Macrophage-derived netrin-1 contributes to endometriosis-associated pain

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Background: Endometriosis-associated pain can be considered a type of neuropathic pain. Netrin-1 is an axon guidance cue that regulates axonal attraction or rejection in neural injury and regeneration. However, whether netrin-1 plays a role in endometriosis-associated pain remains unclear. This study aimed to determine the role of netrin-1 in endometriosis-related pain.

Methods: Peripheral blood, peritoneal fluid, and endometrial tissues were sampled from women with (n=37) and without endometriosis (n=23). Lipopolysaccharide (LPS) and interferon gamma (IFN-γ) were used to stimulate human monocytic cell lines (THP-1) and rat alveolar macrophage-derived cell lines (NR8383) to induce M1 phenotype macrophages. Serum netrin-1 concentrations, endometrial expression levels of netrin-1, and its receptors including deleted in colorectal cancer (DCC), A2B adenosine receptor (A2BAR), uncoordinated B receptor (UNC5B), uncoordinated C receptor (UNC5C) and Down's syndrome cell adhesion molecule (DSCAM) were assessed. The polarization phenotypes of the peritoneal macrophages were identified by detecting the marker expression of M1/M2 macrophages via flow cytometry. The expression levels of M1 markers and netrin-1 in THP-1/NR8383 cells were determined.

Results: The expression levels of netrin-1 in serum and endometriotic lesions were significantly higher in women with endometriosis, and were positively correlated with the severity of endometriosis-associated pain. Netrin-1 was co-expressed with CD68 (a macrophage marker) in endometriotic lesions and was synthesized and secreted by THP-1 and NR8383 cells in the process of M1 polarization. In women with endometriosis, peritoneal macrophages were polarized towards the M1 phenotype. In addition, increased expression of DCC and A2BAR, and decreased expression of UNC5B, UNC5C and DSCAM were found in endometriotic lesions.

Conclusions: These results suggest that netrin-1 production by macrophages in endometriotic lesions may play an important role in endometriosis-associated pain.

Keywords: Endometriosis; pain; netrin-1; macrophage; polarization

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Introduction

Endometriosis is a common gynecological disease characterized by pain and infertility, which affects women of childbearing age (1). Pain symptoms in patients with endometriosis include dysmenorrhea, dyspareunia, dysuria, defecation pain and chronic pelvic pain, all of which have a significant impact on women's quality of life (2). However, despite extensive research efforts, the exact mechanisms of endometriosis-associated pain remain unclear (3,4).

In 2000, Anaf et al. were first to report S100-labeled nerves infiltrating in endometriotic lesions, with the percentage of nerves being significantly higher in the lesions of women who suffered severe endometriosis-associated pain (5). In subsequent studies, elevated specific makers for sensory, sympathetic, and parasympathetic nerves (6-8) and growth factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurite growth factor 2 (NEGF2) (9-13) were identified in different types of endometriotic lesions, all of which correlated with endometriosis-associated pain. Therefore, endometriosis-associated pain was considered to be a type of neuropathic pain. Moreover, in a rat model of endometriosis, auto-transplanted endometriotic lesions were shown to develop autonomic and sensory innervation (14). In other studies, growth-associated protein 43 (GAP-43), a marker for neurite outgrowth and regeneration, was expressed in nerve fibers infiltrating in the endometriotic lesions of peritoneal and deep infiltrating endometriosis (15,16). However, it is still unclear how the nerve fibers in endometriotic lesions sprout abnormally.

Netrins are members of the laminin superfamily which share a similar amino acid terminal sequence (17). The name netrin is derived from the Sanskrit word "netr", meaning "to guide", because of their role in axon guidance (18). Until now, three secreted netrins (netrins 1, 3 and 4), and two glycosylphosphatidylinositol-anchored membrane proteins, netrins G1 and G2, have been identified in mammals. Netrin Gs regulate axon guidance by forming synaptic interactions between neurons with the transmembrane netrin G ligands NGL1 and 2. The secreted netrins can bind to the receptors of deleted in colorectal cancer (DCC), neogenin or uncoordinated (UNC5) A–D, causing axonal attraction or rejection (19,20). Recent studies have shown that netrins also participate in angiogenesis, cell proliferation, migration and tumorigenesis by binding to the receptors of DCC, neogenin, UNC5, Down's syndrome cell adhesion molecule (DSCAM), CD146 and A2B adenosine receptor (A2BAR) (21-24).

It has been demonstrated that netrin-1, the most studied guidance cue, triggers an attraction effect through DCC and neogenin, or a repulsion effect via UNC5 A-D (17). In the central nervous system, netrin 1 is secreted by ventricular zone neural progenitors and floor-plate cells in the ventral embryonic spinal cord (25,26). In the process of peripheral neural injury and regeneration, increased levels of netrin-1 are mainly produced by Schwann cells (27). Netrin-1 expression has also been found to be increased in hypoxic conditions (24), and in inflammatory and other disease conditions such as obesity (28), type 2 diabetes (29), acute lung injury (30), atherosclerosis (31) and abdominal aortic aneurysm (AAA) (32). However, it is not clear whether netrin-1 is involved in the pathogenesis of endometriosis.

Co-localization of netrin-1 and CD68, a marker of macrophages, has been identified in atherosclerotic plaques (31), adipose tissues (28) and inflamed aortic vessel walls (32), suggesting that macrophages may participate in inflammation by secreting netrin-1. In fact, the inflammatory microenvironment of endometriosis can promote macrophage infiltration (33,34), which plays a crucial role in the etiology and pathogenesis of this disease including inflammatory response (35), angiogenesis (34), proliferation (36) and neurogenesis (37). The interaction between macrophages and nerve fibers contributes to neuroinflammation and pain generation in endometriosis (37). A large number of up-regulated molecules released by nerve fibers in endometriotic lesions such as monocyte chemotactic protein-1 (MCP-1), colony-stimulating factor 1 (CSF-1) and leukemia inhibitory factor (LIF), are responsible for the recruitment of macrophages from vessels within the lesions (38,39). Conversely, infiltrating macrophages in the lesions can in turn mediate neurogenesis by secreting neurotrophins, semaphorins, and vascular epithelial growth factor (VEGF) (37,39,40). Macrophage activation is closely associated with endometriosis. However, whether macrophages differentiate into classically activated macrophages (pro-inflammatory, M1) or alternatively activated macrophages (anti-inflammatory, M2) (41) remains highly debated, as does the issue of polarization of M1/M2 macrophages in endometriosis (33,34,42,43), Therefore, it is important to explore the role of macrophage-mediated netrin-1 in the pathogenesis of endometriosis.

In the present study, we aimed to investigate the role of macrophage-mediated netrin-1 in endometriosis-associated pain. Firstly, we detected expressions of netrin-1

and its receptors in endometriotic lesions. Secondly, we localized netrin-1 and macrophages in endometriotic lesions. Thirdly, we determined polarization phenotypes of peritoneal macrophages. Lastly, we observed the expression and secretion of netrin-1 after macrophage M1 polarization was induced *in vitro*.

We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/atm-20-2161).

Methods

Patients

A total of 60 women with endometriosis (case group, n=37) and without endometriosis (e.g., other benign gynecologic diseases excluding chronic pelvic pain, control group, n=23) who were admitted to the Women's Hospital between January 2018 and June 2018 were recruited for this study. All participants underwent laparoscopic surgery to determine the presence and staging of endometriosis. The specimen was confirmed by pathologists after surgery. In the endometriosis group, 17 women were at stage I–II while 20 were at stage III–IV, according to the Revised American Fertility Society Scoring system. The types of pain associated with endometriosis included dysmenorrhea, dyspareunia, dysuria, defecation pain and chronic pelvic pain. The severity of pain was documented using a standardized questionnaire with a Visual Analog Scale (VAS, 0–10) as previously described (44). The pain scale was subdivided into 10 grades, graduated from "no pain" on the left side of the scale to "maximum pain imaginable" on the right side of the scale. Pain symptoms were reported in 48.6% (n=18) and 0% (n=0) of the endometriosis and non-endometriosis groups, while infertility was 24.3% (n=9) and 17.4% (n=4), respectively. None of the participants had received hormone therapy in the 6 months prior to surgery. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was obtained from all participating patients. This study was approved by the Human Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University (no. 20190012).

Samples collection

Peripheral blood samples were obtained 1 day before surgery, and red fresh endometriotic lesions and endometrial tissues were collected during the surgical procedure. The blood or cell supernatants were centrifuged at 1,000 g for 10 minutes, and the supernatants were transferred into 1.5 mL tubes and stored at –80 ℃ until processing. Of the 37 women with endometriosis, 16 (43.2%) had ovarian endometriosis, 11 (29.7%) had peritoneal endometriosis and 10 (27.0%) had deeply infiltrating endometriosis. Half of the endometrial tissue was frozen in –80 ℃ for mRNA detection, and the other half was immersed in formalin (Solarbio) for further immunohistochemical and immunofluorescence staining.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of netrin-1 in serum or culture supernatants were quantified by ELISA kits (Cusabio) according to the manufacturer's instructions.

Quantitative real-time polymerase chain (qRT-PCR) and Western blot analyses

Total RNA was extracted from the endometrial samples or cells with TRIzol reagent (Takara) and reversed by a PrimeScript Reverse Transcription (RT) reagent kit (Takara) according to the manufacturer's recommendations. SYBR Premix Ex Taq kit (Takara) was used for quantitative polymerase chain reaction (PCR) and the fold change was determined through the $2^{-\Delta\Delta Ct}$ method. The primers for netrin-1, receptors, and inflammatory factors were synthesized from Generay and the sequences are listed in *Tables 1,2*. Western blot analysis was used to test the netrin-1 protein expression levels with the netrin-1 antibody in THP-1 and NR8383 cells in the M1 polarization process. In the Western blot, the primary antibodies of netrin-1 (1:1,000, ab126729, rabbit monoclonal, Abcam), and GAPDH (1:1,000, Mab5465-100; mouse monoclonal, Multi Sciences) as well as a secondary antibody (1:5,000, ab97051/ab97023; Abcam) were used and detected using an electrochemiluminescence detection kit (Biological Industries, Beit-Ha'emek).

Immunohistochemical staining

In brief, serial sections of 6 μm thickness were immunostained using the corresponding antibody for 1 hour at room temperature. The specific antibodies of netrin-1 (1:50, ab122903, goat polyclonal, Abcam), DCC

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Table 1 Primer sequences of netrin-1 receptors

Primer	Forward (5'-3')	Reverse (5'-3')
Actin (h*)	AGAAGGATTCCTATGTGGGCG	GGATAGCACAGCCTGGATAGCA
Netrin-1 (h)	CGACCCCAAGAAGGCGCACCCGCCC	CCTCCTGCTCGTTCTGCTTG
DCC (h)	AGCAGGGAGCTCTATGTCCA	ACTGACTTCTTCCTGCTCCG
Neogenin (h)	CAGCCTGTGATTAGTGCCCA	TCATAGGTGGGAGGTCCTGG
UNC5A (h)	CAAGGTTTGCTGAGCTGCTG	GTCCAGGTGGAGTTTCTGGG
UNC5B (h)	TGTGCATGCAAATGCTGGAG	TGTCTGTGTCGAAGTCACGG
UNC5C (h)	GCAAATGCTCGTGCTACCTG	TCAATGCTCACTTCCCGGAC
UNC5D (h)	TCAATGGTGGGGCCTTTTGT	ATTCGCTCCACACTTCCCAG
DSCAM (h)	CAAGAGGTAGTGTTTGCCAGC	AGACGACAGTGATGTACGCC
CD146 (h)	CCCTCACACCAGACTCCAAC	GTTCGCTCTTACGAGACGGG
A2BAR (h)	CTGCAGACGCCCACCAACTA	ATTCGTGGTTCCATCCCAGG

*, h = human.

Table 2 Primer sequences of netrin-1 and M1 phenotype markers

Primer	Forward (5'-3')	Reverse (5'-3')
GAPDH (h*)	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
Netrin-1 (h)	CGACCCCAAGAAGGCGCACCCGCCC	CCTCCTGCTCGTTCTGCTTG
TNF-α (h)	CGAGTGACAAGCCTGTAGCC	TGAAGAGGACCTGGGAGTAGAT
IL-1β (h)	AGCTACGAATCTCCGACCAC	CGTTATCCCATGTGTCGAAGAA
IL-6 (h)	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
MCP-1 (h)	AAACTGAAGCTCGCACTCTCGC	AGGTGACTGGGGCATTGATTG
iNOS (h)	TTCAGTATCACAACCTCAGCAAG	TGGACCTGCAAGTTAAAATCCC
GAPDH (r#)	CTCATGACCACAGTCCATGC	TTCAGCTCTGGGATGACCTT
Netrin-1 (r)	CGTTACGCTCACTCTGTCGC	GCCTCCTGCTCGTTCTGCT
TNF-α (r)	ACCATGAGCACGGAAAGCAT	AACTGATGAGAGGGAGCCCA
IL-1β (r)	AGTGAGGAGAATGACCTGTTC	CGAGATGCTGCTGTGAGATT
IL-6 (r)	GCCAGAGTCATTCAGAGCAATA	GTTGGATGGTCTTGGTCCTTAG
MCP-1 (r)	TCGGCTGGAGAACTACAAGA	GCTGAAGTCCTTAGGGTTGA
iNOS (r)	CTGCTTTGTGCGGAGTGTC	ATTTCTTCCTGATAGAGGTGGT

*, h = human; #, r = rat. TNF, tumor necrosis factor; IL, interleukin; iNOS, nitric oxide synthase 2; LPS, lipopolysaccharide; INF, interferon; MCP-1, monocyte chemotactic protein-1.

(1:300, bs-0592R, rabbit polyclonal, Bioss), UNC5B (1:300, bs-11492R, rabbit polyclonal, Bioss) and A2BAR (1:1,000, PA5-72850, rabbit polyclonal, Invitrogen) were used to access the expression levels and localization of netrin-1 and its receptors in endometrial tissues. Envision-labeled polymer-alkaline phosphatase goat/rabbit (Dako, Glostrup) and diaminobenzidine (K5007; Dako, Glostrup) were used to visualize the antigen-antibody reaction, also known as chromogen. After washing, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted

with a mounting medium. The sum of the percentage (0–3) and intensity scores (0–3) were represented as immunohistochemistry (IHC) scores to show the expression levels of molecules. The detailed immunohistochemical process and scoring have been described previously (10). All slides were analyzed by two blinded observers.

Double immunofluorescence staining

The slides of the endometriotic lesions were incubated with the primary antibody of netrin-1 (1:500, ab126729, rabbit monoclonal, Abcam) and CD68 (1:200, ab201340, mouse monoclonal, Abcam), and then were rinsed in phosphate-buffered saline (PBS) before being mounted with the corresponding fluorescent secondary antibody (Abcam).

Flow cytometric analysis

The peritoneal macrophages were washed with an erythrocyte lysis buffer and incubated with human Fc block (1:200, BD564219, BD Biosciences) on ice. Subsequently, the cells were incubated with the PE-Cy^{TM}7-conjugated anti-CD86 (1:200, BD561128, mouse BALB/c IgG1, κ, BD Biosciences) and PE-conjugated anti-CD163 (1:50, BD556018, mouse BALB/c IgG1, κ, BD Biosciences) antibody for 15 minutes. For intracellular staining, the cells were fixed and permeabilized in fixation and permeabilization solution (BD54722, BD Biosciences) for 20 minutes, and incubated with the FITC-conjugated anti-CD68 (1:200, BD562117, mouse BALB/c IgG2b, κ, BD Biosciences) antibody for 1 hour. The samples were then analyzed with a FACS Verse system and analyzed with the BD FACS DIVA software (BD Biosciences, United States). The PE-Cy^{TM}7-conjugated IgG1 (1: 200, BD557872, BD Biosciences), PE-conjugated IgG1 (1:50, BD555749, BD Biosciences) and FITC-conjugated IgG2b (1:1,000, BD565379, BD Biosciences) antibodies served as control antibodies. CD68$^+$CD86$^+$CD163$^-$ macrophages were identified as M1 macrophages, while the CD68$^+$CD86$^-$CD163$^+$ cells were identified as M2 macrophages.

Cells intervention

Human monocytic cell line THP-1 cells and rat alveolar macrophage-derived cell line NR8383 cells were purchased from the Stem Cell Bank at Chinese Academy of Sciences. The cells were cultured with Dulbecco's Modified Eagle Medium/F-12 containing 15% fetal calf sera (Gibco). The THP-1 cells were induced to the macrophages (M0) by phorbol-12-myristate-13-acetate (PMA, 20 ng/mL, Sigma). Subsequently, the differentiated human THP-1 macrophages and NR8383 cells were treated with lipopolysaccharide (LPS, 20 ng/mL, Sigma) and interferon gamma (IFN-γ, 20 ng/mL, PeproTech) to further induce M1 phenotype macrophages. Next, expression levels of the M1 phenotype markers tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, MCP-1 and nitric oxide synthase 2 (iNOS) were assessed at 1, 3, 6, 12, 24 and 48 hours after stimulation. Meanwhile, mRNA and protein expression levels of netrin-1 as well as secreted netrin-1 levels in cell supernatants were measured using qRT-PCR, Western blot and ELISA respectively.

Statistical analysis

Data were analyzed using SPSS Version 24.0 (IBM). The continuous data variables were quantified as mean ± SEM. Differences in variables between two groups and multiple groups were analyzed using unpaired Student's t-test and one-way ANOVA, respectively. Nonparametric testing was used where sample sizes were insufficient to confirm normality of data distribution. Spearman's analysis was conducted to analyze the correlation between netrin-1 expression and the severity of endometriosis-associated pain. Statistical tests ($χ^2$ and Mann-Whitney U test) were performed to compare the frequency and median among groups. P values <0.05 were considered statistically significant.

Results

Patient characteristics

Except for pain symptoms, there were no significant differences between the endometriosis and non-endometriosis groups with respect to their age, gravidity, parity, abortion, infertility, or cycle stage (*Table 3*).

Netrin-1 concentrations in serum were associated with endometriosis-associated pain

The serum concentrations of netrin-1 in women with endometriosis were significantly higher than those from women without endometriosis (P<0.05, *Figure 1A*), and Spearman's analysis results showed that netrin-1

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Table 3 Characteristics of patients

Parameters	Endometriosis (n=37)	Controls (n=23)	P
Age (years), mean ± SEM	33.5±1.0	30.6±1.2	0.07
Gravidity, median (range)	3 (0–5)	2 (0–5)	0.16
Parity, median (range)	1 (0–2)	1 (0–4)	0.94
Abortion, median (range)	2 (0–4)	1 (0–3)	0.06
Menstrual cycle phase, n (%)			0.40
Proliferative	29 (78.4)	20 (87.0)	
Secretory	8 (21.6)	3 (13.0)	
Pain symptoms, n (%)	18 (48.6)	0 (0)	<0.0001
Infertility, n (%)	9 (24.3)	4 (17.4)	0.53
r-AFS, n (%)			–
I–II	17 (45.9)	–	
III–IV	20 (54.1)	–	

r-AFS, revised American Fertility Society; SEM, standard error of the mean.

concentrations in the serum (r=0.46, P<0.01; *Figure 1B*) were positively correlated with the severity of endometriosis-associated pain.

Netrin-1 was over-expressed in endometriotic lesions

The mRNA expression levels of netrin-1 in endometriotic lesions were significantly higher than those in the eutopic endometrium from women with (P<0.01) or without endometriosis (P<0.01, *Figure 1C*), and were positively correlated with the severity of endometriosis-associated pain (r=0.83, P<0.0001; *Figure 1D*). Immunohistochemical staining showed that netrin-1 was not only expressed in epithelial and interstitial vascular endothelial cells, but also expressed in endometrial stromal cells in endometriotic lesions from women with endometriosis with pain (*Figure 1E*) or without pain (*Figure 1F*), and in endothelial cells in the eutopic endometrium from women with (*Figure 1G*) or without (*Figure 1H*) endometriosis. Moreover, netrin-1 was co-expressed with CD68 in endometriotic lesions (*Figure 1I,J,K,L*).

Endometriosis polarized peritoneal macrophages towards the M1 phenotype

CD68$^+$ cells were recognized as peritoneal macrophages in women with endometriosis (*Figure 2A,B*) or without endometriosis (*Figure 2C,D*). Flow cytometry plots revealed that the proportion of M1 macrophages (CD68$^+$CD163$^-$)

was high in women with endometriosis, but it did not reach statistical significance (*Figure 3A*). Moreover, there were a significantly higher proportion of CD86$^+$CD163$^+$ macrophages (P<0.0001, *Figure 3B*), but a statistically lower proportion of M2 macrophages (CD86$^-$CD163$^+$) (P<0.0001, *Figure 3C*) and CD86$^-$CD163$^-$ macrophages (P<0.05, *Figure 3D*) in women with endometriosis compared to women without endometriosis. In short, the proportion of CD86$^+$ macrophages significantly increased in endometriosis patients compared with those without endometriosis (P<0.0001, *Figure 3E*), while the proportion of CD163$^+$ macrophages did not differ (*Figure 3E,F*).

Netrin-1 was secreted by macrophages

PMA treatment induced significantly higher mRNA expression levels of IL-1β (P<0.01), IL-6 (P<0.05), and iNOS (P<0.01); lower mRNA expression levels of TNF-α (P<0.01); and similar mRNA levels of MCP-1 (P>0.05) and netrin-1 (P>0.05) in human THP-1 macrophages compared with untreated monocytic THP-1 cells. The mRNA expression levels of the M1 phenotype markers TNF-α, IL-1β, IL-6, MCP-1 and iNOS were all significantly elevated after combined stimulation of LPS and IFN-γ within 48 hours, reaching pecks at 1 hour (P<0.05; *Figure 4A*), 1 hour (P<0.01; *Figure 4B*), 6 hours (P<0.05; *Figure 4C*), 24 hours (P<0.01; *Figure 4D*) and 48 hours (P<0.05; *Figure 4E*), respectively. The mRNA, protein expression
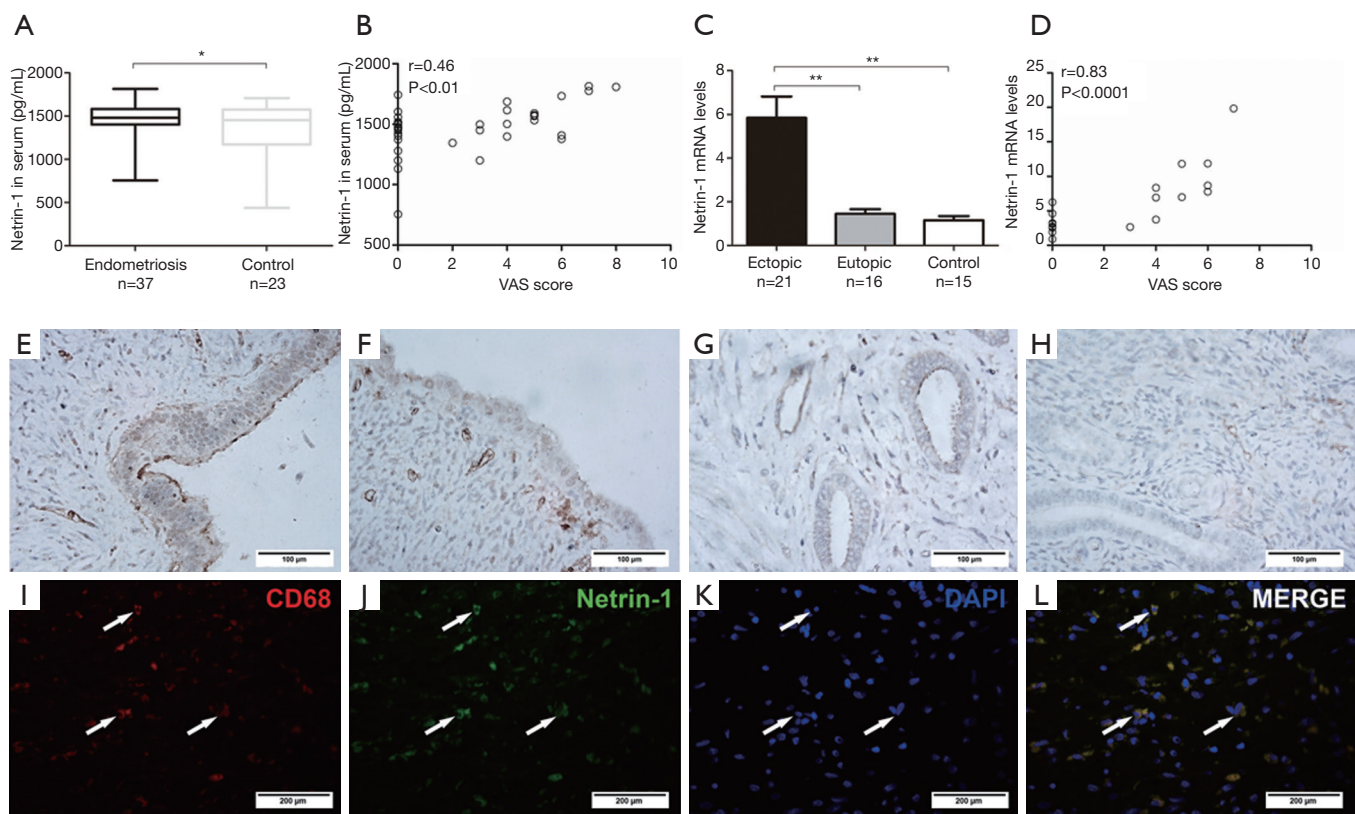
Figure 1 Netrin-1 levels in women with endometriosis. (A,B,C,D) The netrin-1 concentrations in serum (A,B) and mRNA expression levels in endometrial tissues (C,D) were tested using RT-qPCR and ELISA respectively. (E,F,G,H) The location and expression levels of netrin-1 in endometriotic lesions. (E,F) The eutopic endometria with (G) or without (H) endometriosis were analyzed by immunohistochemical staining. (I,J,K,L) The location of netirn-1 and CD68 in endometriotic lesions were tested by immunofluorescence double staining. Error bars show mean ± SEM. *, P<0.05; **, P<0.01 (one-way ANOVA and Student's *t*-test). VAS, Visual Analog Scale; SEM, standard error of the mean.

levels and supernatant concentrations of netrin-1 in PMA-differentiated human THP-1 macrophages were significantly higher at 12 hours (P<0.05; *Figure 4F*), 24 hours (P<0.05; *Figure 4G*) and 48 hours (P<0.01; *Figure 4H*) respectively after M1 polarization.

In the NR8383 cell lines, the mRNA expression levels of TNF-α, IL-1β, IL-6, MCP-1 and iNOS all increased within 48 hours after LPS and IFN-γ treatment, reaching pecks at 6 hours (P<0.01; *Figure 4I*), 6 hours (P<0.05; *Figure 4J*), 3 hours (P<0.05; *Figure 4K*), 6 hours (P<0.05; *Figure 4L*) and 12 hours (P<0.05; *Figure 4M*), respectively. Meanwhile, the mRNA and protein levels and supernatant concentrations of netrin-1 in the NR8383 cells were significantly elevated at 12 hours (P<0.05; *Figure 4N*), 24 hours (P<0.05; *Figure 4O*) and 48 hours (P<0.0001; *Figure 4P*) respectively after M1 polarization.

Netrin-1 receptors were more expressed in endometriotic tissues

The expression levels of different netrin-1 receptors vary greatly in different types of endometrial tissue (*Figure 5*). Specifically, the mRNA expression levels of DCC and A2BAR in endometriotic lesions were significantly higher than those in the eutopic endometrium from women with (P<0.01; P<0.05) or without (P<0.01; P<0.01) endometriosis (*Figure 5A,I*). Also, neogenin mRNA expression levels were statistically higher in endometriotic lesions than those in the eutopic endometrium from women without endometriosis (P<0.01; *Figure 5B*). On the contrary, UNC5B, UNC5C and DSCAM mRNA expression levels in endometriotic lesions were significantly lower than those in the eutopic endometrium from women with (P<0.01; P<0.0001; P<0.01)

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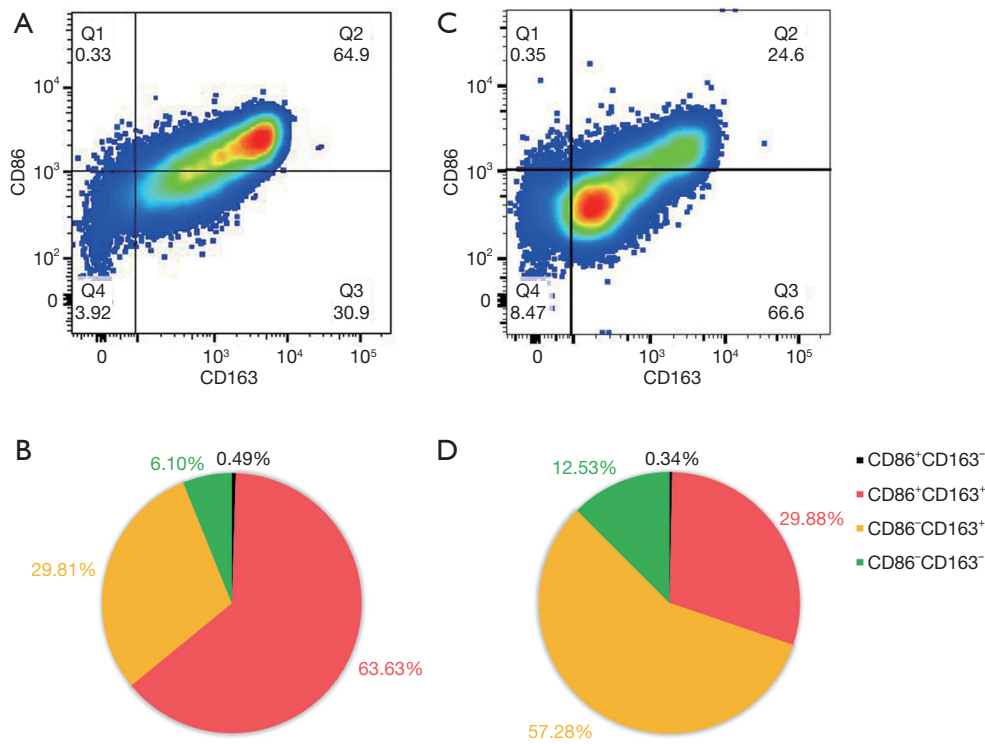
Figure 2 Polarized phenotypes of peritoneal macrophages in women with or without endometriosis. Representative dot plot and statistical chart of CD68$^+$ cells in peritoneal fluids from endometriosis (A,B, n=8) and non-endometriosis (C,D, n=6) women by flow cytometric analysis using CD86 and CD163 markers.
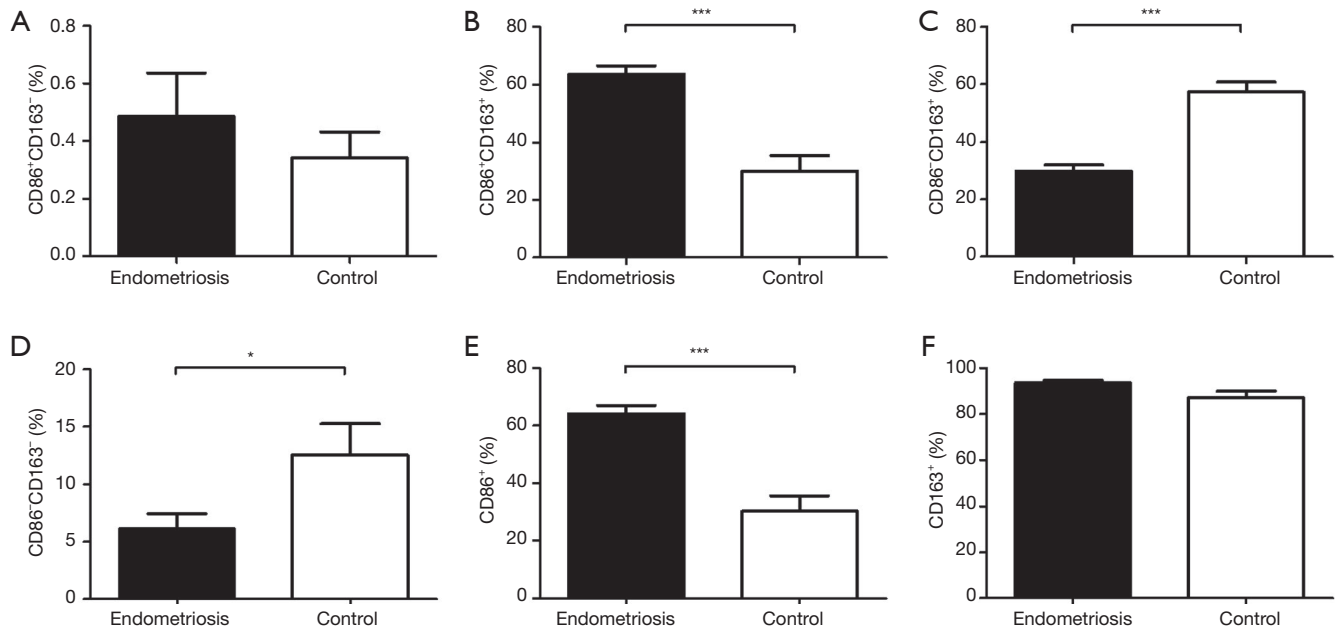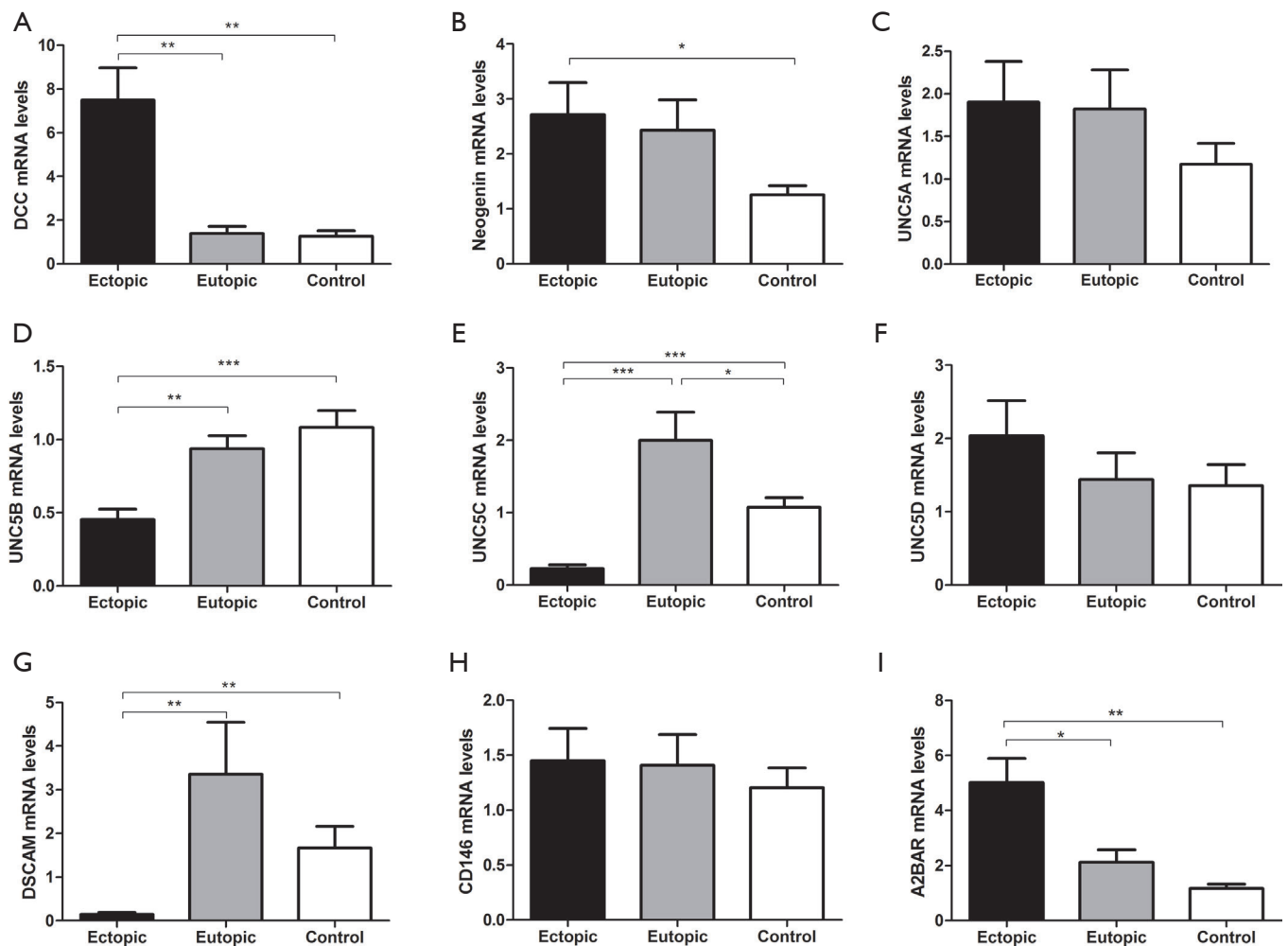

Figure 3 Different polarized phenotypes of peritoneal macrophages in endometriosis and women without endometriosis. The percentages of CD86$^+$CD163$^-$ (A), CD86$^+$CD163$^+$ (B), CD86$^-$CD163$^+$ (C), CD86$^-$CD163$^-$ (D), CD86$^+$ (E), and CD163$^+$ (F) cells in each group. *, $P<0.05$; ***, $P<0.0001$ (Student's t-test).
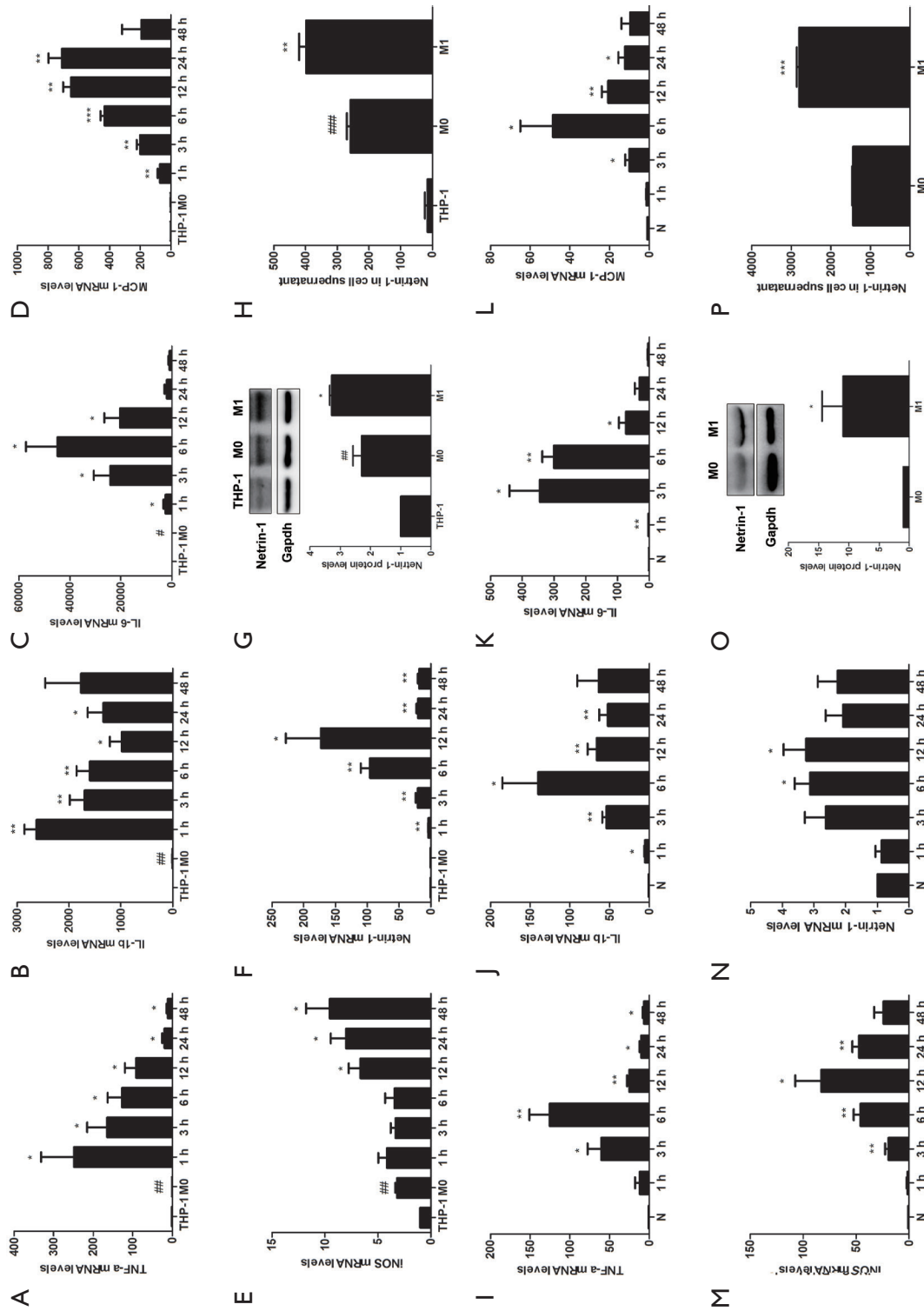
Figure 4 Expression levels of macrophage M1 phenotype markers and netrin-1 after M1 polarization *in vitro*. (A,B,C,D,E,F,G,H) The mRNA expression levels of TNF-α (A), IL-1β (B), IL-6 (C), MCP-1 (D) and iNOS (E) in THP-1 cells were tested using RT-qPCR after PMA stimulation and combined stimulation of LPS and IFN-γ; the mRNA (F) and protein levels (G) as well as supernatant concentrations (H) of netrin-1 in THP-1 were measured using RT-qPCR. Western blot and ELISA, respectively. (I,J,K,L,M,N,O,P) The mRNA expression levels of TNF-α (I), IL-1β (J), IL-6 (K), MCP-1 (L) and iNOS (M) in NR8383 cells were tested using RT-qPCR after combined stimulation of LPS and IFN-γ; The mRNA (N) and protein levels (O) as well as supernatant concentrations (P) of netrin-1 in NR8383 cells were measured using RT-qPCR. Western blot and ELISA, respectively. Error bars show mean ± SEM. #, P<0.05; ##, P<0.01; ###, P<0.0001, compared with macrophage M0 phenotype; *, P<0.05; **, P<0.01; ***, P<0.0001 compared with THP-1; #, P<0.05; ##, P<0.01; ###, P<0.0001, compared with macrophage M0 phenotype (Student's *t*-test). TNF, tumor necrosis factor; IL, interleukin; INF, interferon; MCP-1, monocyte chemotactic protein-1; SEM, standard error of the mean.

Figure 5 Expression levels of netrin-1 receptors in endometrial tissues. The mRNA expression levels of DCC (A), neogenin (B), UNC5A (C), UNC5B (D), UNC5C (E), UNC5D (F), DSCAM (G), CD146 (H) and A2BAR (I) in endometriotic lesions and eutopic endometria from women with or without endometriosis were tested using RT-qRCR. Error bars show mean ± SEM. * $P<0.05$; ** $P<0.01$; *** $P<0.0001$ (one-way ANOVA and Student's t-test). SEM, standard error of the mean.

or without ($P<0.0001$; $P<0.0001$; $P<0.01$) endometriosis (*Figure 5D,E,G*). The eutopic endometrium from women with endometriosis also showed significantly higher UNC5C mRNA expression levels than those from women without endometriosis ($P<0.05$; *Figure 5E*). No statistical differences in UNC5A, UNC5B or CD146 mRNA expression levels among endometriotic lesions and the eutopic endometrium from women with or without endometriosis were found (*Figure 5C,F,H*).

IHC results showed that DCC was mainly expressed in the epithelial and stromal cells and the interstitium in endometriotic lesions (*Figure 6A,B,C*), and showed a higher IHC score in endometriotic lesions than those in

the eutopic endometrium from women with or without endometriosis ($P<0.0001$). UNC5B was mainly expressed in glandular epithelial cells (*Figure 6D,E,F*) and was less expressed in endometriotic lesions than those in eutopic endometrium from women with ($P<0.01$) or without ($P<0.05$) endometriosis. A2BAR was widely expressed in endometriotic lesions (*Figure 6G,H,I*), and the IHC scores were higher than those in the eutopic endometrium from endometriosis ($P<0.05$) and non-endometriosis ($P<0.05$) women.

Discussion

This study demonstrated that netrin-1 is increased in serum
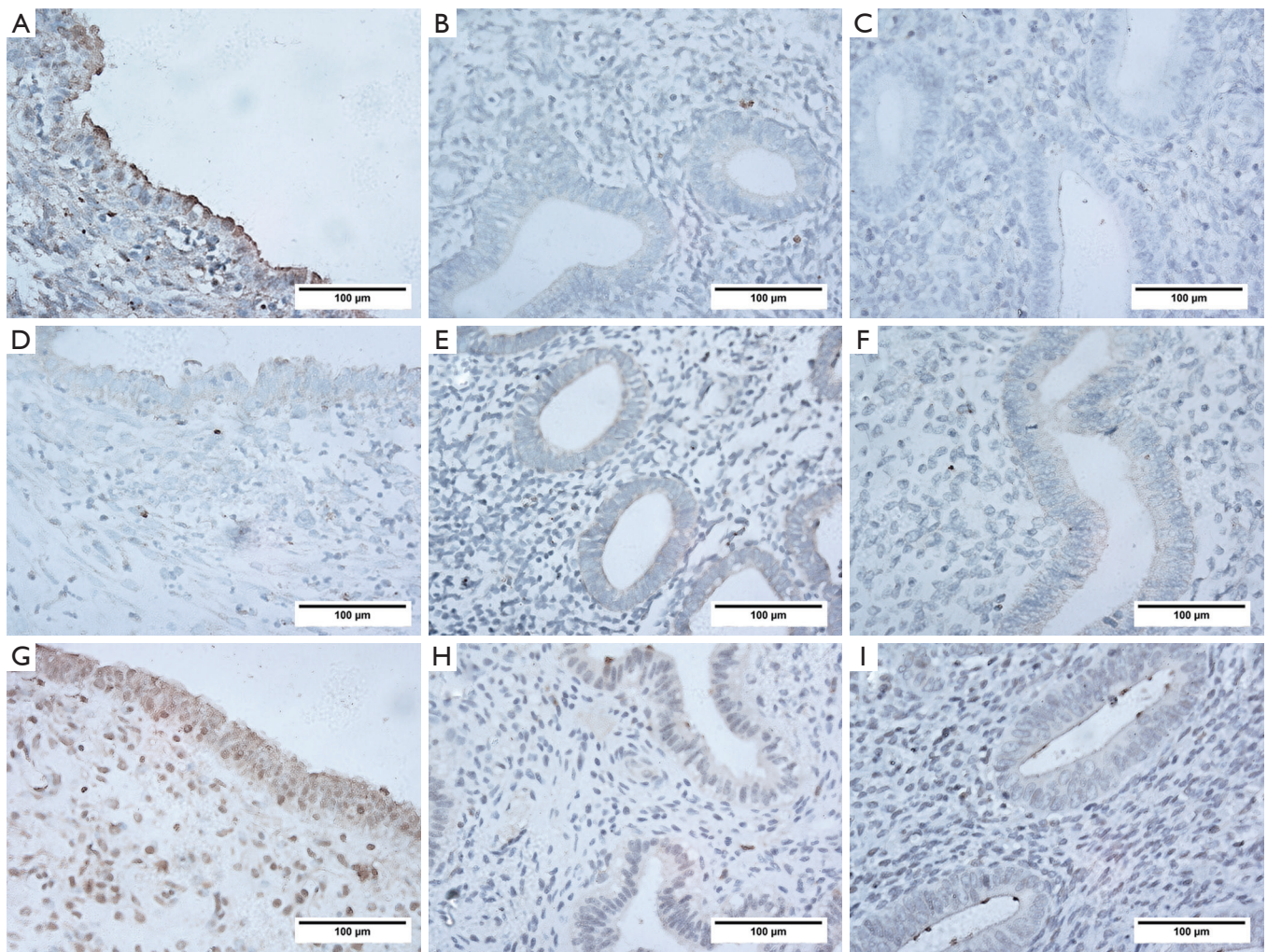
Figure 6 DCC, UNC5B and A2BAR immunoreactive staining in endometrial tissues. The locations and expression levels of DCC (A,B,C), UNC5B (D,E,F) and A2BAR (G,H,I) in endometriotic lesions (A,D,G) and eutopic endometrium from women with (B,E,H) and without (C,F,I) endometriosis were measured using immunohistochemistry analysis.

and endometriotic lesions in women with endometriosis. Since netrin-1 is an axon guide molecule, we speculated that netrin-1 mediates endometriosis-associated pain by promoting nerve fiber infiltration in endometriotic lesions. In fact, netrin-1 has bi-functionality on axonal guidance. It has been reported that netrin-1 leads to axon attraction by binding to the DCC receptor or repulsion by binding to the UNC5A–D receptors in the same cells (17). Neogenin, DSCAM and CD146 also show a promoting effect on axon extension (20). The bi-functionality on axon guidance is based on the crystal structure of netrin-1 and its receptors (45). Plus, cross-links with different receptor types and the charge changes on netrin-1 and receptors also

determines the promotion or inhibition of netrin-1 in axon guidance (20). Our study demonstrated that endometriotic lesions showed significantly higher expression levels of DCC and A2BAR and lower expression levels of UNC5B and UNC5C compared with the eutopic endometrium, which further supports the proposal that netrin-1 is responsible for endometriosis-associated pain by promoting nerve infiltration in endometriotic lesions. Dorsal root ganglion (DRG) neurons express DCC, neogenin and UNC5A–D receptors (46). In a rat model of sciatic nerve transection, the expression of the DCC receptor was found to be up-regulated while the expression of the UNC5B and UNC5C receptors was down-regulated in sensory neurons (46).

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Transplantation of netrin-1 overexpression in bone marrow mesenchymal stem cells promotes axon regeneration and functional recovery of the sciatic nerve after crush injury (47). However, netrin-1 treatment (500 ng/mL) inhibits neurite outgrowth of adult DRG neurons in explant and dissociated cultures, which may be mediated by the UNC5A-C receptors on DRG (48). Thus, the effect of netrin-1 on nerve outgrowth is complex and the mechanism of netrin-1 involvement in endometriosis-associated pain remains to be further studied.

M1 macrophages, activated by IFN-γ, LPS or TNF-α, participate in tissue injury, inflammatory and immune responses by producing pro-inflammatory cytokines and chemokines (49). In contrast, M2 macrophages can be activated by IL-4, IL-10, IL-13, or the transforming growth factor-β (TGF-β), thus participating in tissue repair tumor angiogenesis and vessel normalization (50). Several studies have reported that the macrophages in women with endometriosis are predominantly of the M2 phenotype (CD163$^+$/CD206$^+$), which play an important role in the development of endometriosis (33,43,51-53). Endometriosis is an estrogen-dependent disease, and some researchers have reported that estrogen promotes M2 polarization through the activation of the signal transducers and activators of the transcription (STAT3) and P38-mitogen-activated protein kinases (MAPK) pathway (54,55). However, another study showed the opposite results with 17β-estradiol repressing the suppressor for M2 polarization by inhibiting the JAK1-STAT6 signaling pathway (56). Takebayashi et al. have reported that the macrophage population slants toward M1 in the endometrium of endometriosis patients due to the significantly lower ratio of the number of CD163$^+$ or CD206$^+$ macrophages to CD68$^+$ macrophages (42). In this study, we found that the peritoneal macrophages of endometriosis were mainly of the CD86$^+$CD163$^+$ type, while those of women without endometriosis were mainly of the CD86$^-$CD163$^+$ type (M2). The percentage of CD86$^+$ macrophages in women with endometriosis was significantly higher than that in the control group, which displayed a unique M1/M2 polarization signature that was skewed towards the classical M1 activation phenotype. This was consistent with the subsequent results of experiments in vitro in which M1 polarization induced up-regulation of netrin-1 synthesis and secretion in human and rat cell line. A recent study also reported that netrn-1-enriched macrophages were highly expressed pro-inflammatory markers, as netrin-1 mRNA expression levels were increased in CD68$^+$CD206$^-$ pro-inflammatory

phenotypes rather than CD68$^+$CD206$^+$ samples (32). In this study, we used CD68$^+$CD86$^+$CD163$^-$ to label M1 and CD68$^+$CD86$^-$CD163$^+$ to label M2 when analyzing the polarization phenotypes of the peritoneal macrophages, which is the most commonly used method. In the in vitro cell experiments, we used LPS and IFN-γ to induce M1 phenotype macrophages in the THP-1/NR8383 cells, which is the most widely used method (57), and verified the macrophage polarization by detecting the expression of the inflammatory mediators. In actuality, polarization is a dynamic process as the signals are temporal and dynamic, and the use of the terms M1 and M2 is confusing due to the lack of a specific phenotypic scoring criteria (57,58). Many physiological or pathological macrophages do not show a clear M1 or M2 phenotype (59), and macrophages with combinations of both M1 and M2 markers can be found during M1/M2 polarization (60,61). New methods and technical advances are needed to investigate the activation and classification of macrophage.

The netrin-1 gene is a direct transcriptional target of nuclear factor (NF)-κB, which up-regulates netrin-1 in colorectal carcinoma and mammary epithelial cells in response to inflammation (62). However, previous research has reported that activation of NF-κB represses netrin-1 expression levels in adenocarcinomic alveolar epithelial cells and dermal microvessel endothelial cells (30). In fact, macrophages from endometriosis patients show a statistically significant higher proportion of NF-κB nuclear translocations, and release various cytokines, growth factors and angiogenic factors to participate in endometrial fragment adhesion, proliferation and neovascularization (35). Since we only tested cell lines in vitro, primary macrophages from women with endometriosis would be more valuable in determining the amount of cytokine, time of exposure, and the competition for cytokines (58). Thus, the regulatory mechanism of netrin-1 in endometriosis macrophages needs further study.

Netrin-1 also plays a role in angiogenesis, cell migration, cell proliferation and cell survival. The present study confirmed the higher expression of netrin-1, the constant expression of CD146, and the lower expression of UNC5B in endometriotic lesions. Netrin-1-enriched macrophages also highly express pro-angiogenic markers (32), and treatment of netrin-1 with low doses (50–200 ng/mL) of endothelial cells promotes proliferation, migration and tube formation by binding to the high affinity receptor CD146 (21). However, high concentrations of netrin-1 (1,000–2,000 ng/mL) inhibit the above effects, possibly via

the UNC5B signaling pathway (21). In endometriosis, nerve fibers are accompanied by immature blood vessels within endometriotic lesions (15). Netrin-1 also dose-dependently regulates cell migration of Schwan cell and endothelial cells by activating or inhibiting the MAPK pathway via CD146 or UNC5B receptors (21,63,64). In inflammatory conditions, it has been reported that netrin-1 in endothelial cells inhibits inflammatory cell migration of leukocytes and macrophages (32,65,66). This may be an anti-inflammatory response in the body, but it can lead to the accumulation of inflammatory cells in the lesions, resulting in AAA or atherosclerosis (31,32). In endometriosis, macrophage retention in endometriotic lesions increases the local concentration of netrin-1, which may play a role in the infiltration of nerve fibers. In tumorigenesis, netrin-1 promotes cell survival, proliferation, invasion and migration in different types of cancer, such as prostate carcinoma, hepatocellular carcinoma, gastric cancer, and breast cancer (67-70). Although endometriosis is a benign gynecological disease, it demonstrates malignant behaviors in its adhesion, proliferation, invasion, metastasis and recurrence (71-74). Thus, elevated netrin-1 may also be involved in the growth of endometriotic lesions.

Our study has some limitations, which should be taken into account when interpreting our findings. First, in this study a total of 60 women were recruited. Different sample sizes often lead to differences in results. In order for greater validation of the present results, follow-up studies with increased sample sizes are needed to reduce experimental statistical errors. Secondly, double immunofluorescence staining showed that there were approximately 62.2% of netrin-1-expressing macrophages (CD68$^+$netrin-1$^+$) in the total netrin-1$^+$ cells. Thus, another limitation in our study is that it is unclear which cells are responsible for the increased netrin-1 in endometriotic lesions. Immunohistochemical staining results showed that netrin-1 was not only expressed in epithelial and interstitial vascular endothelial cells, but also expressed in endometrial stromal cells in endometriotic lesions from women with endometriosis. In this study, we demonstrated that the expression levels of netrin-1 in serum and endometriotic lesions were significantly higher in women with endometriosis, and netrin-1 was co-expressed with CD68 in endometriotic lesions, which is consistent with previous studies in which netrin-1 was highly expressed in macrophage infiltration in atherosclerotic plaques (31), adipose tissues (28), and inflamed aortic vessel walls (32). In the *in vitro* studies, we found that netrin-1 was synthesized and secreted by THP-1

and NR8383 cells in the process of M1 polarization. However, it has been reported that endothelial cells (75), as well as epithelial cells (24), can also synthesize and secrete netrin-1. Thus, further research is needed on the origin of the up-regulation of netrin-1 expression in endometriotic lesions. Last but not least, we have not studied the effects of netrin-1 on different receptors; that is, how netrin-1 causes endometriosis-associated pain. In another study, we demonstrated that netrin-1 induced angiogenesis in ovarian endometriomas through interaction with CD146 in vascular endothelial cells and promoted neurite growth and sensitization through another receptor, neogenin (76). This is an ongoing study, and we will further validate and inform our research with additional animal experiments.

In summary, the present study indicates that increased netrin-1 in women with endometriosis may play a role in endometriosis-associated pain. Targeting therapy towards netrin-1 and macrophages may not only reduce endometriosis-associated pain, but may also inhibit the progression of endometriosis.

Acknowledgments

Footnote

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aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Human Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University (grant number 20190012). Informed consent was obtained from all patients.

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