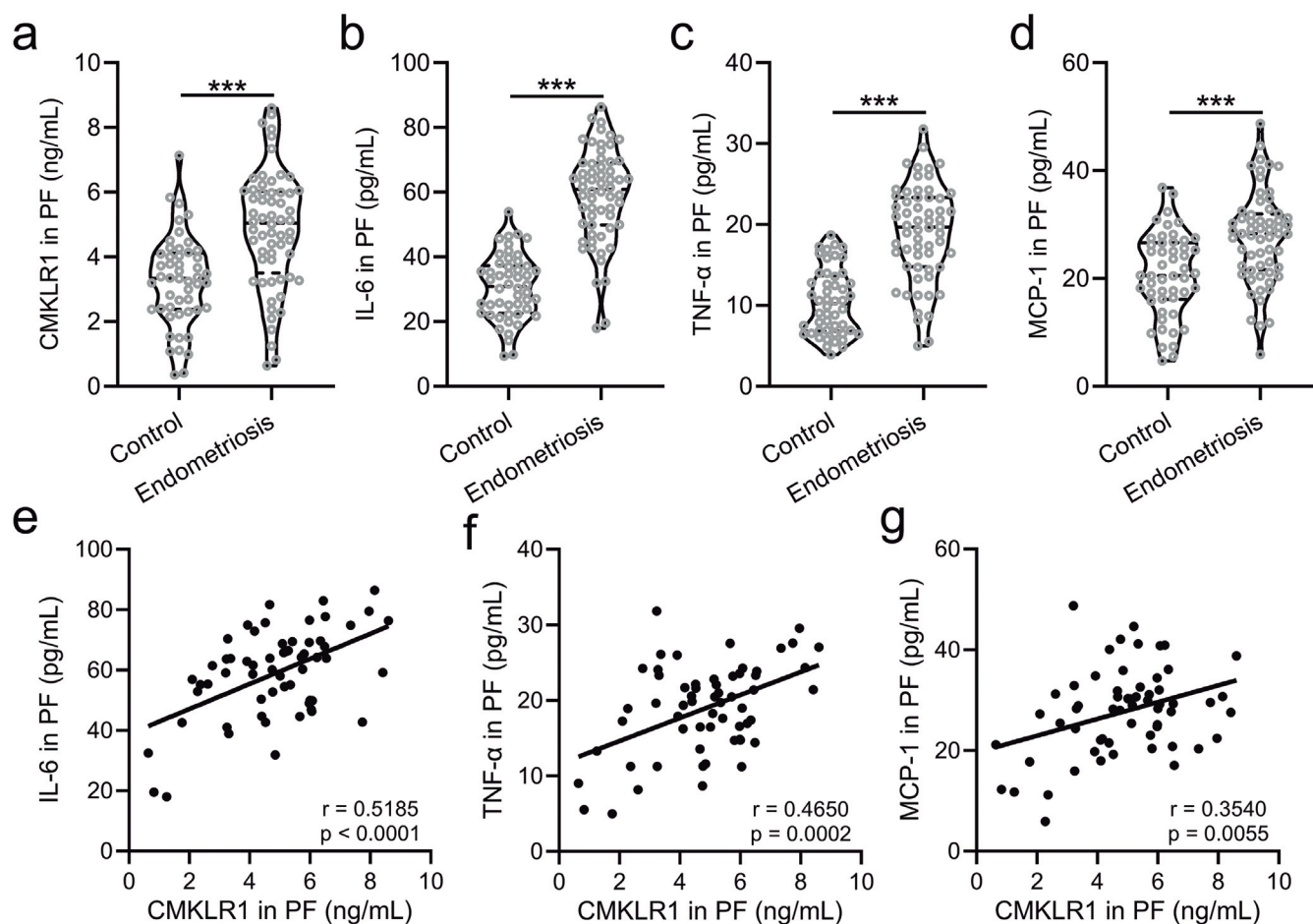
HESCs were lysed using the Beyotime RIPA buffer with fresh protease inhibitor cocktails (Promega, Madison, USA). Total protein concentration of each sample was determined by Pierce™ BCA Protein Assay Kit. Target proteins were detected by Western blot as described previously (Jin et al., 2015). Primary antibody against CMKLR1 (ab230442, 1:1000 dilution), and GAPDH (MA1-16757, 1:1500 dilution) were ordered from Abcam (Cambridge, UK) and Thermo Fisher Scientific (Waltham, USA), respectively.

### Immunohistochemistry analysis

Ten normal, eutopic, and ectopic endometrium tissues (n=10 for each group) were used for CMKLR1 protein expression and localization analyses through immunohistochemistry, following the protocol described in a previous study (Liu et al., 2017). The CMKLR1 polyclonal antibody (Thermo Fisher Scientific, Waltham, USA) was used for immunohistochemical staining. Four to six fields of each tissue sample were imaged by Leica DM6 microscope system (Wetzlar, Germany), and the average value was presented as the data of each sample.

### Statistical analysis

GraphPad Prism 7.0 software was used for all the statistical analyses in this study. One-way analysis of variance (ANOVA) followed by Dunnett's T3 multiple comparisons test methods were used to analyze the differences between groups. Pearson correlation



**Fig. 1.** CMKLR1 was up-regulated in the peritoneal fluid from endometriosis patients. ELISA was used to measure the concentrations of CMKLR1 (a), IL-6 (b), TNF-α (c) and MCP-1 (d) in the peritoneal fluid from endometriosis patients. Violin plot and \*\*\*P<0.001 from unpaired t tests. Pearson correlation coefficient analysis of CMKLR1 concentration with IL-6 (e), TNF-α (f) and MCP-1 (g) in the peritoneal fluid from endometriosis patients.

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coefficient analysis was used to analyze the relation between CMKLR1 and pro-inflammatory cytokines. The data were represented mean  $\pm$  standard deviation (SD). It was regarded as statistically significant when P value was less than 0.05.

### Results

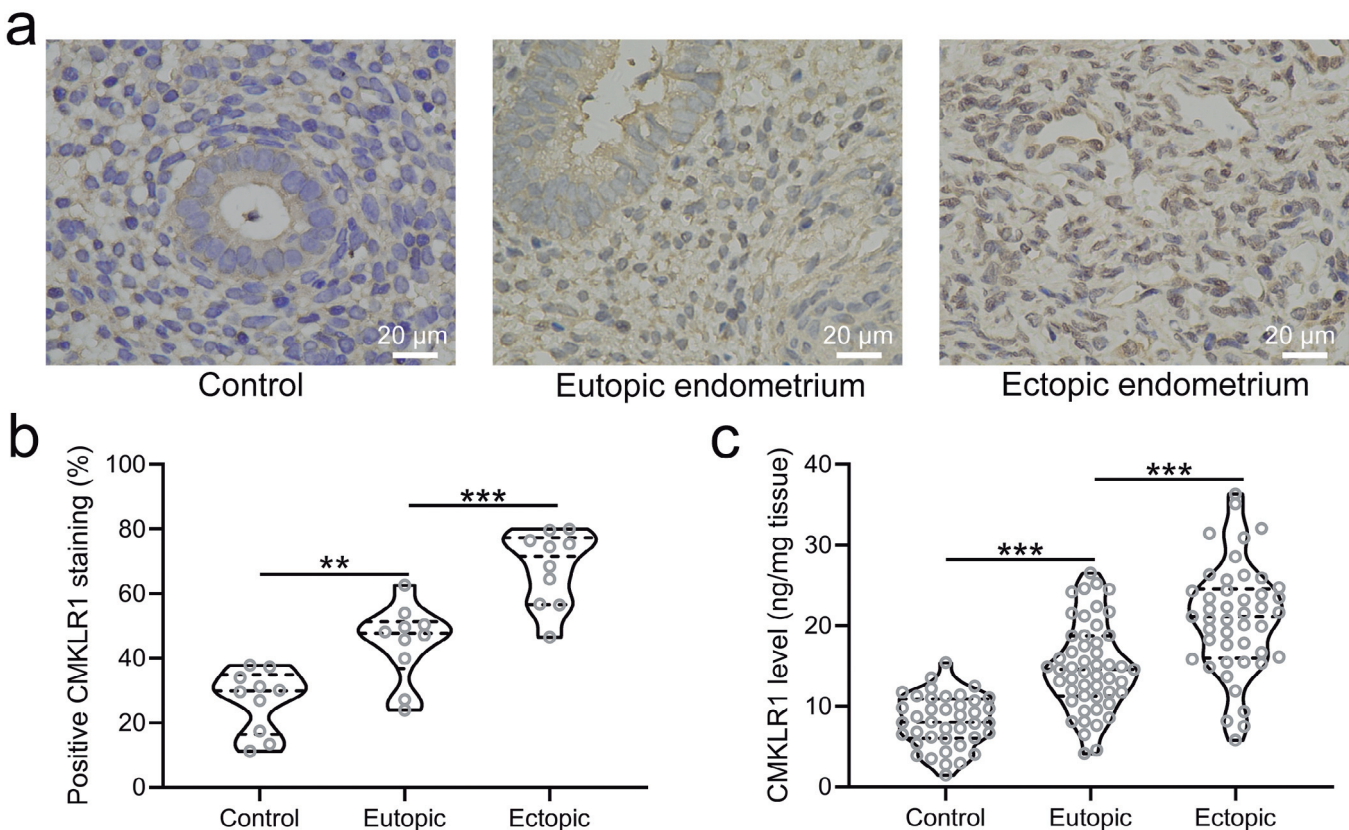
#### *CMKLR1 is up-regulated in peritoneal fluid and endometrium of endometriosis patients*

To investigate the pathological role of CMKLR1 in endometriosis patients, the concentration of CMKLR1, pro-inflammatory cytokines, and chemokine (IL-6, TNF- $\alpha$ , and MCP-1) from peritoneal fluid of patients (n=60) and healthy controls (n=50) were detected using ELISA. Like pro-inflammatory cytokines, CMKLR1 protein level was significantly increased in the peritoneal fluid of endometriosis patients compared to that of healthy controls (Fig. 1a-d). Importantly, Pearson correlation coefficient analysis showed that CMKLR1 protein level positively correlated with pro-inflammatory cytokines and chemokine, IL-6 ( $r=0.5185$ ,  $p<0.0001$ ), TNF- $\alpha$

( $r=0.4650$ ,  $p=0.0002$ ), and MCP-1 ( $r=0.3540$ ,  $p=0.0055$ ), in the peritoneal fluid of endometriosis patients (Fig. 1e-g). Next, we examined the expression level of CMKLR1 in the control, eutopic endometrium, and ectopic endometrium tissues through IHC staining and ELISA. As shown in Figure 2a, the expression and distribution of CMKLR1 were significantly increased in endometrium relative to that in control. Ectopic endometrium had the highest CMKLR1 expression, eutopic endometrium was next after the quantification (Fig. 2b). In line with the IHC data, the ELISA result also showed that CMKLR1 protein was highly expressed in eutopic endometrial tissues and further elevated in ectopic endometrial tissues (Fig. 2c). All these data suggested that CMKLR1 was overexpressed in peritoneal fluid and endometrium, which positively correlated with the increasing level of pro-inflammatory cytokines in endometriosis patients.

#### *CMKLR1 positively correlates with the inflammatory responses in endometriosis tissues*

To confirm the relationship between CMKLR1



**Fig. 2.** CMKLR1 was up-regulated in the endometrium from endometriosis patients. Representative immunohistochemical images of CMKLR1 protein localization in normal endometrium, eutopic endometrium, and ectopic endometrium (a). The quantification was expressed as a percentage of the area occupied by the color of interest (b). n=10 for each group. ELISA was used to analyze the protein levels of CMKLR1 among normal endometrium, eutopic endometrium, and ectopic endometrium (c). n=40 for control, n=50 for eutopic endometrium and n=45 for ectopic endometrium. Violin plot and \*\* $P<0.01$ , \*\*\* $P<0.001$  from One-way ANOVA followed by Dunnett's T3 multiple comparisons test.

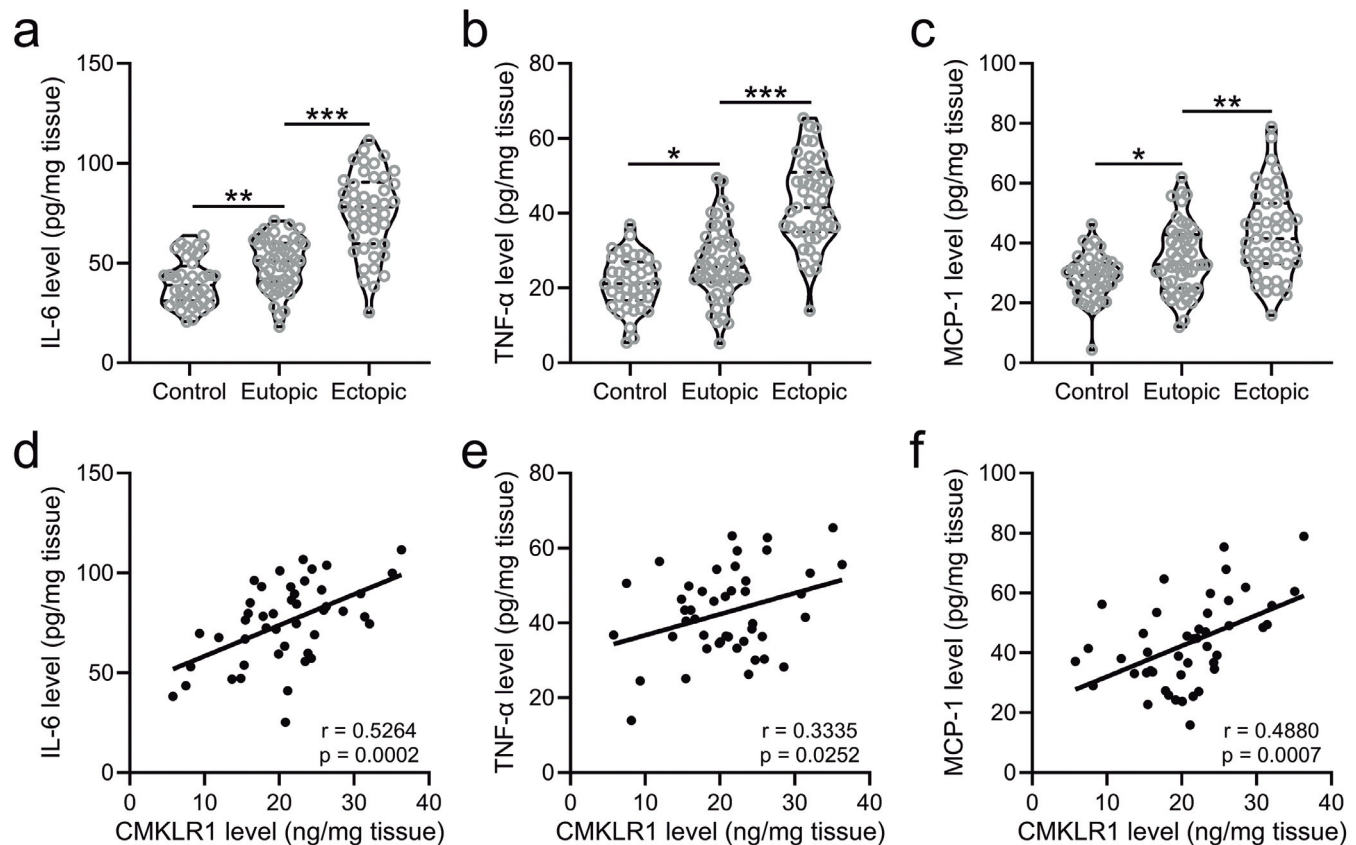
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expression and inflammatory responses in the endometrial tissues of endometriosis tissues, we detected the concentration of pro-inflammatory cytokines and chemokines (TNF- $\alpha$ , IL-6, and MCP-1) in control and endometrial tissues through ELISA. The results showed that all three pro-inflammatory cytokines were highly expressed in eutopic endometrial tissues and further increased in ectopic endometrial tissues (Fig. 3a-c). Interestingly, CMKLR1 overexpression positively correlated with elevated IL-6 ( $r=0.5264$ ,  $p=0.0002$ ), TNF- $\alpha$  ( $r=0.3335$ ,  $p=0.0252$ ), and MCP-1 ( $r=0.4880$ ,  $p=0.0007$ ) in ectopic endometrial tissues (Figure 3d-f) with Pearson correlation coefficient analysis.

#### CMKLR1 activation aggravates inflammatory responses in HESCs

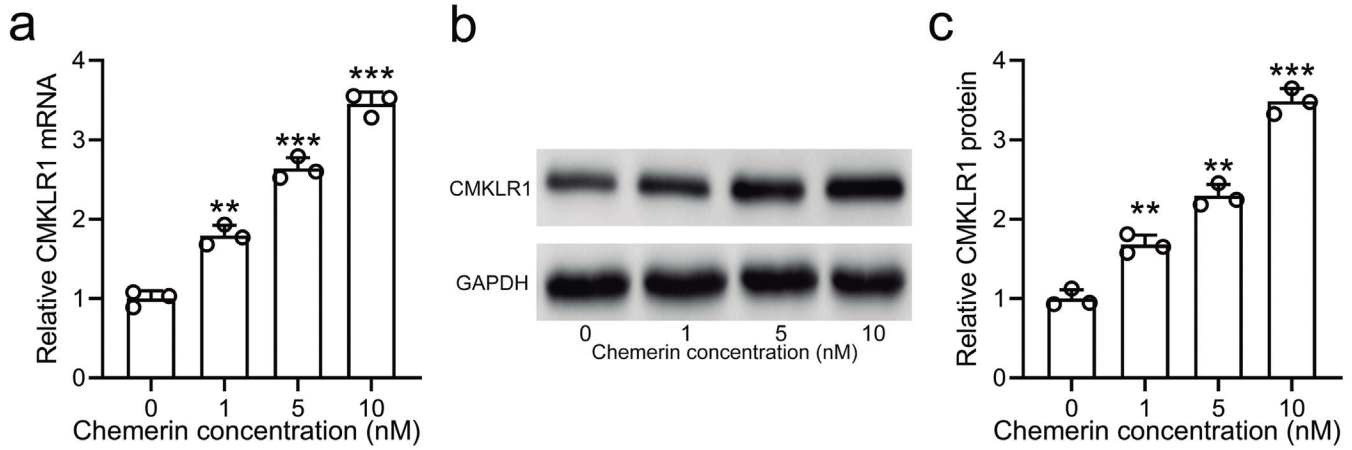
To further explore the underlying regulation of CMKLR1 on endometriosis, we isolated and cultured HESCs, and performed Chemerin treatment, an endogenous ligand of CMKLR1 (Serafin et al., 2019). As expected, Chemerin treatment increased the

expression of CMKLR1 on both mRNA (Fig. 4a) and protein (Fig. 4b-c) levels in a dose-dependent pattern. Next, we detected the production of pro-inflammatory cytokines in conditional medium of Chemerin treated HESCs. In line with the in vivo data, the protein level of TNF- $\alpha$ , IL-6, and MCP-1 in supernatant was significantly elevated after 2 hours recombinant human Chemerin treatment (Fig. 5a-c). Moreover, qRT-PCR results showed that the transcriptional level of all three pro-inflammatory cytokines was significantly increased (Fig. 5d-f). The up-regulation of both mRNA and protein of IL-6, TNF- $\alpha$ , and MCP-1 presented a dose-dependent pattern with Chemerin treatment too. Jin et al. reported that chemerin and its receptor were elevated in the ovarian endometrioma tissue, which were significantly correlated with the TNF- $\alpha$  and IL-6 levels in peritoneal fluid (Jin et al., 2015). To further expand the regulatory role of CMKLR1 in inflammatory responses, we detected IL-1 $\beta$  levels in treated cells and found that upregulation of CMKLR1 aggravated inflammatory responses of IL-1 $\beta$  in HESCs (data not shown). These data indicated that Chemerin mediated CMKLR1

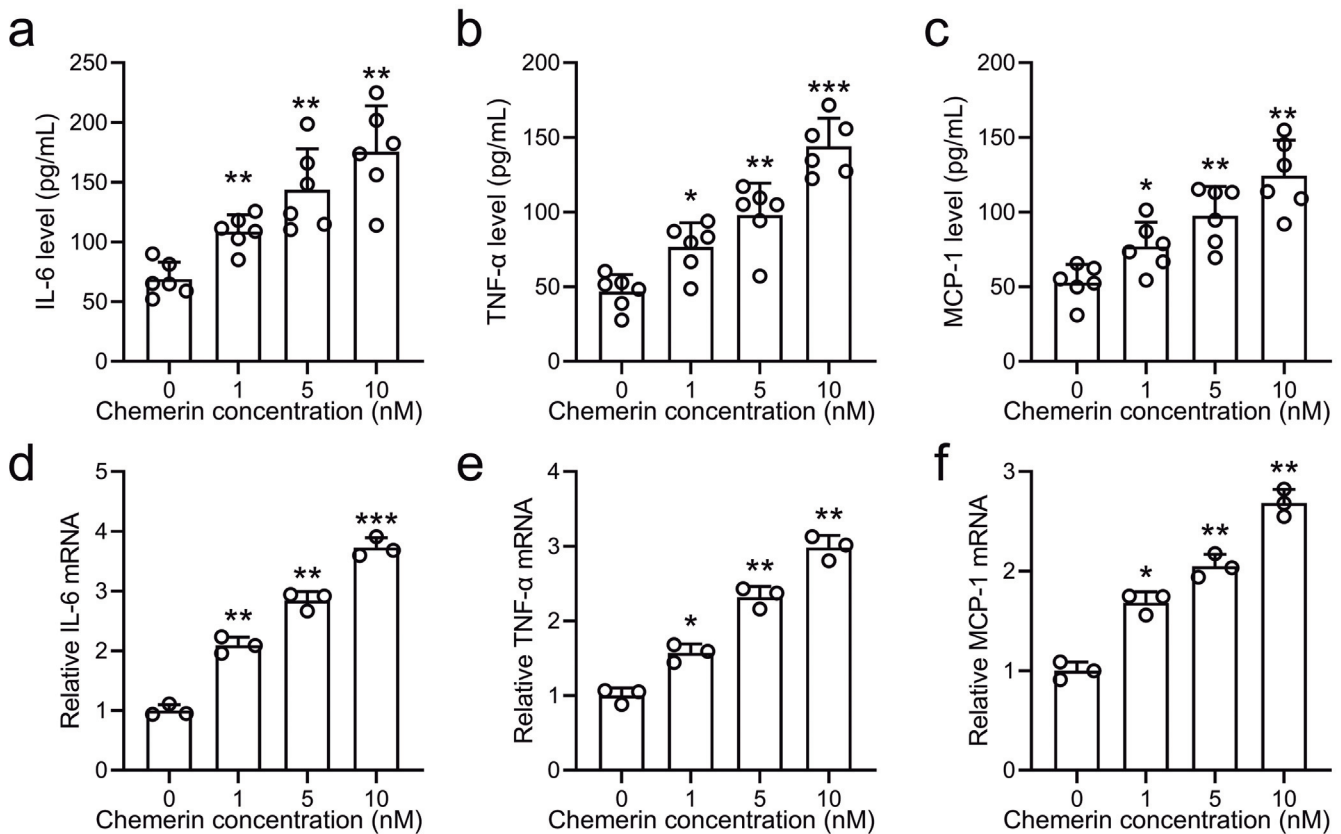


**Fig. 3.** Inflammatory responses in the endometrium from endometriosis patients. ELISA was used to analyze the levels of IL-6 (a), TNF- $\alpha$  (b) and MCP-1 (c) in normal endometrium, eutopic endometrium, and ectopic endometrium.  $n=40$  for control,  $n=50$  for eutopic endometrium and  $n=45$  for ectopic endometrium. Violin plot and \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  from One-way ANOVA followed by Dunnett's T3 multiple comparisons test. Pearson correlation coefficient analysis of CMKLR1 concentration with IL-6 (d), TNF- $\alpha$  (e) and MCP-1 (f) in the ectopic endometrium.

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**Fig. 4.** Chemerin activated CMKLR1 in human endometrial stromal cells (HESCs). HESCs were exposed to different concentrations of recombinant human Chemerin for 2h. RT-qPCR was used to measure the mRNA level of CMKLR1 (**a**) and Western blot was used to measure the protein level of CMKLR1 (**b and c**).  $n=3$  for each group. \*\* $P<0.01$ , \*\*\* $P<0.001$  compared to control. One-way ANOVA followed by Dunnett's T3 multiple comparisons test.



**Fig. 5.** CMKLR1 overexpression aggravated inflammatory responses in HESCs. HESCs were exposed to different concentrations of recombinant human Chemerin for 2h. ELISA was used to analyze the levels of IL-6 (**a**), TNF- $\alpha$  (**b**) and MCP-1 (**c**) in supernatant.  $n=6$  for each group. RT-qPCR was used to measure the mRNA expressions of IL-6 (**d**), TNF- $\alpha$  (**e**) and MCP-1 (**f**) in cell lysis.  $n=3$  for each group. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  from One-way ANOVA followed by Dunnett's T3 multiple comparisons test.

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activation might induce the expression and production of pro-inflammatory cytokines, and then aggravated inflammatory responses in HESCs.

### Discussion

Inflammation is the typical feature of endometriosis, which presents overproduction of cytokines and chemokines of ectopic tissue in the peritoneal cavity (Chung et al., 2001; Kyama et al., 2008; Reis et al., 2013; Graham et al., 2014). In this study, we investigated the expression and distribution of CMKLR1 in peritoneal fluid and endometrial tissues of patients with endometriosis and explored the relationship between CMKLR1 expression and inflammatory response *in vivo* and *in vitro*. Our study is the first to demonstrate the significantly local overexpression of CMKLR1 in endometriosis patients, which positively correlates with the overproduction of pro-inflammatory cytokines and chemokines in peritoneal fluid and ectopic endometrium in patients. Our findings suggest that elevated CMKLR1 level promotes the progression of endometriosis through regulation of the inflammatory responses.

CMKLR1 has been reported to play important roles in chronic inflammatory diseases (Chung et al., 2002; Zabel et al., 2005). Much evidence has demonstrated the critical roles of CMKLR1 in the regulation of pathogenic inflammation. In the central nervous system, CMKLR1 positive dendritic cells are present in the leptomeninges and are able to activate the lesions of multiple sclerosis (Graham et al., 2009). In contrast, in comparison with WT mice, the clinical and histological phenotypes of experimental autoimmune encephalomyelitis in CMKLR1 knockout mice are less severe (Lande et al., 2008). Xie et al. demonstrated that the activation of CMKLR1 by Chemerin treatment in diabetic cardiomyopathy rats promotes inflammation through activating of NLRP3 inflammasome (Xie et al., 2020). Diaz-Rubio et al. also reported that CMKLR1 expression level is positively correlated with insulin resistance in patients with rheumatoid arthritis (Diaz-Rubio et al., 2021). In line with the above findings, this study showed that CMKLR1 expression level was significantly increased in the peritoneal fluid and endometrial tissues of patients with endometriosis compared to that in healthy controls. Importantly, the expression of CMKLR1 positively correlated with the local overproduction of pro-inflammatory cytokines and chemokines in ectopic endometrium. These findings indicate that CMKLR1 overexpression promotes the progression of endometriosis through regulating the inflammatory responses. Elevated Chemerin plays a critical role in the pelvic inflammation processes (Ernst and Sinal, 2010; Jin et al., 2015). As a ligand Chemerin exists in the systemic circulation, which cannot interpret its pro-inflammatory effect in endometriosis because there is no correlation between peritoneal fluid and serum Chemerin concentration (Bondue et al., 2011; Jin

et al., 2015). Interestingly, chemerin and its receptor were elevated in the ovarian endometrioma tissue, which were significantly correlated with the TNF- $\alpha$  and IL-6 levels in peritoneal fluid (Jin et al., 2015). We hypothesized that there is a local effector in endometrial tissues, which can be sensitized by the elevated Chemerin, and then induce local inflammatory responses. Hence, we think CMKLR1 might be the mediator between Chemerin and inflammatory reactions in endometriosis, which can be supported by several pieces of evidence. First, Chemerin treatment can increase the expression level of CMKLR1 significantly in human endometrial stromal cells. Upon Chemerin treatment, HESCs produce high levels of pro-inflammatory cytokines and chemokines, which positively correlate with the overexpression of CMKLR1 in the conditional medium. In addition, CMKLR1 expression positively correlates with the overproduction of IL-6, TNF- $\alpha$ , and MCP-1 in ectopic endometrium of patients with endometriosis. However, there are some limitations to this study. The physiological role of Chemerin-CMKLR1 in endometriosis needs to be further confirmed in rodent models, and the underlying molecular mechanism of Chemerin/CMKLR1-induced inflammatory responses need to be dissected in both *in vivo* and *in vitro*. Elucidating the interactions of the Chemerin/CMKLR1 axis with other inflammatory cytokines in endometriosis is also required in future studies.

### Conclusion

CMKLR1 is up-regulated in the peritoneal fluid and endometrial tissues of endometriosis patients. This study indicates that CMKLR1 overexpression promotes the progression of endometriosis through the regulation of inflammatory responses.

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*Ethics approval and consent to participate.* All participants gave written informed consent before taking part in the study, and the study protocols have been approved by the ethics commitment of Zibo Central Hospital.

*Patient consent for publication.* The written informed consents were collected from patients.

*Conflict of interest.* The authors declare that they have no conflict of interest.

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