CCL18 promotes endometriosis by increasing endometrial cell migration and neuroangiogenesis

Yangying Peng,[1,2] Shaojie Ding,[1] Ping Xu,[1] Xueyan Zhang,[1] Jianzhang Wang,[1] Tiantian Li,[3] Liyun Liao,[4] Xinmei Zhang[1]

[1]Department of Obstetrics and Gynecology, Women's Hospital, Zhejiang University School of Medicine, Hangzhou
[2]Department of Obstetrics and Gynecology, Huangyan Hospital of Wenzhou Medical University, Taizhou First People's Hospital, Taizhou
[3]Assisted Reproduction Unit, Department of Obstetrics and Gynecology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou
[4]Xiangshan First People's Hospital Medical and Health Group, Ningbo, China

ABSTRACT

Endometriosis is an estrogen-dependent inflammatory gynecological disease whose pathogenesis is unclear. C-C motif chemokine ligand 18 (CCL18), a chemokine, is involved in several inflammatory diseases. In this study, we aimed to investigate the role of CCL18 in endometriosis and its underlying mechanisms. Human endometrium and peritoneal fluid were obtained from women with and without endometriosis for molecular studies. The expression level of CCL18 in each tissue sample was examined by RNA sequencing analysis, quantitative PCR analysis and immunohistochemistry staining. The effects of CCL18 on cell migration, tube formation and neurite growth were investigated *in vitro* using primary endometrial cells, human umbilical vein endothelial cells (HUVECs) and dorsal root ganglion (DRG) neurons, respectively. Moreover, the development of endometriosis in mice was studied *in vivo* by blocking CCL18. CCL18 was shown to be overexpressed in endometrial foci and peritoneal fluid in women with endometriosis and was positively correlated with endometriosis pain. *In vitro*, CCL18 promoted the migration of endometrial cells, tube formation of HUVECs, and nerve outgrowth of DRG neurons. More importantly, inhibition of CCL18 significantly suppressed lesion development, angiogenesis, and nerve infiltration in a mouse model of endometriosis. In conclusion, CCL18 may play a role in the progression of endometriosis by increasing endometrial cell migration and promoting neuroangiogenesis.

Key words: CCL18; CCR8; endometriosis; macrophage; neuroangiogenesis.

Introduction

Endometriosis is a common gynecological disease in which endometrial tissue appears outside the uterine cavity, usually resulting in dysmenorrhea, pelvic mass, and infertility in reproductive-age women.[1] The clinical management of endometriosis includes pharmacological and surgical interventions, the most common of which are surgery and adjunctive pharmacologic therapy.[2] However, treatment outcomes are often unsatisfactory, with high recurrence rates and low cure rates.[3] Therefore, further in-depth studies on the pathogenesis and pathology of endometriosis are necessary to explore and develop effective clinical treatments and to assess treatment efficacy and prognosis. Studies have shown that inflammation and the corresponding immune cells are closely associated with endometriosis.[4] Among these factors, macrophages and the immune-inflammatory factors they secrete have been shown to play a key role in the development of endometriosis, but their exact mechanism remains controversial.[4-6]

C-C motif chemokine ligand 18 (CCL18) is a chemokine mainly produced by M2 macrophages and is involved in various inflammatory diseases.[7,8] In pulmonary inflammation, CCL18 overexpression induces the infiltration of T cells and the overexpression of inflammatory factors such as TNF-α, interferon-γ, matrix metalloproteinase (MMP)-2, and MMP-9.[9] In addition to its role in inflammation, CCL18 is involved in cancer cell migration, invasion, and epithelial-mesenchymal transition through its receptor CCR8.[10] Additionally, CCL18 significantly promotes angiogenesis in breast cancer both *in vitro* and *in vivo*.[11] In gynecology, CCL18 plays a critical role in ovarian cancer development by promoting the invasive, migratory, and adhesive capacities of ovarian cancer cells.[12]

Although CCL18 has been extensively studied in various inflammatory diseases, the impact of this molecule on endometriosis development remains unclear. In this study, we investigated the role of CCL18 in the function of endometrial cells as well as in the neuroangiogenesis of endometriotic lesions.

Materials and Methods

Patients and tissue specimens

This study was approved by the Human Ethics Committee of Women's Hospital, School of Medicine, Zhejiang University (No. 20190012). We excluded individuals with diabetes mellitus, prior autoimmune disorders, cancer, or severe medical conditions such as hepatic or thyroid disorders. Informed consent was obtained from each woman with (n=37) and without endometriosis (n=18) who underwent surgery between July 2019 and September 2020.

Peritoneal fluid, eutopic endometrial tissue and ovarian ectopic cystic wall tissue (ectopic endometrial tissue) were collected from 37 patients with endometriosis, and peritoneal fluid and eutopic endometrial tissue were collected from 18 patients without endometriosis. Tissue sections were immediately washed in ice-cold diethylpyrocarbonate-treated PBS (Invitrogen, Carlsbad, CA, USA) and then immersed in RNAlater solution (Invitrogen) for quantitative PCR or snap-frozen in liquid nitrogen for protein blot analysis. Furthermore, another portion of fresh specimens was kept in sterile, serum-free DMEM/F12 medium to culture primary cells. Moreover, a small portion of each tissue sample was fixed and embedded in paraffin for standard hematoxylin and eosin (H&E) staining to allow the pathologist to confirm the cycle stage. Peritoneal fluid was collected from all patients for subsequent ELISAs.

RNA sequencing

Total mRNAs were isolated from ovarian endometriosis tissues and eutopic and control endometrial tissues. Briefly, frozen endometrial tissue was thawed on ice, and the surface blood was washed away with cold PBS. A total of 20 mg of endometrial tissue was placed in a 1.5 mL RNA-free EP tube and homogenized with 1 mL of TRIzol and steel beads. After homogenization for 15 min, the sample was centrifuged, the clear supernatant was aspirated into a 1.5 mL RNA-free EP tube, mixed with an equal volume of isopropanol, and placed on ice for 10 min. The sample was subsequently centrifuged, the supernatant was discarded, and the precipitate was washed with 1 mL of 75% ethanol (cold). The supernatant was discarded, and the precipitate was dissolved in 20-30 μL of RNase-free double-distilled water to obtain RNA. The concentration and purity of the RNA were determined with a Nanodrop2000 system. An RNA sequencing library was constructed, and mRNA sequencing was conducted at Genenergy Biotechnology (Shanghai, China).

Quantitative real-time PCR

RNA was reverse-transcribed into cDNA using a cDNA synthesis kit (TaKaRa Bio, Inc., Shiga, Japan) according to the manufacturer's protocol. Then, qPCR analysis was conducted using a 7500 ABI Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with a SYBR Green kit (TaKaRa Bio, Inc.) under the following thermocycling conditions: 95°C for 30 s, followed by 35 cycles of 95°C for 5 s and 60°C for 30 s. The results obtained from the PCR were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) using the $2^{-\Delta\Delta Ct}$ method. The primers used in this study were as follows: *CCL18* forward, 5′-TCTATACCTCCTGGCAGATTC-3′ and reverse, 5′-TTTCTGGACCCACTTCTTATTG-3′; *GAPDH* forward, 5′-TCGTGGAAGGACTCATGACC-3′ and reverse, 5′-CCAGTGAGCTTCCCGTTCA-3′.

Immunohistochemistry staining

Fresh specimens were fixed in 4% paraformaldehyde solution for 24 h, embedded in paraffin, cut into 4 μm-thick sections, and then immersed in xylene and ethanol for deparaffinization and rehydration, respectively. Antigen recovery was performed in a microwave oven using Tris-EDTA (pH 9.0) (Biosharp, Anhui, China). The following steps were performed using a staining kit (Absin, Shanghai, China). Briefly, the slides were incubated with 3% H_2O_2 to eliminate endogenous peroxidase activity and then blocked with 5% BSA, first by incubation with a primary antibody against CCL18 (dilution 1:300, ab104867; Abcam, Cambridge, UK) overnight at 4°C and then with a secondary antibody (GK600711; GenenTech, South San Francisco, CA, USA) at room temperature for 1 h. Staining was performed using DAB and hematoxylin. The slides were visualized and imaged using a microscope (Leica, Wetzlar, Germany). The intensity of staining was assessed using the following scoring parameters: strong (3×), moderate (2×) and weak (1×). H-scores from 0 to 300 were calculated according to the following formula:[13] H-score = 1* (% cells 1×) + 2* (% cells 2×) + 3* (% cells 3×). Five high-magnification fields of view of each section were randomly selected for statistical scoring, and H scores were assessed independently by two researchers at different times and averaged.

ELISA

Immunoreactivity for CCL18 in the peritoneal fluid was quantified using the RayBio® Human CCL18/PARC ELISA Kit (RayBiotech Life, Inc., Peachtree Corners, GA, USA). ELISAs were performed according to the manufacturer's instructions. Briefly, the standard curve was first plotted with the standards. One hundred microliters of each sample was added to a 96-well plate, two duplicate wells were used for each sample, and the plates were incubated at room temperature for 2.5 h and then washed. Then, 100 µL of 1×biotin-antibody conjugate solution was added, and the mixture was incubated at room temperature for 1 h. After washing, 100 µL of 1×HRP solution was added, and the mixture was incubated at room temperature for 45 min. Subsequently, 100 µL of TMB solution was added, and the mixture was incubated for 15-30 min in the dark. Optical densities were measured at 450 nm with a Varioskan Flash (Thermo Fisher Scientific, Inc).

Isolation and culture of primary eutopic endometrial cells

Eutopic endometrial epithelial cells and stromal cells from the eutopic endometrium were isolated through 100 µm and 40 µm nylon meshes after digestion with 0.1% type I collagenase. The isolated cells were resuspended in DMEM/F12 supplemented with 12% fetal bovine serum (FBS; Gibco, Waltham, MA, USA) and cultured in a 5% CO_2 incubator at 37°C.

Cell culture

Human umbilical vein endothelial cells (HUVECs) from ScienCell Research Laboratories (Carlsbad, CA, USA) were cultured in endothelial cell medium (Gibco) and used at passages 4-7. The rat embryonic dorsal root ganglion (DRG) hybrid cell line F11 was obtained from the European Collection of Authenticated Cell Cultures (ECACC 08062601; Salisbury, UK).

Cell proliferation assay

HUVECs (1000 per well) were cultured in serum-free medium for 24 h and seeded into a 96-well plate with different doses of CCL18. After culture for 24-96 h, 10 µL of CCK-8 solution (Dojindo, Tabaru, Japan) was added to each well. The cells were incubated with 10% CCK-8 medium at 37°C in 5% CO_2 for 2 h according to the manufacturer's instructions.

Migration assay

Migration assays were conducted in 24-well Transwell chambers with a pore size of 8 µm (Corning, Inc., Corning, NY, USA). The cells were pretreated with serum-free DMEM/F12 or the corresponding cell culture medium containing 0-10 ng/mL CCL18. After incubation for 48 h, $5×10^4$ cells were detached and resuspended in 200 µL of DMEM/F12 or cell culture medium without serum and seeded in the upper chamber. The lower chamber contained 500 µL of DMEM/F12 supplemented with 3% FBS or cell culture medium supplemented with 1% FBS containing 0-10 ng/mL CCL18. The cells at the bottom of the membrane were stained with crystal violet solution after 4-24 h. The invading cells were then photographed and counted.

Tube formation

For the tube formation assay, 96-well plates were precoated with Matrigel (50 µL/well). HUVECs were pretreated with CCL18 at the indicated doses for an additional 48 h, seeded at 10^4 per well, and cultured for up to 6 h. After the formation of tube networks, network structures were analyzed and photographed with a digital camera (Nikon, Tokyo, Japan).

Immunofluorescence

Neurite growth analysis was performed as previously described.[14] F11 cells (10^4 per well) were seeded directly on µ-Slide 8-well chamber slides (IBID) in complete DMEM/F12 medium. After culture with 10 ng/mL CCL18 for 24 h, the cells were fixed in 4% buffered formalin, fixed at room temperature for 15 min, blocked and permeabilized in 0.1% Triton X-100 before they were incubated with primary TUBB3 antibody (1:1000 dilution) at 4°C overnight. Then, the cells were incubated with a secondary goat anti-mouse antibody (1:1000 dilution) at room temperature for 2 h in the dark. F11 cells not incubated with CCL18 were used as negative controls. DAPI (1:500) was used as a double stain, and the slides were stained at room temperature for 5 min. Images were obtained with an Olympus FV1200 confocal microscope (Olympus, Tokyo, Japan) at 1000× magnification. With ImageJ software, neurite growth was analyzed by calculating the length and number of axonal projections from five regions obtained from three independent experiments.

Animals and treatment

The treatment of the mice in this study was approved by the Ethics Committee of Animal Laboratory, Zhejiang University (ZJU20200015). According to Dodds et al.,[15] endometriotic lesions in BALB/c mice are more likely to develop into cystic lesions after allograft transplantation, and the immune response they generate is dominated by Th2/M2 macrophages, so we chose BALB/c mice to construct an animal model. A total of 23 female BALB/C mice aged 6-8 weeks were intraperitoneally injected with 50 mg/kg pentobarbital sodium. Ovariectomy and subcutaneous injection of estradiol benzoate (500 ng/mouse/5 days) were performed 7 days before endometriosis induction. We induced endometriosis as previously described.[16] Eight mice were randomly selected as donors, and 15 mice were included as recipient mice. After surgery, the mice were randomly assigned to three groups and referred to Wang et al. for selection of drug dosage.[17] Mice were intraperitoneally injected with vehicle control (DMSO), 0.3 mg/kg ML604086 (a potent CCR8 antagonist, n=5), or 1.0 mg/kg ML604086 (n=5) every day. After 21 days of treatment, the mice were anesthetized and sacrificed by cervical dislocation, the number of endometriotic lesions was counted, and lesion sizes and weights were measured at two perpendicular diameters (d, D). We determined the lesion size according to the following formula: $V = (4/3) \pi r^2 R$ (r and R are the radii, r, R).[18,19] Sections of mouse paraffin-embedded endometriotic lesions were first subjected to H&E staining for confirmation of the diagnosis of endometriosis; subsequently, immunohistochemistry (IHC) staining for CCL18 (dilution 1:100, ab233099; Abcam), CCR8 (dilution 1:100, NB100-709; Novus Biologicals, Centennials, CO, USA), CD31 (dilution 1:200, ab28364; Abcam), and protein-encoding gene product (PGP) 9.5 (dilution 1:200, ab8189; Abcam) was performed.

Statistical analysis

Statistical analysis was conducted using SPSS Version 24.0 (IBM). The data are presented as the means ± standard devia-
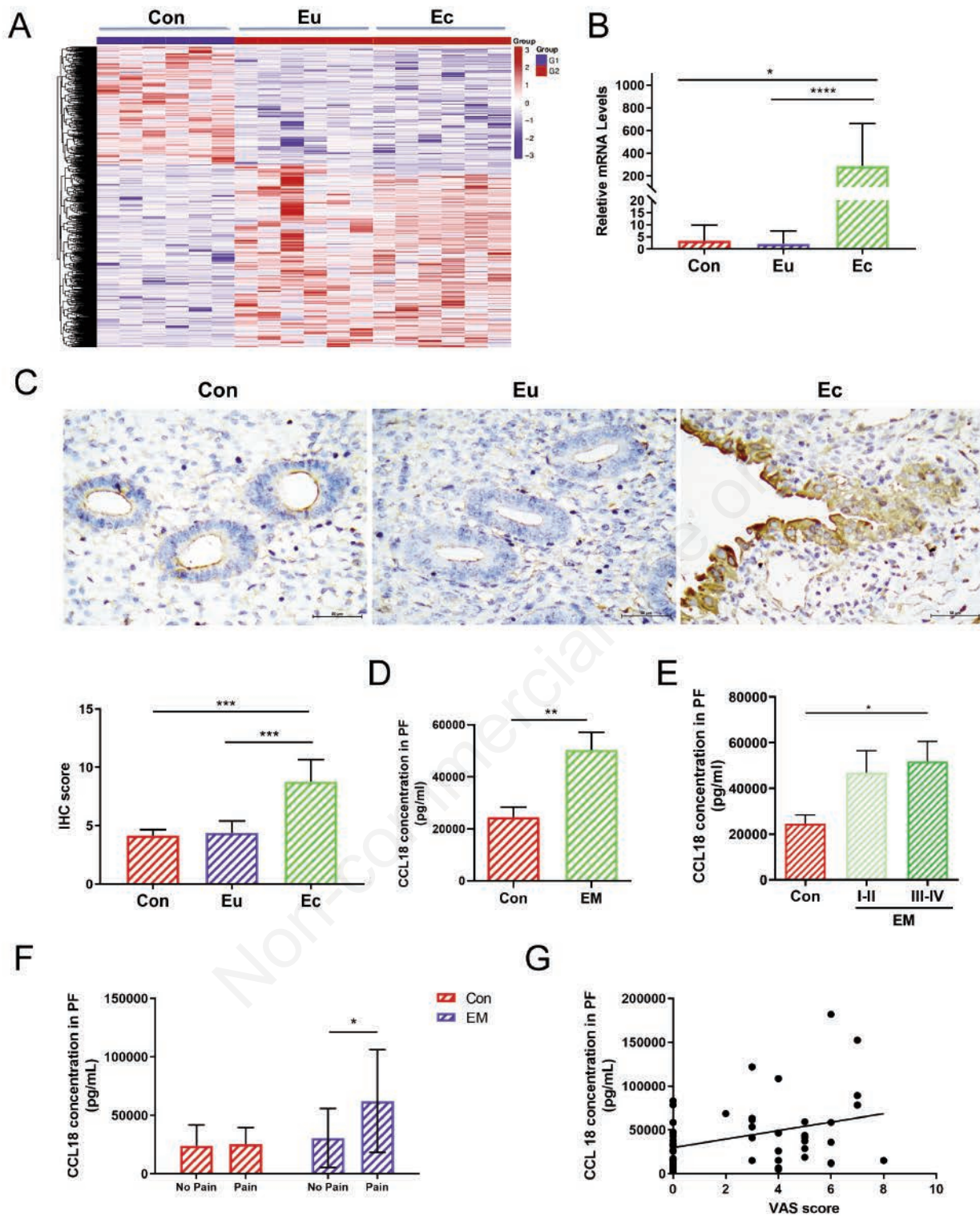
Figure 1. CCL18 was overexpressed in endometriotic lesions. **A**) RNA sequencing analysis of differentially expressed genes in the control, eutopic and ectopic endometria. **B**) Relative mRNA expression levels in the control endometrium (n=8), eutopic endometrium (n=9) and endometriotic lesions (n=10) were determined by qRT–PCR. **C**) Immunohistochemical staining of CCL18 in the control (n=10), eutopic (n=10) and ectopic endometria (n=12); scale bar: 50 μm (400× magnification). **D**) CCL18 concentrations in peritoneal fluid from patients with (n=37) or without endometriosis (n=18). **E**) The concentrations of CCL18 in the PF were measured and analyzed according to the stage of endometriosis (n=55). **F**) The concentrations of CCL18 in the PF from individuals with pain symptoms were analyzed. **G**) The correlation of the CCL18 concentration in peritoneal fluid with the visual analog scale (VAS) score. The data are shown as the means ±SD; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

tions from at least three independent experiments. *p*-values were determined using two-tailed Student's *t*-tests or Mann-Whitney U tests when comparing two groups and using one-way ANOVA when comparing more than two groups. Correlations between two variables were assessed using Spearman's analysis. A $p<0.05$ was considered to indicate statistical significance.

Results

CCL18 was overexpressed in the ectopic lesions of endometriosis patients

RNA sequencing of endometrial tissues was conducted, and the differentially expressed genes were clustered (Figure 1A). A total of 1,206 mRNAs were differentially expressed between the ectopic group and the control and eutopic groups (n=6). CCL18 was a representative upregulated gene in endometriotic lesions. Subsequently, CCL18 expression levels in the three groups were measured using qPCR and IHC staining. In the ectopic endometria (n=10) of the endometriosis patients, the mRNA expression levels of CCL18 were significantly greater than those in the eutopic (n=9, $p<0.001$) and control (n=8, $p<0.05$) endometria (Figure 1B). The IHC results showed that CCL18 was mainly expressed in the epithelium of the ectopic endometrium (Figure 1C), and the expression levels of CCL18 in the ectopic endometrium (n=12) were also significantly greater than those in both the control (n=10, $p<0.001$) and eutopic (n=10, $p<0.001$) endometrium (Figure 1C). Consistently, the CCL18 concentration was significantly greater in the endometriotic peritoneal fluid (n=37) than in the control peritoneal fluid (n=18, $p<0.01$; Figure 1D).

Furthermore, CCL18 concentrations were significantly greater only in the advanced endometriosis (III-IV) group than in the control group ($p<0.05$; Figure 1E).

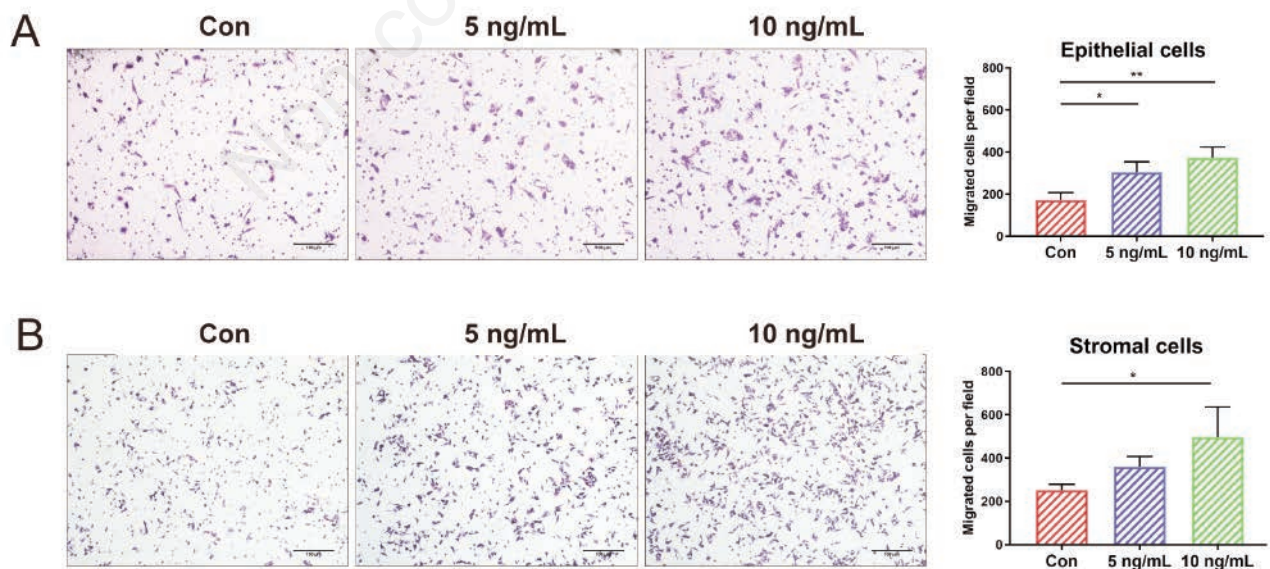### CCL18 promoted the migration of human primary eutopic endometrial epithelial and stromal cells

Eutopic endometrial epithelial or stromal cells were treated with different doses of CCL18 for 36 h. Compared with those in the control group, eutopic endometrial epithelial and stromal cells in the treatment group exhibited increased migration after treatment with 5 ng/mL ($p<0.05$, $p<0.01$) or 10 ng/mL CCL18 ($p>0.05$, $p<0.05$; Figure 2 A,B).

CCL18 induced tube formation and migration in endothelial cells

Compared with those in the control group, the number of branches in the HUVECs treated with a high dose of 10 ng/mL CCL18 significantly increased ($p<0.05$; Figure 3A). The migratory ability of HUVECs was also evaluated. Transwell assays revealed that the number of migrated HUVECs significantly increased after pretreatment with 5 ng/mL and 10 ng/mL CCL18 ($p<0.01$, $p<0.001$; Figure 3B), particularly at 10 ng/mL CCL18. However, incubation of HUVECs with CCL18 had no effect on HUVEC proliferation (Figure 3C).

CCL18 induced neurite growth and infiltration of neurons

After treatment with different doses of CCL18 for 36 h, the Transwell assay showed increased migration of F11 cells at a high dose of 10 ng/mL CCL18 compared with that in the control group ($p<0.01$; Figure 4A). Furthermore, we obtained F11 cell morphology images using immunofluorescence analysis of

Figure 2. CCL18 promoted the migration of primary eutopic endometrial cells. **A**) Representative images of the Transwell migration assay of primary endometrial epithelial cells treated with different concentrations of CCL18; scale bar: 100 μm. **B**) Representative images of the Transwell migration assay of primary endometrial stromal cells treated with different concentrations of CCL18; scale bar: 100 μm. The data are shown as the means ± SD; n=3; *$p<0.05$, **$p<0.01$.

TUBB3 to assess neurite development. Statistical analyses revealed that both the number and length of neurites were greater in the F11 cells treated with CCL18 than in the control cells ($p<0.05$, $p<0.05$; Figure 4 B-D).

CCL18 was positively correlated with endometriosis pain

To investigate the relationship between CCL18 and endometriosis pain, we used a visual analog scale (VAS) to assess patients' preoperative pain levels.[20] The presence and severity of pain symptoms potentially related to the diagnosis were evaluated by administering a symptom-oriented questionnaire to each patient. The severity of symptoms was graded according to the VAS (from 0=no pain to 10=unbearable pain). CCL18 concentrations were significantly different between women with endometriosis with or without clinical pain symptoms ($p<0.05$; Figure 1F). Spearman analysis revealed that CCL18 concentrations in the peritoneal fluid were positively correlated with the VAS score in the patients with and without endometriosis ($p<0.05$; Figure 1G).

Blocking CCL18/CCR8 inhibited the development of endometriosis *in vivo*

H&E staining confirmed that the transplanted uterine tissues of all groups had developed into typical endometriotic lesions (Figure 5A). The IHC results showed that the expression levels of both CCL18 ($p<0.05$; Figure 5B) and CCR8 ($p<0.01$; Figure 5C) were significantly greater in the ectopic endometrium of the model mice than in the eutopic endometrium. Compared with those in the vehicle group, the mice treated with both low and high doses of ML604086 had a decreased number ($p<0.01$, $p<0.05$; Figure 5D), weight ($p<0.01$, $p>0.05$; Figure 5E), and size ($p<0.01$, $p>0.05$; Figure 5F) of endometriotic lesions. These data demonstrated that blocking CCL18/CCR8 suppressed endometriosis development *in vivo*. Furthermore, IHC staining revealed fewer CD31-positive blood vessels in the lesions of the ML604086-treated mice ($p<0.01$, $p<0.01$; Figure 5G), which indicated that the CCR8 antagonist significantly reduced vessel density in endometriotic lesions *in vivo*. Consistently, PGP9.5 staining revealed a lower
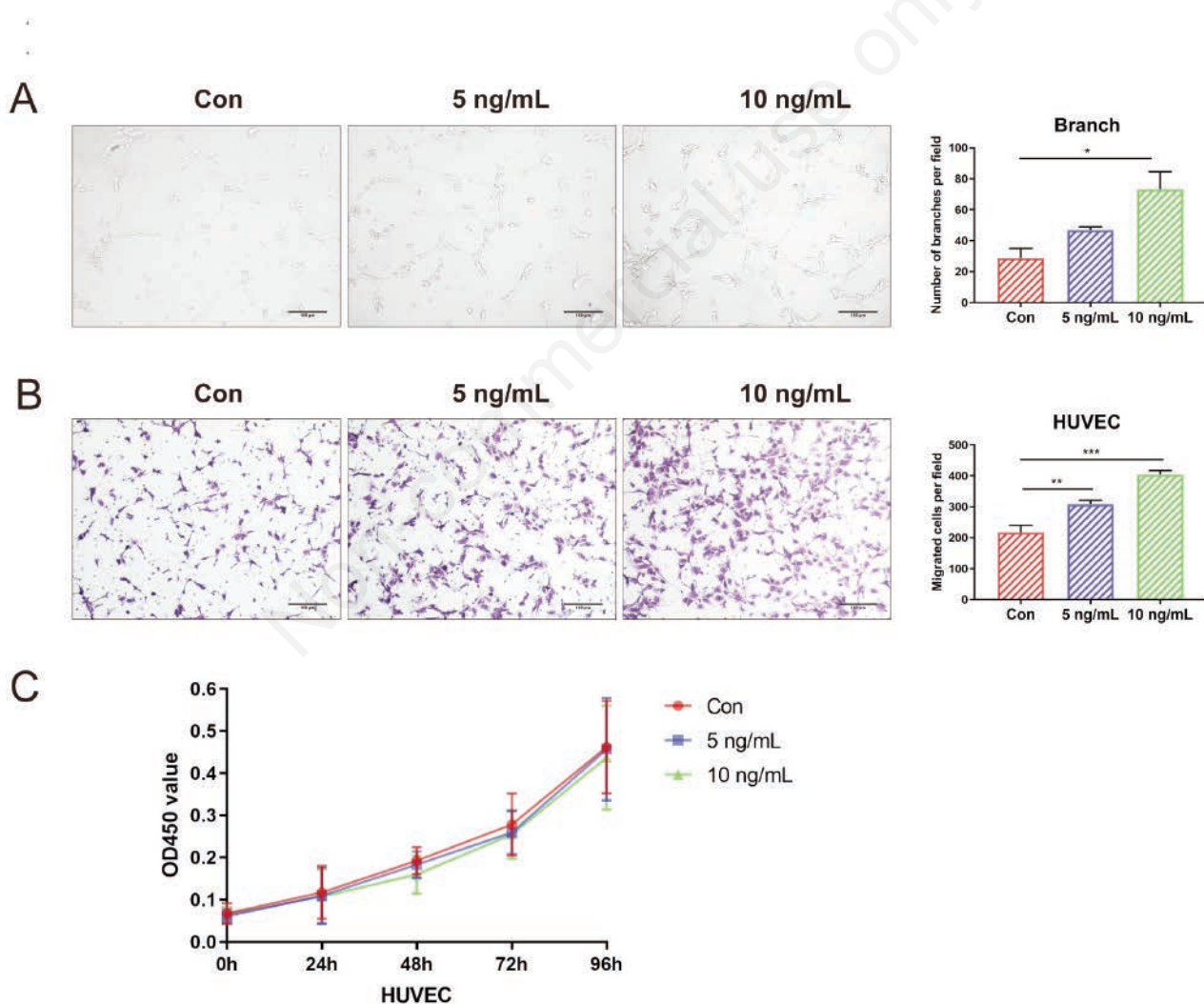
Figure 3. CCL18 promoted the migration of human endothelial cells and facilitated angiogenesis. **A**) Quantitative analysis of tube formation was performed by measuring the branch points of the tubular structures formed; scale bar: 100 μm. **B**) Representative images of the Transwell migration assay of HUVECs treated with CCL18; scale bar: 100 μm. **C**) The CCK-8 assay was used to evaluate the proliferation of HUVECs incubated with CCL18. The data are shown as the means ± SD; n=3; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

nerve infiltration density in the mice treated with a low dose of the CCR8 antagonist (*p*<0.05; Figure 5H).

Discussion

In this study, we employed RNA sequencing analysis to screen crucial transcription factors in endometriotic lesions compared with those in control endometrial tissues. Among these genes, CCL18 was confirmed to be highly expressed in endometriotic lesions. CCL18, a chemokine produced primarily by M2 macrophages, is involved in various inflammatory diseases.[15,16] In addition, CCL18 is involved in cancer cell migration, invasion, and epithelial mesenchymal transition through its receptor, CCR8, in breast cancer,[11] ovarian cancer[12] and endometrial cancer.[21] However, the impact of CCL18 on the development of endometriosis remains unclear.

The migratory and invasive ability of endometriotic cells is substantially increased in altered environments.[22,23] This study is the first to show that CCL18 can significantly promote the migration of human endometrial epithelial cells and stromal
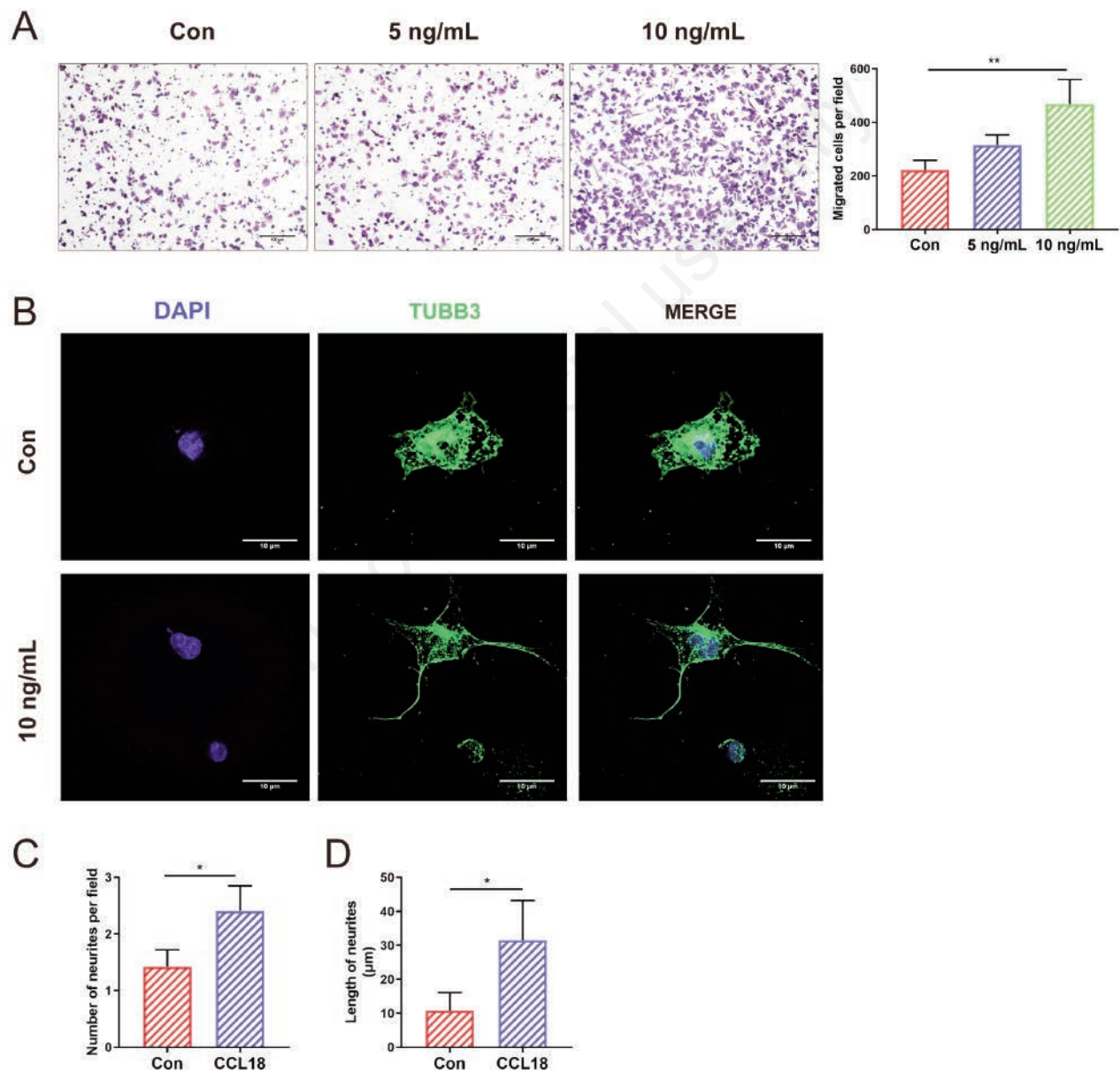
Figure 4. CCL18 promoted neurite growth in neurons. **A**) Representative images of Transwell migration assays in F11 cells incubated with 10 ng/mL CCL18 for 36 h; scale bar: 100 μm. **B-D**) Representative TUBB3 immunofluorescence-stained images of neurite growth in the F11 cell line incubated with 10 ng/mL CCL18 for 36 h; scale bar: 10 μm. The data are shown as the means ± SD; n=3; *p<0.05, **p<0.01.
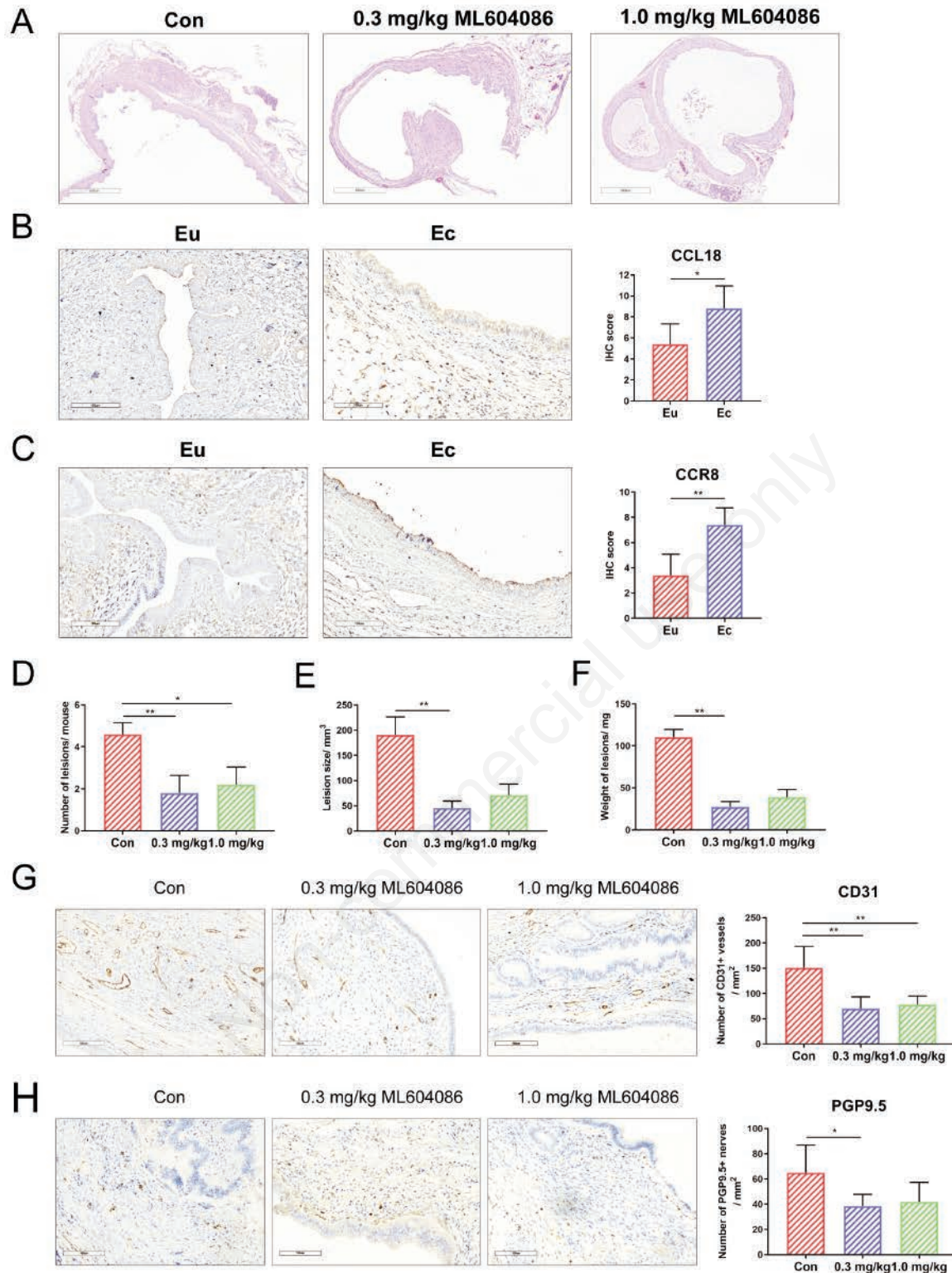
Figure 5. ML604086 (a CCR8 antagonist) restricted the development of endometriosis *in vivo*. **A**) Representative images of H&E staining of endometriotic lesions from different ML604086-treated groups of endometriosis model mice; scale bar: 600 μm. **B**) IHC staining of CCL18 in eutopic and ectopic endometria from endometriosis model mice; scale bar: 100 μm. **C**) IHC staining of CCR8 in eutopic and ectopic endometria from endometriosis model mice; scale bars: 100 μm. Effect of ML604086 on the number (**D**), cyst size (**E**) and weight (**F**) of endometriotic lesions in the control, 0.3 mg/kg ML604086 and 1 mg/kg ML604086 groups. IHC staining and IHC scores of CD31-positive vessels (**G**) and PGP9.5-positive nerve fibers (H) in endometriotic lesions in a mouse model; scale bar: 100 μm. The data are expressed as the means ± SD; n= ; *$p<0.05$, **$p<0.01$.

cells. This result is consistent with previous reports that CCL18 promotes ovarian cancer development by increasing cell migration and invasion.[12] In addition, compared with that of normal endometrial tissue, a denser vascularization during the angiogenic process contributes to endometriotic implantation in the pelvic cavity.[2]. In this study, human endothelial cells displayed more branch points and a more rapid migration after CCL18 treatment *in vitro*, which suggests that CCL18 may induce more blood sprouts to support the growth of endometriotic lesions. In endometriosis patients, dysmenorrhea is the most common symptom that impairs patients' quality of life. Brett *et al*. reported that menstrual pain is directly associated with the density of nerve fibers in endometriotic lesions.[25] This study demonstrated that CCL18 can increase the migratory ability and modulate nerve fiber growth of F11 cells *in vitro*. These results suggest that CCL18 may influence endometriosis by affecting the migration of uterine endothelial cells, blood vessel formation, and nerve fiber density. Interestingly, we also observed a positive correlation between CCL18 concentrations and clinically diagnosed endometriosis pain. This finding fur-

ther confirms that CCL18 may be a promising new approach for the clinical treatment of endometriosis.

In this study, we did not investigate the molecular mechanism by which CCL18 affects endometriosis. However, CCL18 was reported to promote the migration of cancer cells by activating its receptor CCR8.[10] Therefore, in our study, the role of CCL18 was evaluated in a mouse model of endometriosis by using ML604086, an inhibitor of CCR8. Blocking CCL18/CCR8 inhibited lesion development, angiogenesis and nerve fiber growth in endometriosis *in vivo*. However, how CCL18 is involved in the regulation of neuroangiogenesis during the development of endometriosis, as well as the optimal concentration of CCR8 inhibitors, has yet to be determined, and we hope to focus on these aspects in future studies.

In summary, this study revealed that CCL18 plays a role in endometriotic progression by increasing the migration of endometrial cells and promoting neuroangiogenesis. Thus, targeting CCL18 through its specific receptor CCR8 may provide a potential therapeutic approach for endometriosis.
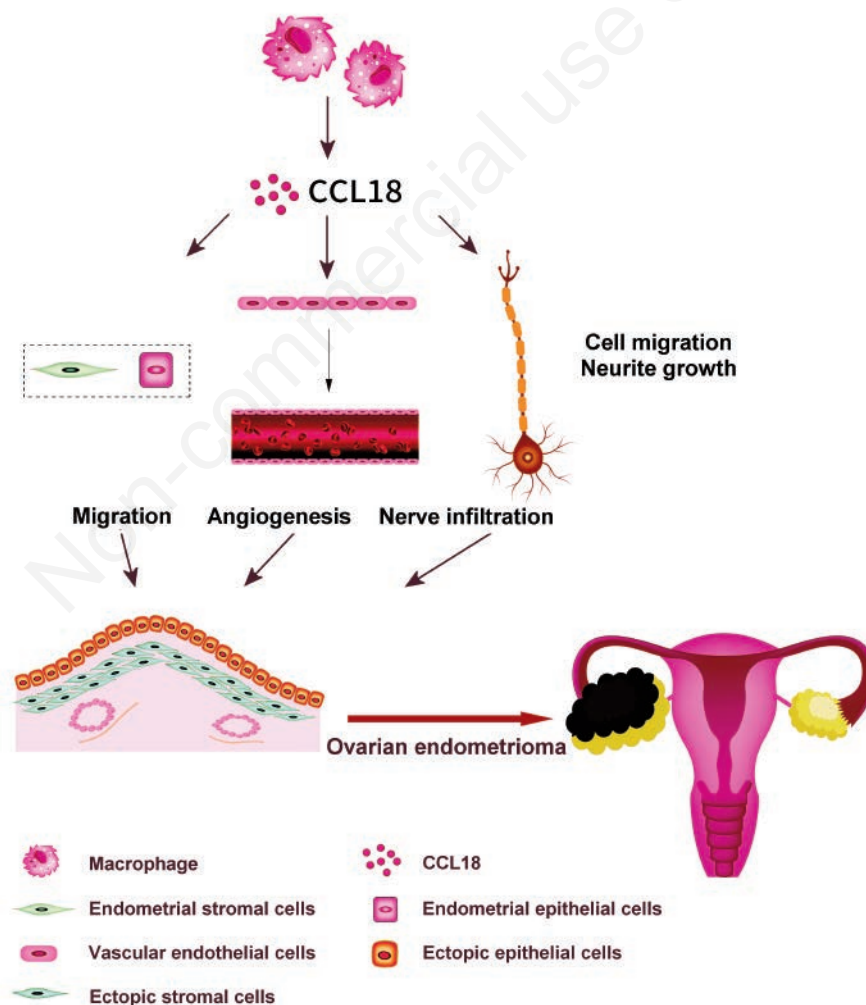
Figure 6. Schematic diagram of CCL18 in endometriosis. This study demonstrated that CCL18 promotes the migration of endometrial epithelial and stromal cells, angiogenesis and neurite growth, thereby contributing to the development of endometriosis.

References

1. Taylor HS, Kotlyar AM, Flores VA. Endometriosis is a chronic systemic disease: clinical challenges and novel innovations. Lancet 2021;397:839-52.
2. La Rosa VL, De Franciscis P, Barra F, Schiattarella A, Tropea A, Tesarik J, et al. Sexuality in women with endometriosis: a critical narrative review. Minerva Med 2020;111:79-89.
3. Rizk B, Fischer AS, Lotfy HA, Turki R, Zahed HA, Malik R, et al. Recurrence of endometriosis after hysterectomy. Facts Views Vis Obgyn 2014;6:219-27.
4. Vallvé-Juanico J, Houshdaran S, Giudice LC. The endometrial immune environment of women with endometriosis. Hum Reprod Update 2019;25:564-91.
5. Laskin DL, Sunil VR, Gardner CR, Laskin JD. Macrophages and tissue injury: agents of defense or destruction? Annu Rev Pharmacol Toxicol 2011;51:267-88.
6. Chen P, Bonaldo P. Role of macrophage polarization in tumor angiogenesis and vessel normalization. Int Rev Cell Mol Biol 2013;301:1-35.
7. Schmid S, Le U-T, Haager B, Mayer O, Dietrich I, Elze M, et al. Local concentrations of CC-Chemokine-Ligand 18 correlate with tumor size in non-small cell lung cancer and are elevated in lymph node-positive disease. Anticancer Res 2016;36:4667-71.
8. Mertens JS, Jong EMGJd, Hoogen LLvd, Wienke J, Thurlings RM, Seyger MMB, et al. The identification of CCL18 as biomarker of disease activity in localized scleroderma. J Autoimmun 2019;101:86-93.
9. Pochetuhen K, Luzina IG, Lockatell V, Choi J, Todd NW, Atamas SP. Complex regulation of pulmonary inflammation and fibrosis by CCL18. Am J Pathol 2007;171:428-37.
10. Liu X, Xu X, Deng W, Huang M, Wu Y, Zhou Z, et al. CCL18 enhances migration, invasion and EMT by binding CCR8 in bladder cancer cells. Mol Med Rep 2019;19:1678-86.
11. Lin L, Chen Y-S, Yao Y-D, Chen J-Q, Chen J-N, Huang S-Y, et al. CCL18 from tumor-associated macrophages promotes angiogenesis in breast cancer. Oncotarget 2015;6:34758-73.
12. Yang Y-Z, Zhang W, Zhao B-B, Li L. Expression of CCL18 gene in ovarian cancer and its impact on the biologic function of ovarian cancer cells. Int J Clin Exp Pathol 2019;12:2572-84.
13. Li G, Lin Y, Zhang Y, Gu N, Yang B, Shan S, et al. Endometrial stromal cell ferroptosis promotes angiogenesis in endometriosis. Cell Death Discov 2022;8:29.
14. Greaves E, Collins F, Esnal-Zufiaurre A, Giakoumelou S, Horne AW, Saunders PTK. Estrogen receptor (ER) agonists differentially regulate neuroangiogenesis in peritoneal endometriosis via the repellent factor SLIT3. Endocrinology 2014;155:4015-26.
15. Dodds KN, Beckett EAH, Evans SF, Hutchinson MR. Lesion development is modulated by the natural estrous cycle and mouse strain in a minimally invasive model of endometriosis. Biol Reprod 2017;97:810-21.
16. Somigliana E, Viganò P, Rossi G, Carinelli S, Vignali M, Panina-Bordignon P. Endometrial ability to implant in ectopic sites can be prevented by interleukin-12 in a murine model of endometriosis. Hum Reprod 1999;14:2944-50.
17. Wang L, Jenkins TJ, Dai M, et al. Antagonism of chemokine receptor CCR8 is ineffective in a primate model of asthma. Thorax 2013;68:506-12.
18. Ricci AG, Olivares CN, Bilotas MA, Bastón JI, Singla JJ, Meresman GF, et al. Natural therapies assessment for the treatment of endometriosis. Hum Reprod 2013;28:178-88.
19. Ricci AG, Olivares CN, Bilotas MA, Meresman GF, Barañao RI. Effect of vascular endothelial growth factor inhibition on endometrial implant development in a murine model of endometriosis. Reprod Sci 2011;18:614-22.
20. Gardella B, Dominoni M, Gritti A, Arrigo A, Antonucci S, Carletti GV, et al. Endometriosis pain and epithelial neutrophil activating peptide-78 levels. Sci Rep 2022;12:3227.
21. Jing X, Peng J, Dou Y, Sun J, Ma C, Wang Q, et al. Macrophage ERα promoted invasion of endometrial cancer cell by mTOR/KIF5B-mediated epithelial to mesenchymal transition. Immunol Cell Biol 2019;97:563-76.
22. Liu J, Wang Y, Chen P, Ma Y, Wang S, Tian Y, et al. AC002454.1 and CDK6 synergistically promote endometrial cell migration and invasion in endometriosis. Reproduction 2019;157:535-43.
23. Li J, Ma J, Fei X, Zhang T, Zhou J, Lin J. Roles of cell migration and invasion mediated by Twist in endometriosis. J Obstet Gynaecol Res 2019;45:1488-96.
24. Rocha ALL, Reis FM, Taylor RN. Angiogenesis and endometriosis. Obstet Gynecol Int 2013:859619.
25. McKinnon B, Bersinger NA, Wotzkow C, Mueller MD. Endometriosis-associated nerve fibers, peritoneal fluid cytokine concentrations, and pain in endometriotic lesions from different locations. Fertil Steril 2012;97:373-80.

OPEN ACCESS