Human Reproduction, pp. 1–14, 2017
doi:10.1093/humrep/dex067

human reproduction

ORIGINAL ARTICLE *Reproductive biology*

Synergistic effect of regulatory T cells and proinflammatory cytokines in angiogenesis in the endometriotic milieu

Xiao-Qiu Wang†, Wen-Jie Zhou†, Xue-Zhen Luo, Yu Tao, and Da-Jin Li*

Laboratory for Reproductive Immunology, Key Laboratory of Reproduction Regulation of NPFPC, SIPPR, Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Hospital and Institute of Obstetrics and Gynecology, IRD, Fudan University Shanghai Medical College, Shanghai, China

*Correspondence address. Laboratory for Reproductive Immunology, Key Laboratory of Reproduction Regulation of NPFPC, SIPPR, Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Hospital and Institute of Obstetrics and Gynecology, IRD, Fudan University Shanghai Medical College, Shanghai, China.Tel/Fax: +86-21-6345-7331; E-mail: djli@shmu.edu.cn

Submitted on September 16, 2015; resubmitted on February 21, 2017; accepted on March 19, 2017

STUDY QUESTION: Do regulatory T cells (Tregs) contribute to angiogenesis in endometriosis?

SUMMARY ANSWER: High levels of CCL17 and CCL22 cause the recruitment of Tregs, upregulate the immunosuppression of Tregs and, in turn, may promote angiogenesis in endometrial cells in synergy with proinflammatory cytokines.

WHAT IS ALREADY KNOWN: The peritoneal fluid of patients with endometriosis has a higher percentage of Tregs than that of normal individuals; however, the regulatory role of Tregs in the disease remains unclear.

STUDY DESIGN, SIZE, DURATION: This study used primary human endometrial stromal cells (ESCs), monocytes (Mo), Tregs and human umbilical vein endothelial cells (HUVECs). All experiments were performed at least three times.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The migration of Tregs was evaluated by the transwell migration assay. The activation of extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase and p38 signaling pathways was examined using the In-Cell Western™ (LI-COR®) western blot analysis system, as well as by traditional western blot analysis. Changes in the expression of CCL22, CCL17, transforming growth factor-beta 1 (TGF-β1), Interleukin (IL)-1β, tumor necrosis factor alpha (TNF-α), IL-8 and vascular endothelial growth factor (VEGF) in cell-culture supernatant were detected by ELISA. We analyzed the Tregs by multicolor flow cytometry to directly test the expression of CCR4, CD4, CD25, Foxp3, CTLA-4, CD39 and CD73.

MAIN RESULTS AND THE ROLE OF CHANCE: Our results showed that ESCs-Mo co-culture produced significantly higher levels of CCL22 and CCL17 than ESCs or Mo cultured alone, and that estradiol (E2) or progesterone (P) further promoted this upregulation, demonstrating stronger chemotaxis on Tregs. The co-culture of ESCs with Mo stimulated TGF-β1 secretion by Tregs, which could be inhibited by anti-CCL22 or/and anti-CCL17 neutralizing antibodies (Abs). The expression of CCR4 by Tregs was upregulated in ESCs-Mo co-culture, especially by treatment with E2 and/or P, and this effect could be abolished by anti-CCL22 and/or anti-CCL17-neutralizing Abs. The Treg-ESCs-Mo co-culture treated with E2 (10^{-8} mol/l) and P (10^{-8} mol/l) could enhance the immunosuppression of Tregs, as proved by the elevated expression of Foxp3, CTLA-4, CD39 and CD73 on Tregs. ESCs-Mo co-culture could significantly promote the secretion of IL-1β and TNF-α. TGF-β1 from Tregs could activate p38/ERK1/2 signaling pathways in ESCs, and IL-1β and TNF-α produced by ESCs-Mo co-culture had synergistic roles with TGF-β1. TGF-β1 and the proinflammatory cytokines IL-1β or TNF-α could synergistically promote IL-8 and VEGF expression in ESCs via the p38/ERK1/2 signaling pathways. The high levels of IL-8 and VEGF in the supernatant of ESCs stimulated the angiogenesis of HUVECs.

LARGE SCALE DATA: None.

†The authors consider that the first two authors should be regarded as joint first authors.

© The Author 2017. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology. All rights reserved.
For Permissions, please e-mail: journals.permissions@oup.com

LIMITATIONS, REASONS FOR CAUTION: This study was only performed *in vitro* using eutopic ESCs, instead of ectopic cells, from endometriosis patients. Therefore, it is necessary to do further experiments to determine whether Tregs promote angiogenesis in the endometriotic milieu in synergy with proinflammatory cytokines *in vivo*.

WIDER IMPLICATIONS OF THE FINDINGS: Co-targeting Tregs and proinflammatory cytokines may be an effective treatment for endometriosis.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by Ministry of Science and Technology of China 2015CB943300 to L.D.-J.; National Natural Science Foundation of China, item number 81200425 to W.X.-Q.; National Natural Science Foundation of China, item number 81471548 to L.D.-J.; and The Research Fund for the Doctoral Program of Higher Education of China to W.X.-Q. (20110071120093). The authors have no conflicts of interest to declare.

Key words: endometriosis / regulatory T cells / proinflammatory cytokines / angiogenesis / ectopic milieu

Introduction

Endometriosis is a frequent benign gynecological disorder characterized by the presence of endometrial tissue outside the uterine cavity. Although Sampson's theory of retrograde menstruation is by far the most widely accepted explanation, the exact pathogenic mechanisms of endometriosis are not clearly understood.

As observed in tumor growth, angiogenesis appears to be essential for the survival and development of endometrial implants in the ectopic lesions. Similar to tumor metastasis, neovascularization is required for endometriotic implants to survive and proliferate, invade the extracellular matrix and establish endometriotic lesions (Laschke and Menger, 2007). Accordingly, there is a typical dense vascular network in endometriotic lesions. Angiogenic cytokines levels have been shown to be elevated in the peritoneal fluid, serum and endometriotic lesions of patients with the disease (Laschke and Menger, 2007). Vascular endothelial growth factor (VEGF) has been shown to be a potent angiogenic factor, and several studies have reported that endometriosis patients have increased levels of VEGF, which indicates that VEGF plays an important role in disease progression (Gilabert-Estelles *et al.*, 2007). Interleukin (IL)-8 levels are also elevated in the peritoneal fluid of endometriosis patients, and this correlates positively with disease severity. IL-8 is involved in endometrial cell proliferation and neovascularization in endometriotic lesions (Barcz *et al.*, 2002).

The potential role of immune regulation in the pathophysiology of endometriosis is increasingly regaining attention. Our group has found that eutopic endometrium from patients with endometriosis has stronger invasion and growth capacity compared to normal endometrium, and immune cells such as macrophages have important roles in the formation of ectopic foci (Shi *et al.*, 2007; Yu *et al.*, 2008; Wang *et al.*, 2010a, 2010b).

Autoimmune disorders might arise as a consequence of the dysregulation of regulatory T cells (Tregs), which have been proven to play a critical role in the control and suppression of immune cells such as macrophages, natural killer cells, dendritic cells and cytotoxic T cells. Tregs are a subpopulation of $CD4^+CD25^+$ T cells and these $CD4^+CD25^+$ Tregs specifically express the transcription factor *FOXP3*, which is of great importance for their development and function (O'Garra and Vieira, 2004). Although the role of Tregs anomalies in the etiopathogenesis of several autoimmune disorders has already been well documented, little is known about the possible role of Tregs in endometriosis. Recently, Tregs were reported to be present in eutopic and ectopic endometrial tissues of endometriosis patients (Basta *et al.*, 2010; Berbic *et al.*, 2010). Increased levels of Foxp3 mRNA were also detected in human endometriotic lesions (Budiu *et al.*, 2009). The proportion of $CD25^{high}Foxp3^+$ Tregs was significantly high in the peritoneal fluid of women with endometriosis (Olkowska-Truchanowicz *et al.*, 2013). These reports suggest that Tregs might play an important role in the pathogenesis of endometriosis. However, to the best of our knowledge, the functional regulation of Tregs and its possible role in angiogenesis in an ectopic milieu has not yet been elucidated.

Thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) are the ligands for the CC chemokine receptor 4 (CCR4), which is expressed on Tregs (Iellem *et al.*, 2001; Curiel *et al.*, 2004). Some tumor and immune cells can produce CCL17 and CCL22 and in turn mediate the trafficking of CCR4-positive Tregs to the tumor focus, and this specific recruitment of Tregs may be a mechanism by which the tumor suppresses the host antitumour response (Ishida *et al.*, 2006). B-cell chronic lymphocytic leukemia cells were found to produce CCL22 and attract $CCR4^+$T cells (Ghia *et al.*, 2002) and patients with B-cell chronic lymphocytic leukemia showed significantly increased frequencies of $CD4^+CD25^{high}$ Tregs in their peripheral $CD4^+$T cells compared with healthy individuals (Beyer *et al.*, 2005). The chemokine receptor CCR4 is also observed in Treg trafficking and function at inflammation sites (Iellem *et al.*, 2001). Ahern *et al.* 2009 demonstrated that the decreased expression of CCR4 upon allergen stimulation of $CD4^+CD25^+$ Tregs precipitates the failure of their retention at inflammation sites to attenuate T-cell activation.

Endometriosis is a perplexing chronic inflammatory disease. IL−1β is a multifunctional cytokine that may facilitate the development of the disease. The endometriotic cells stimulated by IL-1β can express a variety of cytokines and growth factors, which promote growth, adhesion, invasion, inflammation and vascularization in endometriotic lesions (Kao *et al.*, 2011; Yoshino *et al.*, 2011). Tumor necrosis factor alpha (TNF-α), a 17.3-kDa peptide, is also a versatile inflammatory cytokine and angiogenic factor that could stimulate production of some cytokines and chemokines, such as IL-6, IL-8, granulocyte-macrophage colony-stimulating factor and monocyte chemotactic protein-1 (Fahey *et al.*, 2005; Meter *et al.*, 2005). TNF-α mRNA is upregulated in both the endometrium and peritoneum of endometriosis patients compared to healthy control individuals (Kyama *et al.*, 2006). It is known that TNF-α is important in early tumourigenesis, which includes a cascade of cytokines, chemokines

and adhesion molecules. TNF-α can enhance mitogenic activity and up-regulate IL-8 gene and protein expression through NF-κB activation in endometriotic stromal cells (Sakamoto *et al.*, 2003).

How Tregs are involved in endometriosis needs to be elucidated. Therefore, the aim of our present study was to investigate the functional regulation and the possible pro-angiogenic role of Tregs, and to determine whether the inflammatory cytokines IL-1β and TNF-α have a synergistic effect with Tregs in the ectopic milieu.

Materials and Methods

Tissue collection

The protocol of this study was approved by the institutional review board of Fudan University, Obstetrics and Gynecology Hospital, Shanghai. Written informed consent was obtained from all the patients before the samples were collected. All the patients had regular menstrual cycles and had not received hormonal treatment for at least 3 months before surgery. Eutopic endometrium (n = 27) was obtained from fertile women (aged 27–42 years) with endometriosis. The stage of endometriosis was determined according to the revised classification of the American Society for Reproductive Medicine (r-ARSM), for eutopic endometrial tissues with endometriosis. On the basis of this classification, 16 patients had stage one endometriosis, and 11 patients had stage two endometriosis. The characteristics of the 27 recruited patients with endometriosis are shown in Table I which demonstrates the clinical heterogeneity of the patients. The collected tissues were transported to the laboratory in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, CA, USA) on ice under sterile conditions. The tissues were processed for cell isolation. All the samples were obtained in the proliferative phase of the cycle, as histologically confirmed according to established criteria (Noyes *et al.*, 1975).

Table I Characteristics of 27 recruited patients with endometriosis.

Variable name	Statistics/distribution
Age, year	Mean = 34 (SD = 6.2)
	Median = 32
	Range = 22–43
Gravidity	
0	12 (44.4%)
1	10 (37%)
≥2	5 (18.5%)
rASRM score	Mean = 23.2 (SD = 19.4)
	Median = 15
	Range = 6–58
Severity of dysmenorrhea	
None	11 (40.7%)
Mild	7 (25.9%)
Moderate	5 (18.5%)
Severe	4 (14.8%)
Complaint of infertility	
No	22 (81.5%)
Yes	5 (18.5%)
Cyst size (diameter, cm)	Mean = 5.9 (SD = 1.7)
	Median = 6.2
	Range = 2–9
History of previous gynecologic surgery	
No	22 (81.5%)
Yes	5 (18.5%)
Co-occurrence of deep infiltrating endometriosis	
No	23 (85.2%)
Yes	4 (14.8%)
Co-occurrence of peritoneal endometriosis	
No	20 (74%)
Yes	7 (26%)

SD, standard deviation; rASRM, revised American Society for Reproductive Medicine.

Isolation and culture of endometrial stromal cells

The fresh endometriotic tissue collected in sterile medium was rinsed to remove blood cells, then was minced and incubated in DMEM/F-12 containing collagenase Type IV (0.1%; Sigma, USA) for 20 min at 37°C with constant agitation. The resultant dispersed endometriotic cells were separated by filtration through a 200-mesh sieve to remove debris. After gentle centrifugation, the supernatant was discarded, and the cells were resuspended in 1:1 DMEM/F-12. Endometrial stromal cells (ESCs) were separated from epithelial cells by passing them over a 400-mesh sieve. Stromal cells remaining in the filtrate were collected by centrifugation at 300×*g* for 10 min and resuspended in DMEM/F-12 containing 10% fetal bovine serum (FBS; Gibco, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 mg/ml amphotericin B. The ESCs were plated in 75-cm^2 flasks and allowed to adhere at 37°C in a humidified atmosphere with 5% CO_2 for 20 min. The purity of ESCs was found to be 95%, as determined by positive staining for vimentin and negative staining for cytokeratin.

Isolation and culture of monocytes

Peripheral blood samples were obtained from healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation by using Ficoll, after which they were magnetically labeled with CD14 microbeads. The cell suspension was loaded onto an MS column (Miltenyi Biotec, Germany) placed in the magnetic field of a magnetic-activated cell sorting (MACS) separator. The magnetically labeled $CD14^+$ monocytes (Mo) were retained within the column. The isolated $CD14^+$ Mo were fluorescently stained with fluorescein isothiocyanate (FITC)-coupled anti-CD14 Ab (eBioscience, USA) to identify final purity.

Isolation and *in vitro* expansion of CD4+CD25+ Tregs

$CD4^+CD25^+$ Tregs were purified from peripheral blood of healthy volunteers by using a MACS separation kit (Miltenyi Biotec, Germany). Briefly, PBMCs were isolated by density gradient centrifugation using Ficoll, and $CD4^+$ T cells were enriched by immunomagnetic depletion using a $CD4^+$ T-cell biotin-Ab cocktail and anti-biotin microbeads with an LD column (Miltenyi Biotec, Germany). Enriched $CD4^+$ T cells were incubated with CD25 microbeads and then applied to an MS column to obtain $CD4^+CD25^+$ Tregs. The isolated $CD4^+CD25^+$ Tregs were fluorescently stained with allophycocyanin (APC)-conjugated anti-human CD4 (BD Biosciences, USA) and FITC-conjugated anti-human CD25 (BD Biosciences, USA) Abs to identify final purity. The isolated $CD4^+CD25^+$ Tregs were then expanded for 2–3 weeks with a Treg expansion kit (Miltenyi Biotec,

Germany) at a bead-to-cell ratio of 4:1 and recombinant interleukin 2 (rIL-2) (Peprotech, USA) concentration of 500 U/ml in round-bottom 96-well plates.

Cell co-culture units and *in vitro* cell treatment

To establish an ESCs-Mo co-culture unit, the ESCs were cultured in 96-well plates at a concentration of 1×10^5 cells/well until the cells adhered to the plastic. The media were removed, and Mo were applied over ESCs at the same concentration. After serum starvation for 12 h, ESCs cultured alone, Mo cultured alone, and the co-cultured ESCs-Mo were treated with E2 at 10^{-10}–10^{-7} mol/l (Sigma, USA), P at 10^{-10}–10^{-7} mol/l (Sigma, USA), or a combination of E2 (10^{-8} mol/l) and P (10^{-8} mol/l) for 48 h. Lipopolysaccharides at 10 ng/ml was added to the cell-culture units 18 h before the termination of the experiment. Each experiment was carried out in triplicate and repeated three times ($n = 6$).

To establish an ESCs-Mo-Treg co-culture unit, the ESCs were cultured in 48-well plates at a concentration of 2×10^5 cells/well until they adhered to the plastic. The media were removed, and Mo and Tregs were applied over ESCs at the same concentration, while Tregs cultured alone in the same media were used as controls. 4a-phorbol-12-myristate-13-acetate (PMA) at 50 ng/ml and ionomycin at 2.5 μg/ml were added to the cell-culture units 6 h before the termination of the experiment. The co-culture units were treated, respectively, with anti-CCL17 neutralizing Ab (R&D Systems, USA) at 1.5 μg/ml, anti-CCL22 neutralizing Ab (R&D Systems, USA) at 3 μg/ml or the combination of anti-CCL17 and anti-CCL22 neutralizing Abs for 48 h.

To analyze the intracellular signaling pathway, the ESCs in 96-well plates at a concentration of 1×10^5 cells/well were treated with recombinant human transforming growth factor-beta 1 (rhTGF-β1; Peprotech, USA) at a concentration of 20 ng/ml, recombinant human interleukin one beta (rhIL-1β; Peprotech, USA) at a concentration of 20 ng/ml, recombinant human tumor necrosis factor alpha (rhTNF-α; Peprotech, USA) at a concentration of 20 ng/ml, a combination of rhTGF-β1 and rhIL-1β at the same concentrations or a combination of rhTGF-β1 and rhTNF-α at the same concentrations for 48 h.

To detect the secretion of IL-8 and VEGF, the ESCs were treated with rhTGF-β1 at a concentration of 20 ng/ml, rhIL-1β at a concentration of 20 ng/ml, rhTNF-α at a concentration of 20 ng/ml, a combination of rhTGF-β1 and rhIL-1β at the same concentrations, or a combination of rhTGF-β1 and rhTNF-α at the same concentrations for 48 h. The cells were incubated with or without the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)1/2 inhibitor U0126 (30 μM; Cell Signaling Technology, USA), p38 inhibitor SB202190 (20 μM; Cell Signaling Technology) or c-Jun N-terminal kinase (JNK) inhibitor SP600125 (20 μM; Cell Signaling Technology), with vehicle as the control.

Transwell migration assay

In the first group, the supernatant from ESCs, Mo or the ESCs-Mo co-culture, which were treated for 48 h with 10^{-8} mol/l E2, 10^{-8} mol/l P, or the combination thereof, were placed in the lower chamber, and the Tregs (2×10^5 cells/well) were put into the upper chamber. In the second group, the supernatant from the ESCs-Mo co-culture units treated with E2 at 10^{-8} mol/l, P at 10^{-8} mol/l, or the combination thereof for 48 h were placed in the lower chamber, and the Tregs (2×10^5 cells/well) were put into the upper chamber. Anti-CCL17 neutralizing Abs at 1.5 μg/ml, anti-CCL22 neutralizing Abs at 3 μg/ml, or the combination thereof was added to several lower chambers half an hour before the chemotaxis assay. After 4 h of incubation, the Tregs in the lower chamber were counted and collected for flow cytometry.

In-cell western™ blot analysis

In this study, we used the In-Cell Western™ (LI-COR®)-blot analysis to determine the in-cell protein levels of interest. The eutopic ESCs in 96-well plates were treated as above. The ESCs were treated with 20 ng/ml rhTGF-β1, 20 ng/ml rhIL-1β, 20 ng/ml rhTNF-α, a combination of rhTGF-β1 and rhIL-1β at the same concentrations, or a combination of rhTGF-β1 and rhTNF-α at the same concentrations for 48 h. Thereafter, the cells were immediately fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. After cells were washed with 0.1% Triton, they were blocked by adding 150 μl/well blocking buffer with 10% FBS for 90 min at room temperature. The cells were incubated with rabbit anti-human phospho-ERK1/2 (1:10, Santa Cruz Biotechnology, USA), mouse anti-human ERK1/2 (1:10, Santa Cruz Biotechnology), mouse anti-human phospho-JNK (1:10, Santa Cruz Biotechnology), rabbit anti-human JNK (1:10, Santa Cruz Biotechnology), mouse anti-human phospho-p38 (1:10, Santa Cruz Biotechnology) and rabbit anti-human p38 (1:10, Santa Cruz Biotechnology) Abs. After overnight treatment at 4°C, the wells were incubated with corresponding second IRDye 700DX-conjugated affinity purified (red fluorescence) anti-mouse (phospho-p38 and phospho-JNK) or anti-rabbit (phospho-ERK1/2) Ab and IRDye 800DX-conjugated affinity purified (green fluorescence) anti-rabbit (p38 and JNK) or anti-mouse (ERK1/2) fluorescence Ab recommended by the manufacturer (Rockland, USA). This procedure was carried out in the dark. Images of target proteins were obtained by using the Odyssey Infrared Imaging System (LI-COR Biosciences). The expression levels of the corresponding molecules were calculated as the ratio of the intensity of target proteins to total signal molecules (such as total p38, total ERK1/2). The experiments were carried out in triplicate and repeated three times ($n = 6$).

Western blot analysis

To verify the results of in-cell western blot analysis, we also conducted traditional western blotting. The ESCs were treated as described above. Cells were lysed in RIPA lysis buffer (Pierce) and protease inhibitor cocktail (Roche, Switzerland) on ice for 30 min. After centrifugation at 10 800×*g* for 20 min, protein concentrations were determined with a bovine serum albumin (BSA) protein assay kit (Beyotime, China). Protein extract (30 μg per lane) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% gel (Beyotime) and then transferred to polyvinylidene difluoride membranes (Millipore, USA). The membranes were incubated overnight at 4°C with rabbit Abs for anti-phosphorylated p38, ERK and JNK (Cell Signaling Technology), followed by anti-rabbit-horseradish peroxidase Ab (Bioworld, USA) for 1 h at room temperature. Ab-bound proteins were visualized with enhanced chemiluminescence chemiluminescent reagent (Millipore). The membranes were then stripped and re-probed with rabbit anti-total p38, ERK and JNK Abs (Cell Signaling) or with rabbit anti-GAPDH monoclonal antibody (mAb) (Bioworld, USA). Band intensities were analyzed with Image J software.

ELISA

In ESCs, Mo and Tregs cultured alone or different two-cell or three-cell co-culture units, the culture supernatant was collected for detecting the presence of cytokines and growth factors by commercially available ELISA kits for CCL22, CCL17, TGF-β1, IL-1β, TNF-α, IL-8 and VEGF (R&D Systems), following the protocols supplied by the manufacturers.

Flow cytometry

We analyzed the Tregs by multicolor flow cytometry to directly test the expression of CCR4, CD4, CD25, Foxp3, CTLA-4, CD39 and CD73. The fluorescence-conjugated Abs used in this study were APC-conjugated anti-human CCR4 (eBioscience, USA), APC-conjugated anti-human CD4 (BD

Biosciences, USA), FITC-conjugated anti-human CD25 (BD Biosciences, USA), phycoerythrin (PE)-conjugated anti-human Foxp3 (eBioscience, USA), PE-conjugated anti-human CTLA-4 (eBioscience, USA), APC-conjugated anti-human CD39 (eBioscience, USA) and PE-conjugated anti-human CD73 (eBioscience, USA) Abs. IgG1 immunoglobulin conjugated with the respective fluorochromes served as isotype controls. The Tregs were blocked by treatment with 10% normal horse serum in PBS for 20 min at room temperature before staining. All the fluorescence-conjugated Abs and isotype-matched controls were incubated with the cells at recommended usage for 35 min at room temperature in the dark. For staining Foxp3, 1 ml of 1 × BioLegend's Foxp3 Fix/Perm solution was added to each tube, vortexed, incubated at room temperature in the dark for 20 min, and then spun down to remove the supernatant. The resuspended cells in 1 ml 1 × BioLegend's Foxp3 Perm buffer were incubated at room temperature in the dark for 15 min and spun down. The supernatant was discarded, and the resuspended cells were added to an appropriate amount of flurochrome-conjugated anti-Foxp3 Ab and incubated at room temperature in the dark for 35 min. The cells were then washed once with 1 ml of PBS by centrifugation at 300×*g* for 5 min and analyzed by FACS Calibur flow cytometry and CellQuest software (BD Biosciences).

Tube formation assay

Tube formation was studied as described by Klein *et al.*, 2008. In brief, ~5000 human umbilical vein endothelial cells (HUVECs) were seeded on growth factor-reduced Matrigel in normal growth medium, with the supernatant of ESCs treated with rhTGF-β1 (20 ng/ml), rhIL-1β (20 ng/ml), rhTNF-α (20 ng/ml), rhTGF-β1 combined with rhIL-1β or rhTGF-β1 combined with rhTNF-α, in 96-well plates. After 12 h incubation at 37°C, endothelial tube formation was observed using an inverted microscope. Capillary-like tube formation was quantified by counting the junctions/enclosed circles in five randomly chosen optical fields with light microscopy.

Cell viability assay

Cell viability was assessed using CCK8 (Dojindo, Japan). In brief, ESCs, Mo and Tregs cultured alone, or the ESCs-Mo co-culture were seeded on 96-well plates at a density of 1×10^4 cells/well and cultured in DMEM/F-12 containing 1% FBS at 37°C for 12 , 24 and 48 h. The culture medium was replaced with 100 µl DMEM/F-12 containing 10 µl CCK8, and the plates were incubated for 3 h at 37°C. Absorbance at 450 nm was measured in a multiwell spectrophotometer. Each experiment was performed in triplicate and repeated three times ($n = 6$).

Bromodeoxyuridine proliferation assay

The proliferation of the cells was determined using bromodeoxyuridine (BrdU) cell proliferation assay kits (Merck Millipore, Germany) according to the manufacturer's instructions. ESCs, Mo and Tregs cultured alone, or the ESCs-Mo co-culture, which had been treated with E2 (10^{-8} mol/l), P (10^{-8} mol/l) or the combination thereof, were seeded at a density of 1×10^4 cells/well in 96-well flat-bottom microplates. The cells were cultured in DMEM/F-12 containing 1% FBS for 12, 24 and 48 h. Each experiment was performed in triplicate and repeated three times ($n = 6$).

Statistical analysis

All values are shown as the mean ± SD. Data were analyzed by one-way ANOVA. Calculations were performed using the Statistical Package for the Social Sciences software (version 11.5). Differences were considered statistically significant at $P < 0.05$.

Results

ESCs-Mo co-culture promoted the secretion of CCL22 and CCL17, which was further enhanced by E2 and P

Mo secreted higher levels of CCL22 than ESCs, and the ESCs-Mo co-culture units promoted significantly higher secretion of CCL22 than ESCs or Mo cultured alone ($n = 6$, $P < 0.05$). We found that E2 at 10^{-9}–10^{-7} mol/l, P at 10^{-8}–10^{-7} mol/l and E2 (10^{-8} mol/l) combined with P (10^{-8} mol/l) increased ESCs secreting CCL22 ($P < 0.05$). E2 at 10^{-9}–10^{-7} mol/l, P at 10^{-10}–10^{-8} mol/l and E2 (10^{-8} mol/l) combined with P (10^{-8} mol/l) increased Mo secretion of CCL22 ($P < 0.05$). E2 at 10^{-10}–10^{-7} mol/l, P at 10^{-10}–10^{-7} mol/l and E2 (10^{-8} mol/l) combined with P (10^{-8} mol/l) promoted ESCs-Mo co-culture units to secret CCL22 ($P < 0.05$) (Fig. 1a).

Although Mo secreted more CCL17 than ESCs did, their CCL17 production was not affected by E2 or P. E2 at 10^{-10}–10^{-7} mol/l, P at 10^{-10}–10^{-7} mol/l and E2 (10^{-8} mol/l) combined with P (10^{-8} mol/l) increased the CCL17 secretion of ESCs ($n = 6$, $P < 0.05$). The ESCs-Mo co-culture had significantly higher levels of CCL17 secretion than ESCs or Mo cultured alone ($n = 6$, $P < 0.05$), and E2 at 10^{-10}–10^{-7} mol/l, P at 10^{-10}–10^{-7} mol/l and E2 (10^{-8} mol/l) plus P (10^{-8} mol/l) stimulated a higher secretion of CCL17 by the ESCs-Mo co-culture ($n = 6$, $P < 0.05$) (Fig. 1b).

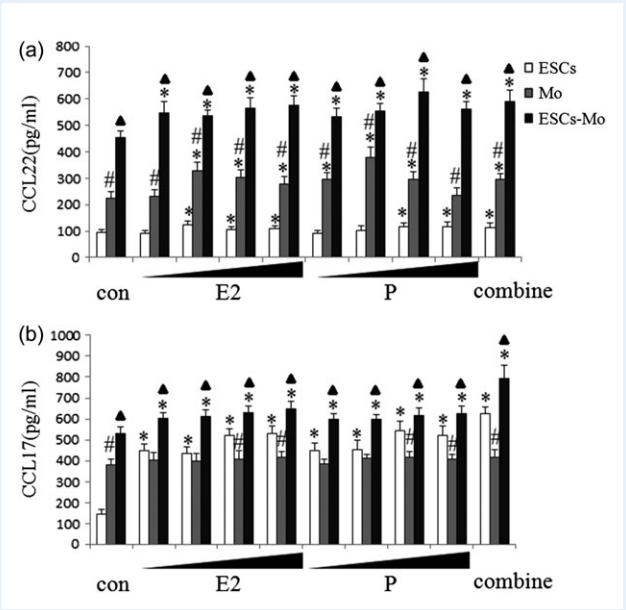

Figure 1 Effect of ESCs-Mo co-culture and sex hormones on CCL22 and CCL17 secretion. ESCs and Mo cultured alone or together were treated with various concentrations of E2 (10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} mol/l successively), P (10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} mol/l successively), or E2 (10^{-8} mol/l) plus P (10^{-8} mol/l), respectively, for 48 h. At the end of the culture, the supernatant was collected. CCL22 (**a**) and CCL17 (**b**) levels were determined by ELISA. Data are expressed as mean ± SEM. *$P < 0.05$ compared to ESCs, Mo or ESCs-Mo control; #$P < 0.05$ compared to ESCs treated in the same group; ▲$P < 0.05$ compared to ESCs or Mo treated in the same group. The result is representative of three repeated experiments ($n = 6$). Mo, monocytes.

The CCK8 cell viability assay and BrdU cell proliferation assay showed that the viability of the cells (ESCs, Mo and ESCs-Mo) did not reduce after a 48 h culture. Moreover, the ESCs and co-cultured ESCs-Mo proliferated slightly under E2 treatment for 48 h (~1.06–1.08-fold) (Supplementary data, Figs S2 and S3).

High levels of CCL22 and CCL17 caused Tregs recruitment in the ectopic milieu

The supernatant from Mo conferred a stronger chemotactic ability in Tregs than that from ESCs, and the supernatant from the ESCs-Mo co-culture conferred a stronger chemotactic ability than that from ESCs or Mo cultured alone ($n = 6$, $P < 0.05$). E2 (10^{-8} mol/l), P (10^{-8} mol/l) or their combination could significantly enhance the chemotaxis compared with the control ($n = 6$, $P < 0.05$), (Fig. 2a, left).

The supernatant from the ESCs-Mo co-culture treated with E2 (10^{-8} mol/l) and/or P (10^{-8} mol/l) could significantly enhance the chemotaxis of Tregs compared with the control ($n = 6$, $P < 0.05$). The anti-CCL17 neutralizing Ab and/or anti-CCL22 neutralizing Ab partly inhibited this effect ($n = 6$, $P < 0.05$) (Fig. 2a, right).

ESCs-Mo co-culture stimulated Tregs to secrete TGF-β1

We examined the secretion of TGF-β1 in different culture units. The Tregs co-cultured with ESCs-Mo treated with E2 (10^{-8} mol/l) plus P (10^{-8} mol/l) produced much more TGF-β1 than Tregs cultured alone ($n = 6$, $P < 0.05$), and the anti-CCL17 neutralizing and anti-CCL22 neutralizing Abs could decrease, but not block, the increased TGF-β1 secretion completely ($n = 6$, $P < 0.05$) (Fig. 2b).

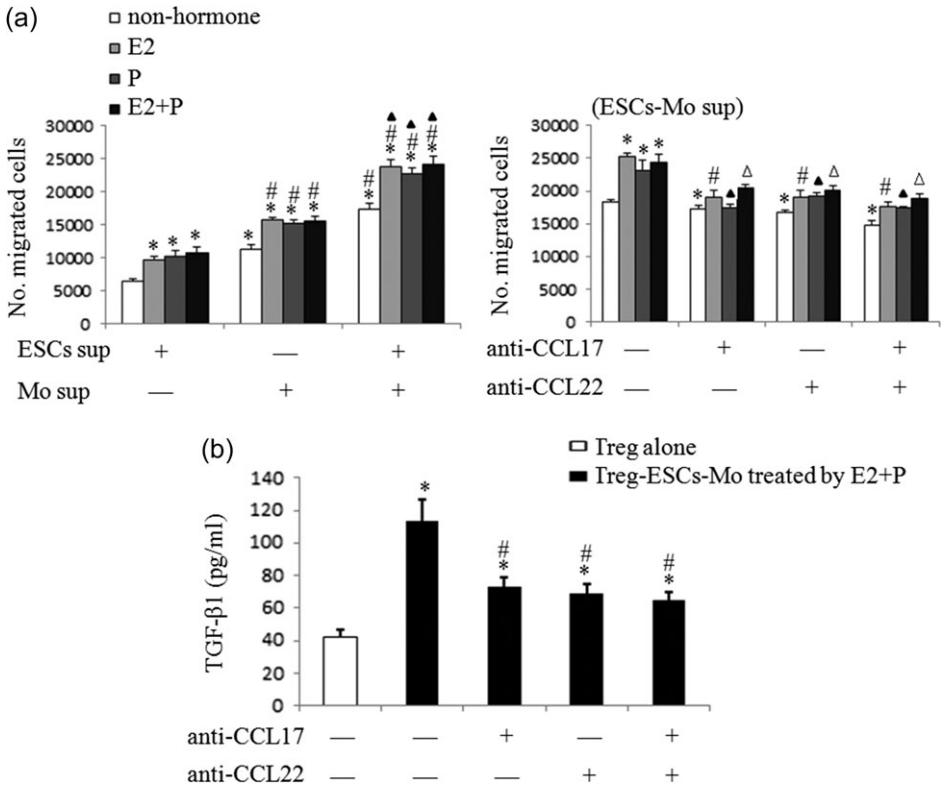

Figure 2 Effect of co-culture and CCL17 and CCL22 on recruitment and TGF-β1 secretion of Tregs. (**a**) Left: The supernatant from ESCs cultured alone, Mo cultured alone or the ESCs-Mo co-culture, which were treated with E2 at 10^{-8} mol/l, P at 10^{-8} mol/l or the combination of E2 and P for 48 h, were placed in the lower chamber of transwell plates. Tregs (2×10^5 cells/well) were put into the upper chamber. After 4 h incubation, the Tregs in the lower chamber were counted. *$P < 0.05$ compared to ESCs, Mo or the ESCs-Mo supernatant control; #$P < 0.05$ compared to ESCs supernatant treated in the same group; ▲$P < 0.05$ compared to ESCs or Mo supernatant treated in the same group. Right: The supernatant from the ESCs-Mo co-culture, which was treated with E2 at 10^{-8} mol/l, P at 10^{-8} mol/l or the combination of E2 and P for 48 h, was placed in the lower chamber of transwell plates. Tregs (2×10^5 cells/well) were put into the upper chamber. Anti-CCL17 neutralizing antibody, anti-CCL22 neutralizing antibody or the combination thereof was added to several lower chambers half an hour before the chemotaxis assay. After 4 h incubation, the Tregs in the lower chamber were counted. *$P < 0.05$ compared to the control, #, ▲, Δ, $P < 0.05$ compared to ESC-Mo supernatant treated with E2 and/or P in the same group. (**b**) Tregs were cultured alone or co-cultured with ESCs-Mo treated with E2 at 10^{-8} mol/l plus P at 10^{-8} mol/l. Anti-CCL22 neutralizing antibody, anti-CCL17 neutralizing antibody or the combination thereof was added. PMA at 50 ng/ml and ionomycin at 2.5 μg/ml were added to the cell-culture units 6 h before the termination. The supernatant was collected. TGF-β1 levels were determined by ELISA. The result is representative of three repeated experiments ($n = 6$). Data are expressed as mean ± SEM. *$P < 0.05$ compared to Tregs cultured alone, #$P < 0.05$ compared to Treg-ESCs-Mo co-culture units treated with E2 plus P. Tregs, regulatory T cells; PMA, 4a-phorbol-12-myristate-13-acetate.

CCL22 and CCL17 produced by ESCs-Mo co-culture up-regulated CCR4 expression on Tregs

We investigated the expression levels of CCR4, the receptor of CCL17 and CCL22, and found that CCR4 expression on Tregs could be regulated by the supernatant from ESCs-Mo co-culture and further increased when treated with the hormone-treated ESCs-Mo co-culture supernatant ($n = 6$, $P < 0.05$) (Fig. 3a). Notably, the anti-CCL17 neutralizing and anti-CCL22 neutralizing Abs inhibited this effect ($n = 6$, $P < 0.05$) (Fig. 3b).

ESCs-Mo co-culture enhanced the immunosuppressive function of Tregs

Tregs were cultured alone or co-cultured with the ESCs-Mo co-culture treated with the combination of E2 (10^{-8} mol/l) and P (10^{-8} mol/l). The expression, including the positive rate and mean fluorescence intensity (MFI) of CD4, CD25, Foxp3, CTLA-4, CD39 and CD73 on Tregs was analyzed by flow cytometry. We found that the expression of CD4 and CD25 was not affected by the co-culture, and anti-CCL17 neutralizing and anti-CCL22 neutralizing Abs had no effect on CD4 and CD25 expression on Tregs ($n = 6$, Fig. 4a). However, we found that the expression of Foxp3, CTLA-4, CD73 and CD39 was up-regulated by the ESCs-Mo co-culture treated with the combination of E2 (10^{-8} mol/l) and P (10^{-8} mol/l). Anti-CCL17 neutralizing and anti-CCL22 neutralizing Abs could inhibit the effect partly ($n = 6$, $P < 0.05$) (Fig. 4b).

Interaction of ESCs with Mo enhanced secretion of the proinflammatory cytokines IL-1β and TNF-α

The peritoneal fluid of women with endometriosis has high concentrations of IL-1β and TNF-α (Cheong *et al.*, 2002; Skrzypczak *et al.*, 2005). To search for the source of IL-1β and TNF-α, we constructed ESCs-Mo co-culture units and found that Mo secreted higher levels of IL-1β and TNF-α than ESCs did ($n = 6$, $P < 0.05$). The ESCs-Mo co-culture significantly promoted the secretion of IL-1β and TNF-α than that of ESCs and Mo cultured alone, respectively ($n = 6$, $P < 0.05$). E2 and P had no effect on IL-1β secretion (Fig. 5a). E2 at 10^{-7} mol/l, P at 10^{-10}–10^{-7} mol/l and E2 (10^{-8} mol/l) combined with P (10^{-8} mol/l) increased TNF-α secretion by Mo ($n = 6$, $P < 0.05$). E2 at 10^{-7} mol/l,

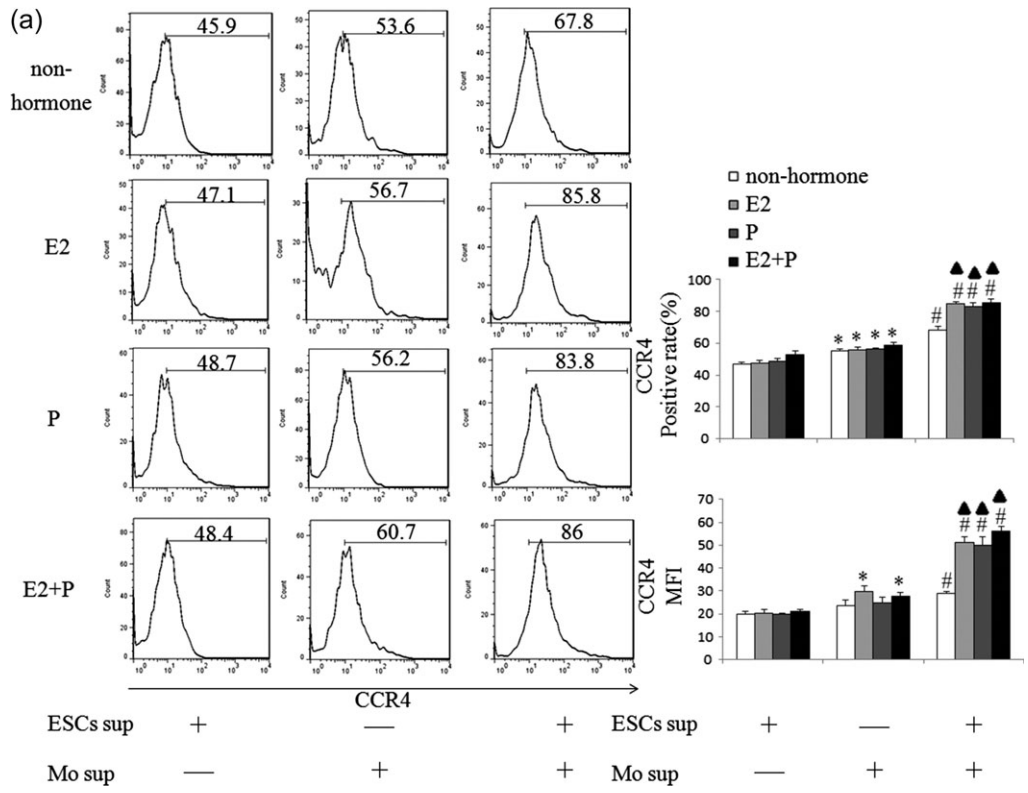

Figure 3 CCL17 and CCL22 in the supernatant from the ESCs-Mo co-culture up-regulate the expression of CCR4 on Tregs. Tregs were treated as in Figure 2a. The expression of CCR4 on Tregs was analyzed by flow cytometry. Data are expressed as mean ± SEM. (**a**) *$P < 0.05$ compared to ESCs supernatant in the same group; #$P < 0.05$ compared to ESCs or Mo supernatant in the same group; ▲$P < 0.05$ compared to ESCs-Mo co-culture supernatant treated without hormones. (**b**) *$P < 0.05$ compared to ESCs-Mo co-culture supernatant treated without hormone but with or without anti-CCL17 or anti-CCL22 neutralizing Abs or the combination thereof; #P < 0.05 compared to ESCs-Mo co-culture supernatant in the same group without neutralizing Abs; ▲P < 0.05 compared to ESCs-Mo co-culture supernatant treated with anti-CCL17 or anti-CCL22 neutralizing antibody in the same group. The result is representative of three repeated experiments ($n = 6$). Abs, antibodies.

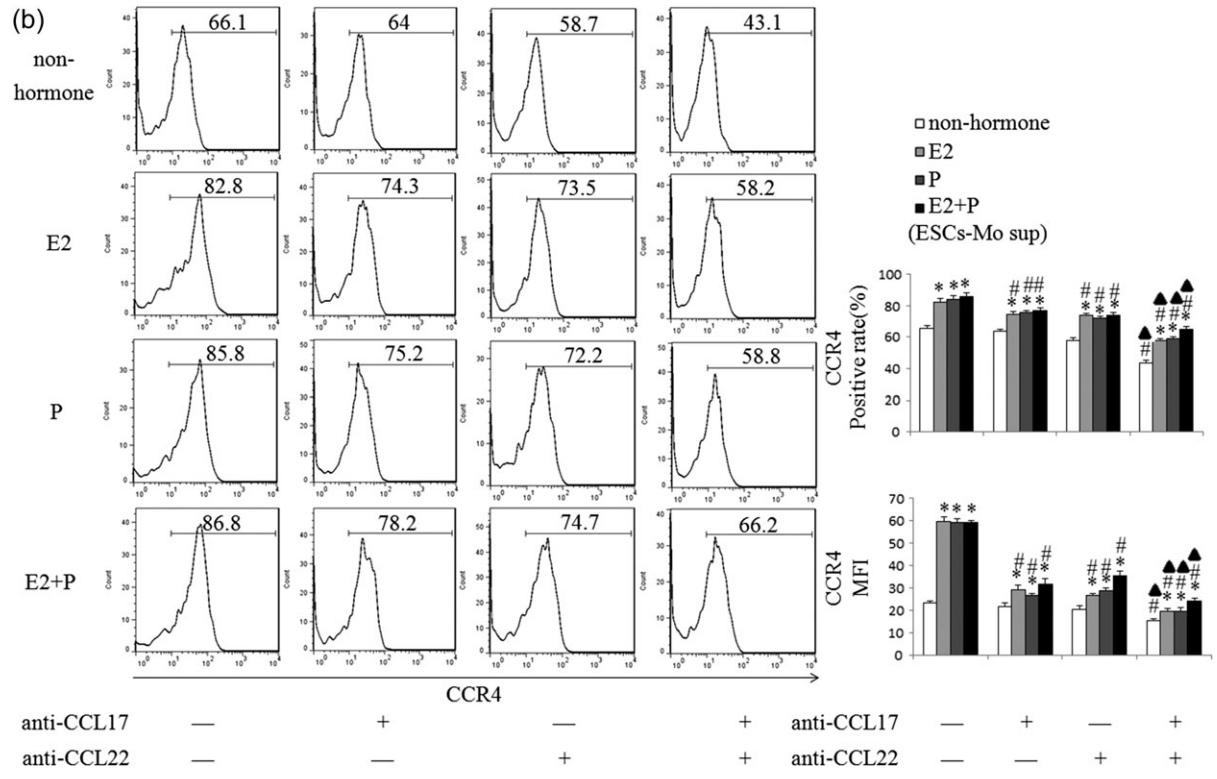

Figure 3 *Continued*

P at 10^{-10}–10^{-7} mol/l and E2 (10^{-8} mol/l) combined with P (10^{-8} mol/l) also enhanced TNF-α secretion by the ESCs-Mo co-culture ($n = 6$, $P < 0.05$) (Fig. 5b). These results suggest that the high levels of IL-1β and TNF-α in the peritoneal fluid of women with endometriosis arise from the interaction of ESCs with Mo.

Combination of TGF-β1 with IL-1β or TNF-α activated the ERK1/2 and p38 signaling pathways in ESCs

ESCs were treated *in vitro* with rhTGF-β1, rhIL-1β, rhTNF-α, a combination of rhTGF-β1 and rhIL-1β, or a combination of rhTGF-β1 and rhTNF-α. We found that rhTGF-β1, rhIL-1β or rhTNF-α could activate p38 and ERK1/2 signaling pathways, and the combination of rhTGF-β1 and rhIL-1β or the combination of rhTGF-β1 and rhTNF-α further enhanced this effect ($n = 6$, $P < 0.05$). rhTGF-β1 and rhIL-1β in combination could activate the JNK signaling pathway ($n = 6$, $P < 0.05$) but had no effect alone. rhTGF-β1 and rhTNF-α either alone or in combination had no effect on the JNK signaling pathway (Fig. 6 and Supplementary data, Fig S1).

IL-1β and TNF-α presented a synergistic effect with TGF-β1 in stimulating ESCs to secrete the pro-angiogenic factors IL-8 and VEGF

ESCs were treated as described in the methods section. The supernatant was collected and analyzed by ELISA. The results showed that rhTGF-β1, rhIL-1β and rhTNF-α alone could stimulate the ESCs to secret IL-8 and VEGF. rhIL-1β and rhTNF-α had a synergistic role with rhTGF-β1 in promoting IL-8 production ($n = 6$, $P < 0.05$), but the combination of rhTGF-β1 and rhIL-1β or the combination of rhTGF-β1 with rhTNF-α had no additional role in stimulating ESCs to produce VEGF (Fig. 7).

Blocking ERK1/2 and p38 signaling pathways by U0126 and SB202190 could significantly downregulate the promotion of rhTGF-β1, rhIL-1β or rhTNF-α on IL-8 and VEGF secretion by ESCs ($n = 6$, $P < 0.05$). However, we found that blocking the JNK signaling pathway by SP600125 not only inhibited the role of rhTGF-β1 in promoting IL-8 secretion but also decreased the role of rhIL-1β and rhTNF-α on promoting VEGF secretion in ESCs ($n = 6$, $P < 0.05$) (Fig. 7).

Supernatant of ESCs treated with rhTGF-β1, rhIL-1β, and/or rhTNF-α stimulated angiogenesis of HUVECs

To probe whether the supernatant of ESCs can promote angiogenesis, we performed the tube formation assay. As shown in Fig. 8, the supernatant of ESCs treated with rhTGF-β1, rhIL-1β or rhTNF-α alone promoted angiogenesis ($n = 6$, $P < 0.05$); the supernatant of ESCs treated with the combination of rhTGF-β1 and rhIL-1β or the combination of rhTGF-β1 and rhTNF-α could further promote angiogenesis ($n = 6$, $P < 0.05$). This finding demonstrates that the supernatant of ESCs stimulated by rhTGF-β1, rhIL-1β and/or rhTNF-α could promote angiogenesis, which is involved in the pathogenesis of endometriosis. Taking into

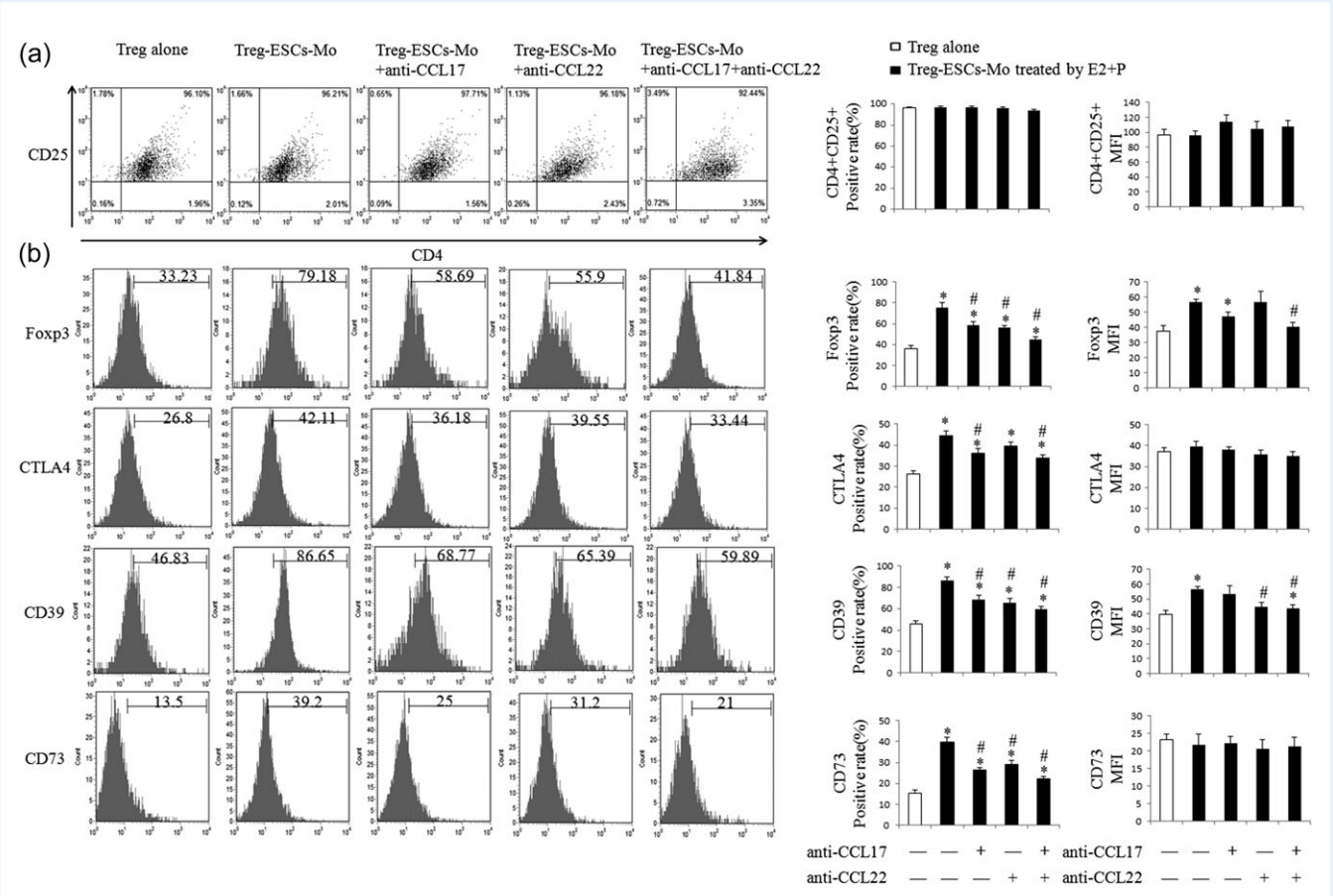

Figure 4 Treg-ESCs-Mo co-culture enhances the immunosuppressive function of Tregs through CCL17 and CCL22. We established the following cell-culture units: Tregs cultured alone and Tregs co-cultured with ESCs-Mo, which were treated with the combination of E2 (10^{-8} mol/l) and P (10^{-8} mol/l) for 48 h. Anti-CCL17 neutralizing antibody, anti-CCL22 neutralizing antibody or both were added to the ESCs-Mo-Treg co-culture. PMA at 50 ng/ml and ionomycin at 2.5 μg/ml were added to the culture 6 h before the end of the culture. Flow cytometry was used to analyze the expression of (**a**) CD4, CD25, (**b**) Foxp3, CTLA-4, CD39 and CD73 on Tregs including the positive rate and MFI. Data are expressed as mean ± SEM. *$P < 0.05$ compared to Tregs cultured alone. #$P < 0.05$ compared to Tregs co-cultured with ESCs-Mo treated with E2 plus P. The result shown is representative of three repeated experiments ($n = 6$). MFI, mean fluorescence intensity.

account the results in Fig. 7, we propose that the high levels of IL-8 and VEGF secreted by ESCs stimulated by rhTGF-β1, rhIL-1β and/or rhTNF-α might be the main factor promoting angiogenesis in the ectopic milieu.

Discussion

Tregs are known to be important immunosuppressive cells that play an important role in maintaining the immune balance *in vivo*. The number and the percentage of Tregs have been reported to be significantly increased in the peritoneal fluid of patients with endometriosis (Podgaec *et al.*, 2012; Olkowska-Truchanowicz *et al.*, 2013), which suggests that Tregs may be involved in the pathogenesis of endometriosis. Tregs may favor the ectopic implantation of endometrium by inhibiting the function of effector T cells (Teff). The inhibitory cytokine TGF-β1 has gained considerable attention as a mediator of Tregs in immunosuppression. Angiogenesis is essential for the pathogenesis of many diseases such as cancer, chronic inflammation and endometriosis. Endometriotic lesions have been shown to be highly vascularized, and angiogenesis is critical for the establishment and growth of ectopic lesions (Groothuis *et al.*, 2005). Endometriosis is an inflammatory disease, and the levels of a lot of inflammatory cytokines (such as IL-1β and TNF-α) are elevated in the peritoneal fluid of patients with endometriosis (Cheong *et al.*, 2002; Skrzypczak *et al.*, 2005). Human Tregs highly express the chemokine receptor CCR4, and thus, their ligands CCL17 and CCL22 might modulate the homing of Tregs to tissues. Foxp3 expression in Tregs is positively correlated with the levels of CCL22 and its receptor CCR4 (Qin *et al.*, 2008), implying that CCL17 and CCL22 can regulate the function of Tregs. CCL17 expression is considerably higher in the eutopic endometrium of patients with endometriosis compared with that in normal fertile women (Bellelis *et al.*, 2013), and tumor cell and macrophage-derived chemokine CCL22 mediates the trafficking of Tregs to the tumor, representing a mechanism by which the tumor may foster immune privilege (Curiel *et al.*, 2004).

Our data showed that the high levels of CCL22 and CCL17 in the ESCs-Mo co-culture units, particularly those treated with E2 or P, could recruit Tregs, promote TGF-β1 secretion, increase CCR4

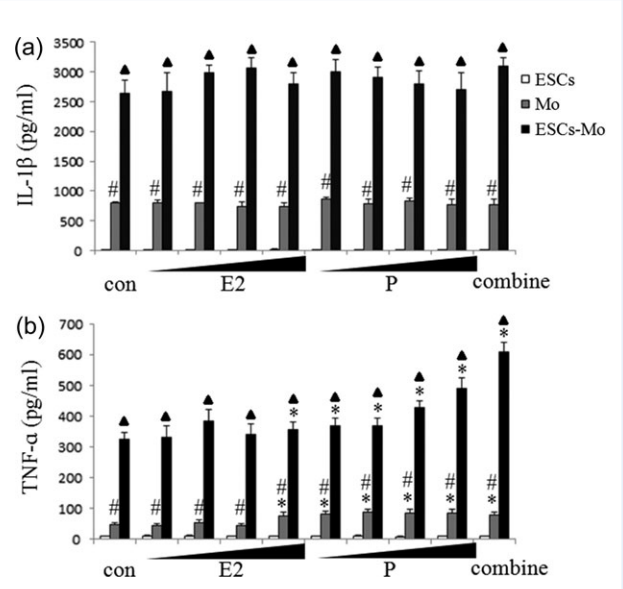

Figure 5 High levels of the inflammatory cytokines IL-1β and TNF-α in the peritoneal cavity of patients with endometriosis may arise from the interaction of ESCs and Mo. ESCs and Mo cultured alone or together were treated with various concentrations of E2 (10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} mol/l successively), P (10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} mol/l successively) or E2 (10^{-8} mol/l) plus P (10^{-8} mol/l) for 48 h. The supernatant was collected. IL-1β (**a**) and TNF-α (**b**) levels were determined by ELISA. The result is representative of three repeated experiments ($n = 6$). Data are expressed as mean ± SEM. *$P < 0.05$ compared to ESCs, Mo or the ESCs-Mo control; #$P < 0.05$ compared to ESCs treated in the same group; ▲$P < 0.05$ compared to ESCs or Mo treated in the same group. TNF-α, tumor necrosis factor alpha; Interleukin, IL-1β.

expression and upregulate the immunosuppressive function of Tregs, which was supported by the increased expression of Foxp3, CTLA-4, CD39 and CD73 (Fig. 9). The migrated Tregs may suppress the activation or proliferation of Teffs in an autologous setting in ectopic foci. In view of the importance of CCR4 in the migration of Tregs, an anti-CCR4 mAb might be a novel treatment strategy for patients with endometriosis to overcome the suppressive effect of $CCR4^+$ Tregs on the host local immune response to ectopic endometrial cells. Ishida *et al.* developed a novel chimeric mAb, KM2760, which binds specifically to CCR4. They found that the Ab could induce effective anti-tumor immunity by reducing the infiltration of CCR4-positive Tregs in the Hodgkin lymphoma tumor cell environment (Ishida *et al.*, 2004). Considering that ESCs and Mo are the key component cells in ectopic tissues, although we used eutopic ESCs in this study, if similar effects to what we have seen occur at ectopic sites, it is likely that the abnormal immune microenvironment in ectopic foci could recruit and regulate Treg functions, which may have an important immunopathological role in endometriosis.

Our research indicates that the interaction of the endometriosis-associated cells ESCs and Mo may be the important source of the high levels of IL-1β and TNF-α in the peritoneal cavity of patients with endometriosis (Fig. 9). Exogenous rhTGF-β1, rhIL-1β or rhTNF-α could activate p38 and ERK1/2 signaling pathways, and rhIL-1β and rhTNF-α have a synergistic role with rhTGF-β1. Although rhTGF-β1 and rhIL-1β in conjunction promoted the activation of the JNK signaling pathway, rhTGF-β1 or rhIL-1β alone had no effect. On the other hand, rhTGF-β1 or rhTNF-α alone or in combination had no effect on the JNK signaling pathway. Our results suggest that ERK1/2/p38 signaling pathways may mediate TGF-β1 signaling, which regulates cell proliferation and growth (Fig. 9).

We also conducted an experiment to exclude possible side-effects caused by reduced viability or enhanced cell proliferation, which may act as confounding factors, and found that the viability of ESCs, Mo and Tregs cultured alone had no obvious change after 48 h. In fact, the viability of the ESCs-Mo co-culture increased slightly. This means that the viability of the cells did not reduce after 48 h culture. The cell proliferation assay showed that the primary cultured Mo and Tregs hardly proliferate *in vitro*, regardless of the use of hormone treatment. ESCs-Mo co-culture showed a higher proliferation compared with ESCs or Mo cultured alone. E2 could promote the proliferation of ESCs and ESCs-Mo slightly after 48 h (~1.06–1.08-fold). Thus, the increased production of certain factors may be partly related to increased cell numbers, but would mainly reflect their increased expression.

Angiogenesis is required for ectopic tissue growth and may constitute an important point in the control of endometriosis progression. We found that rhTGF-β1, rhIL-1β or rhTNF-α alone, the combination of rhTGF-β1 and rhIL-1β, or the combination of rhTGF-β1 and rhTNF-α promoted the ESCs to secrete the angiogenesis factors IL-8 and VEGF (Fig. 9). Furthermore, rhIL-1β and rhTNF-α had a synergistic angiogenesis-promoting role with rhTGF-β1. U0126 or SB202190 inhibited the promoting effect of rhTGF-β1, rhIL-1β or rhTNF-α alone, the combination of rhTGF-β1 and rhIL-1β, and the combination of rhTGF-β1 and rhTNF-α on IL-8 and VEGF secretion by ESCs. SP600125, as an inhibitor of the JNK signaling pathway, downregulated the stimulating role of rhTGF-β1 alone or in combination with rhTNF-α to secrete IL-8, and also decreased the promoting role of rhIL-1β and rhTNF-α on VEGF secretion in ESCs. The results above suggest that blocking ERK1/2 and p38 signaling pathways reduced the levels of IL-8 and VEGF secreted by ESCs. U0126 or SB202190 may simultaneously block some autocrine role in ESCs, and the JNK signaling pathway is also involved in regulating the secretion of IL-8 and VEGF by ESCs through other ways. It is well established that IL-8 and VEGF play a critical role in regulating physiological and pathological angiogenesis. They are probably the most crucial angiogenic factors involved in the pathophysiology of endometriosis. The perturbation of TGF-β1 signaling by aberrant activation of ERK1/2 and p38 underlies the critical role of TGF-β1 in pro-angiogenesis behavior in advanced endometriosis. In summary, the results confirm that ERK1/2/p38 signaling pathways mediate the pro-angiogenesis of TGF-β1 in ESCs, and the inflammatory cytokines IL-1β or TNF-α have a synergistic effect with TGF-β1. Findings of the tube formation assay further confirmed our results, that is, the high levels IL-8 and VEGF secreted by ESCs stimulated by TGF-β1 have a strong angiogenic effect, and IL-1β and TNF-α have a synergistic effect with TGF-β1.

Nevertheless, our study does have certain limitations. Our use of eutopic ESCs instead of ectopic ESCs of endometriosis patients might be better for describing intercellular interactions in eutopic endometrium. The model system we used cannot be easily seen as a model for ectopic lesions. If interactions and effects similar to what we have seen in this study occur at ectopic sites, it could be envisaged that

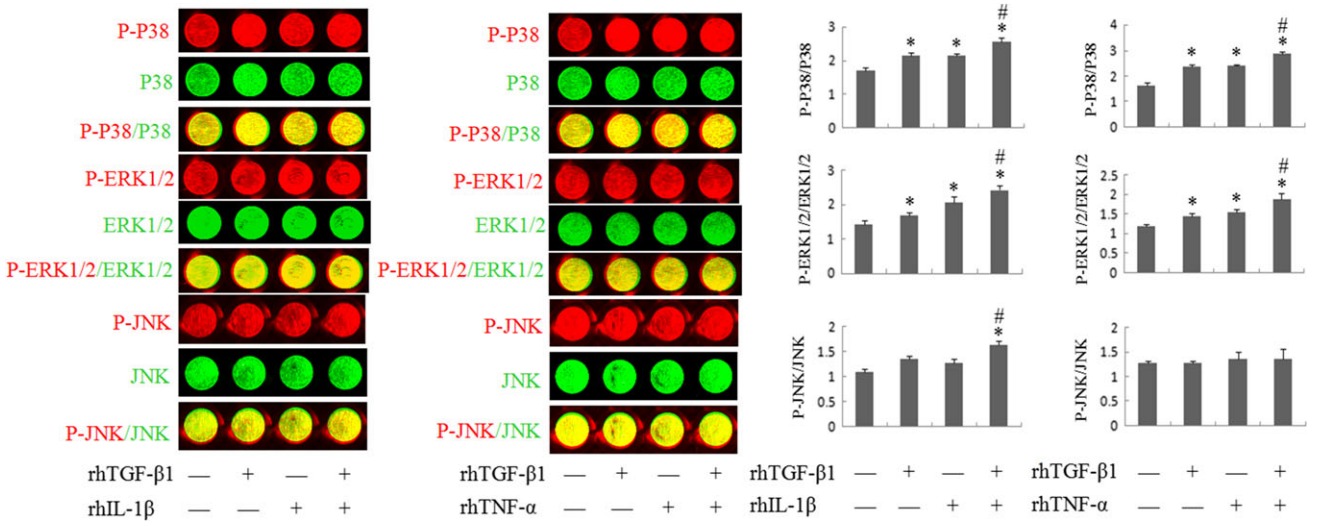

Figure 6 Effect of TGF-β1, IL-1β and TNF-α on p38, ERK1/2 and JNK signaling pathways in ESCs. Eutopic ESCs were treated *in vitro* with: rhTGF-β1 (20 ng/ml), rhIL-1β (20 ng/ml), rhTNF-α (20 ng/ml); the combination of rhTGF-β1 and rhIL-1β; or the combination of rhTGF-β1 and rhTNF-α for 48 h, with vehicle as the control. The In-cell Western™ western blot analysis system was used to analyze the phosphorylation and total levels of p38, ERK1/2 and JNK. Phospho-p38, phospho-ERK1/2, phospho-JNK = red; p38, ERK1/2, JNK = green. These pictures are representative of thre individual experiments ($n = 6$). *$P < 0.05$ compared to the control, #$P < 0.05$ compared to rhTGF-β1, rhIL-1β or rhTNF-α treated alone. ESCs, endometrial stromal cells; ERK, extracellular signal-regulated kinase1/2; JNK, c-Jun N-terminal kinase; rhTGF-β1, recombinant human transforming growth factor-beta 1; rhIL-1β, recombinant human interleukin one beta; rhTNF-α, recombinant human tumor necrosis factor alpha.

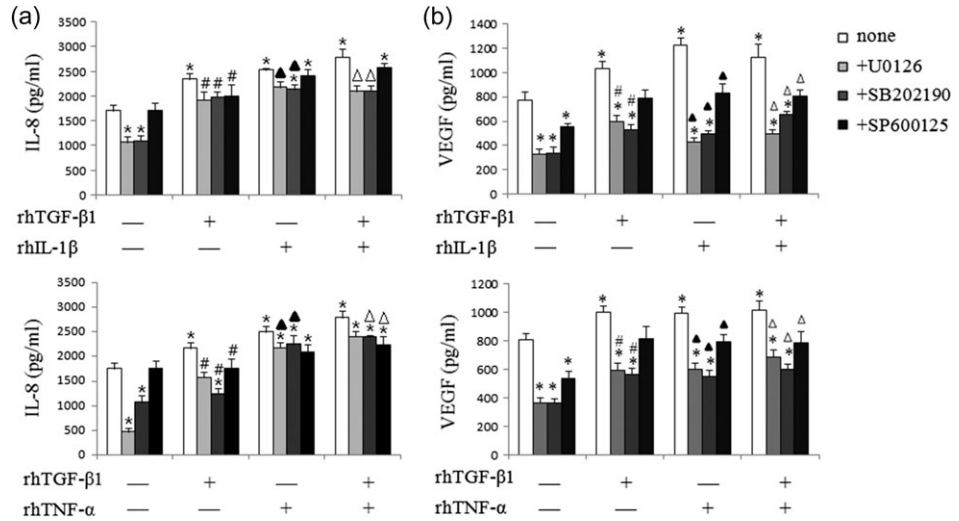

Figure 7 TGF-β1, IL-1β and TNF-α alone or in combination stimulate ESCs to secrete the pro-angiogenic factors IL-8 and VEGF by activating ERK1/2/p38 signaling pathways. ESCs were treated with: rhTGF-β1 (20 ng/ml), rhIL-1β (20 ng/ml), rhTNF-α (20 ng/ml); a combination of rhTGF-β1 and rhIL-1β with the same concentrations; or a combination of rhTGF-β1 and rhTNF-α with the same concentrations for 48 h. The cells were incubated with or without the MAPK/ERK1/2 inhibitor U0126 (30 μM), p38 inhibitor SB202190 (20 μM) or JNK inhibitor SP600125 (20 μM), with vehicle as the control. The supernatant was collected and detected for the expression of IL-8 (**a**) and VEGF (**b**) by ELISA assay. *$P < 0.05$ compared to the control. #, ▲, Δ, $P < 0.05$ compared to rhTGF-β1, rhIL-1β, rhTNF-α treated alone or in combination without inhibitors. These pictures are representative of three individual experiments ($n = 6$). VEGF, vascular endothelial growth factor. MAPK, mitogen-activated protein kinase.

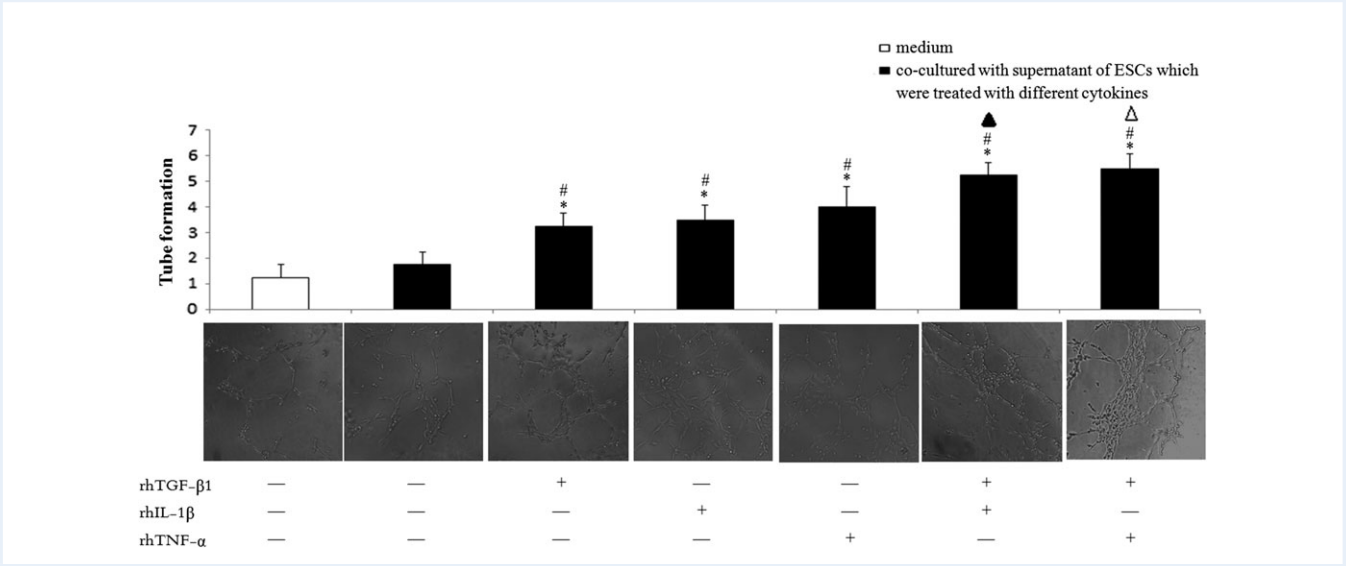

Figure 8 Supernatant of ESCs treated with rhTGF-β1, rhIL-1β and/or rhTNF-α stimulates the angiogenesis of HUVECs. HUVECs (5000 cells/well) were incubated with the supernatant of ESCs treated with rhTGF-β1 (20 ng/ml), rhIL-1β (20 ng/ml) and/or rhTNF-α (20 ng/ml). Next, the tube formation assay was used to analyze the tube formation of HUVECs. Original magnification: ×100. Results were highly reproducible in three independent experiments ($n = 6$). Error bars depict the SEM. *$P < 0.05$ compared to HUVECs cultured in medium, #$P < 0.05$ compared to HUVECs cultured with the supernatant of ESCs with no cytokine treatment. ▲$P < 0.05$ compared to HUVECs cultured with the supernatant of ESCs treated with rhTGF-β1 or rhIL-1β; Δ$P < 0.05$ compared to HUVECs cultured with the supernatant of ESCs treated with rhTGF-β1 or rhTNF-α. HUVECs, human umbilical vein endothelial cells.

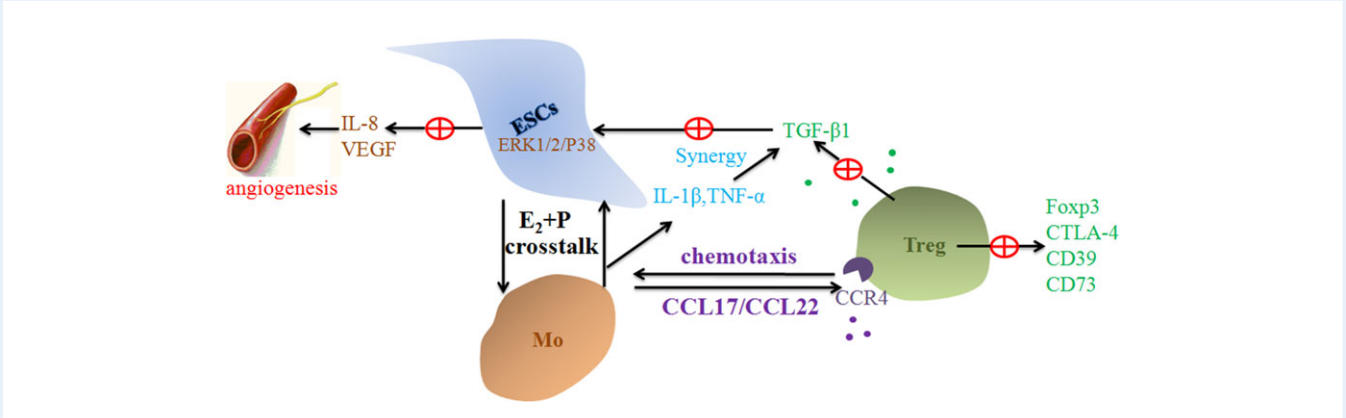

Figure 9 Schematic diagram of key findings. This study has shown that high levels of CCL17 and CCL22 produced by the interaction between ESCs and Mo can recruit Tregs, increase the expression of CCR4 on them, and upregulate the immunosuppression of Tregs, which in turn promotes angiogenesis by TGF-β1 secretion. The abundant IL-1β or TNF-α in an ectopic milieu has a synergistic role with TGF-β1. If similar interactions to what we have seen in this study occur at ectopic sites, it could be envisaged that angiogenesis is strongly promoted, which contributes to the pathogenesis of endometriosis.

angiogenesis is strongly promoted. Furthermore, such interactions in the eutopic endometrium are important and may have a role in poor receptivity and miscarriage. It has been shown that there is a close link between endometriosis and infertility. Some studies indicate that 30–50% of patients who suffer from endometriosis are infertile and 25–50% of infertile women have endometriosis (Meuleman *et al.*, 2009). Women with endometriosis display significantly higher rates of miscarriages which are reported range from 11 to 63% (Santulli *et al.*, 2016; Hjordt Hansen et al., 2014; Gupta *et al.*, 2008). However, although there is a high correlation between endometriosis and infertility, little is known about the mechanisms through which endometriosis impair fertility. Unsuitable endometrial receptivity has been suggested to contribute to endometriosis-associated infertility. In women with endometriosis, the microenvironment of the endometrium becomes proinflammatory, E2-dominant, and P4-resistant, and thereby impairs receptivity for establishment of pregnancy. Endometriosis patients also have altered gene

expression in the eutopic endometrium (such as HOXA10, P receptors, matrix metalloproteinases, immunoinflammatory factors and integrin) that may impair endometrial receptivity (Hurst *et al.*, 2014). An aberrant immunological mechanism might be one of potent pathophysiological mechanisms of endometriosis-related infertility (Inagaki *et al.*, 2011). The interactions between eutopic ESCs, Mo and Tregs shown in this study may affect the expression of receptivity-related molecules and the local immune microenvironment, which may impair endometrial receptivity and increase rates of miscarriages in endometriosis patients.

At present, surgical removal of endometriotic tissues and inhibition of ovarian steroid hormone production remain the main treatment for endometriosis. But the curative effect is not satisfactory because of the high recurrence rate of the disorder and the adverse side-effects of the different treatments. Endometriosis is a consequence of the provision of a fertile environment (the 'soil', so to speak), in which compatible endometrium cells (the 'seed') can proliferate. Tregs may play an important role in creating the suitable environment.

Our present results exhibit for the first time that the abnormal immune microenvironment can account for the recruitment and functional regulation of Tregs, which partly explains the high levels of CCL22 and CCL17 in ectopic foci. Furthermore, excessive sex hormones levels found in patients with endometriosis are attributed to CCL22 and CCL17 overexpression. We found that TGF-β1 secreted by Tregs was enhanced by ESCs-Mo co-culture, and IL-1β or TNF-α had a synergistic effect with TGF-β1 in activating the ERK1/2/p38 signaling pathways to promote the secretion of IL-8 and VEGF by ESCs. If the effects seen in this study occur at ectopic sites, then our study has illustrated the mechanisms of recruitment and functional regulation of Tregs in endometriotic lesions and a new mechanism of angiogenesis in ectopic foci, which shows a new role of Tregs involved in the disease. These findings offer the exciting opportunity to develop novel Treg-based diagnostic and therapeutic approaches for endometriosis, such as therapy with anti-CCR4 mAbs.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

We acknowledge Xiao-Yong Zhu and Xiao-Ying Yao for their assistance with sample collection. We thank Professor Mei-Rong Du and Doctor Ming-Qing Li for their excellent technical support.

Authors' roles

W.X.-Q. and Z.W.-J. conducted all experiments and prepared the figures and the manuscript. T.Y. assisted with cell culture. L.X.-Z. examined patients, obtained specimens and generated clinical data. L.D.-J. was in charge of the study design, overseeing the completion of the study, and editing and finalizing the manuscript.

Funding

Ministry of Science and Technology of China (2015CB943300 to L.D.-J.); National Natural Science Foundation of China (item number 81200425 to W.X.-Q.); National Natural Science Foundation of China (item number 81471548 to L.D.-J.) and The Research Fund for the Doctoral Program of Higher Education of China (20110071120093 to W.X.-Q.).

Conflict of interest

None declared.

References

Ahern D, Lloyd CM, Robinson DS. Chemokine responsiveness of CD4+CD25+ regulatory and CD4+CD25- T cells from atopic and nonatopic donors. *Allergy* 2009;**64**:1121–1129.

Barcz E, Rozewska ES, Kaminski P, Demkow U, Bobrowska K, Marianowski L. Angiogenic activity and IL-8 concentrations in peritoneal fluid and sera in endometriosis. *Int J Gynaecol Obstet* 2002;**79**:229–235.

Basta P, Majka M, Jozwicki W, Lukaszewska E, Kafel A, Grabiec M, Stasienko E, Wicherek L. The frequency of CD25+CD4+ and FOXP3+ regulatory T cells in ectopic endometrium and ectopic decidua. *Reprod Biol Endocrinol* 2010;**8**:116.

Bellelis P, Barbeiro DF, Rizzo LV, Baracat EC, Abrao MS, Podgaec S. Transcriptional changes in the expression of chemokines related to natural killer and T-regulatory cells in patients with deep infiltrative endometriosis. *Fertil Steril* 2013;**99**:1987–1993.

Berbic M, Hey-Cunningham AJ, Ng C, Tokushige N, Ganewatta S, Markham R, Russell P, Fraser IS. The role of Foxp3+ regulatory T-cells in endometriosis: a potential controlling mechanism for a complex, chronic immunological condition. *Hum Reprod* 2010;**25**:900–907.

Beyer M, Kochanek M, Darabi K, Popov A, Jensen M, Endl E, Knolle PA, Thomas RK, von Bergwelt-Baildon M, Bebey S. Reduced frequencies and suppressive function of CD4(+)CD25(hi) regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. *Blood* 2005;**106**:2018–2025.

Budiu RA, Diaconu I, Chrissluis R, Dricu A, Edwards RP, Vlad AM. A conditional mouse model for human MUC1-positive endometriosis shows the presence of anti-MUC1 antibodies and Foxp3+ regulatory T cells. *Dis Model Mech* 2009;**2**:593–603.

Cheong YC, Shelton JB, Laird SM, Richmond M, Kudesia G, Li TC, Ledger WL. IL-1, IL-6 and TNF-alpha concentrations in the peritoneal fluid of women with pelvic adhesion. *Hum Reprod* 2002;**17**:69–75.

Curiel TJ, Coukos G, Zou LH, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;**10**:942–949.

Fahey JV, Schaefer TM, Channon JY, Wira CR. Secretion of cytokines and chemokines by polarized human epithelial cells from the female reproductive tract. *Hum Reprod* 2005;**20**:1439–1446.

Ghia P, Strola G, Granziero L, Geuna M, Guida G, Sallusto F, Ruffing N, Montagna L, Piccoli P, Chilos M. Chronic lymphocytic leukemia B cells are endowed with the capacity to attract $CD4^+$, $CD40L^+$T cells by producing CCL22. *Eur J Immunol* 2002;**32**:1403–1413.

Gilabert-Estelles J, Ramon LA, Espana F, Gilabert J, Vila V, Reganon E, Castello R, Chirivella M, Estelles A. Expression of angiogenic factors in endometriosis: its relation to fibrinolytic and metalloproteinase systems. *Hum Reprod* 2007;**22**:2120–2127.

Groothuis PG, Nap AW, Winterhager E, Gruemmer R. Vascular development in endometriosis. *Angiogenesis* 2005;**8**:147–156.

Gupta S, Goldberg JM, Aziz N, Goldberg E, Krajcir N, Agarwal A. Pathogenic mechanisms in endometriosis-associated infertility. *Fertil Steril* 2008;**90**:247–257.

Hjordt Hansen MV, Dalsgaard T, Hartwell D, Skovlund CW, Lidegaard O. Reproductive prognosis in endometriosis. A national cohort study. *Acta Obstet Gynecol Scand* 2014;**93**:483–489.

Hurst BS, Shimp KE, Elliot M, Marshburn PB, Parsons J, Bahrani-Mostafavi Z. Molecular evaluation of proliferative-phase endometrium may provide insight about the underlying causes of infertility in women with endometriosis. *Arch Gynecol Obstet* 2014;**289**:1119–1124.

Iellem A, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Simigaglia F, D'Ambrosio D. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 2001;**194**:847–853.

Inagaki J, Hao L, Nakatsuka M, Yasuda T, Hiramatsu Y, Shoenfeld Y, Matsuura E. A possible mechanism of autoimmune-mediated infertility in women with endometriosis. *Am J Reprod Immunol* 2011;**66**:90–99.

Ishida T, Ishii T, Inagaki A, Yano H, Komatsu H, Iida S, Inagaki H, Ueda R. Specific recruitment of CC chemokine receptor 4-positive regulatory T cells in Hodgkin lymphoma fosters immune privilege. *Cancer Res* 2006; **66**:5716–5722.

Ishida T, Iida S, Akatsuka Y, Ishii T, Miyazaki M, Komatsu H, Inagaki H, Okada N, Fujita T, Shitara K. The CC chemokine receptor 4 as a novel-specific molecular target for immunotherapy in adult T-cell leukemia/lymphoma. *Clin Cancer Res* 2004;**10**:7529–7539.

Kao AP, Wang KH, Long CY, Chai CY, Tsai CF, Hsieh TH, Hsu CY, Chang CC, Lee JN, Tsai EM. Interleukin-1 beta induces cyclooxygenase-2 expression and promotes the invasive ability of human mesenchymal stem cells derived from ovarian endometrioma. *Fertil Steril* 2011;**96**:678–U207.

Klein D, Demory A, Peyre F, Kroll J, Augustin HG, Helfrich W, Kzhyshkowska J, Schledzewski K, Arnold B, Goerdt S. Wnt2 acts as a cell type-specific, autocrine growth factor in rat hepatic sinusoidal endothelial cells cross-stimulating the VEGF pathway. *Hepatology* 2008;**47**:1018–1031.

Kyama CM, Overbergh L, Debrock S, Valckx D, Vander Perre S, Meuleman C, Mihalyi A, Mwenda JM, Mathieu C, D'Hooghe TM. Increased peritoneal and endometrial gene expression of biologically relevant cytokines and growth factors during the menstrual phase in women with endometriosis. *Fertil Steril* 2006;**85**:1667–1675.

Laschke MW, Menger MD. In vitro and in vivo approaches to study angiogenesis in the pathophysiology and therapy of endometriosis. *Hum Reprod Update* 2007;**13**:331–342.

Meter RA, Wira CR, Fahey JV. Secretion of monocyte chemotactic protein-1 by human uterine epithelium directs monocyte migration in culture. *Fertil Steril* 2005;**84**:191–201.

Meuleman C, Vandenabeele B, Fieuws S, Spiessens C, Timmerman D, D'Hooghe T. High prevalence of endometriosis in infertile women with normal ovulation and normospermic partners. *Fertil Steril* 2009;**92**:68–74.

Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. *Am J Obstet Gynecol* 1975;**122**:262–263.

O'Garra A, Vieira P. Regulatory T cells and mechanisms of immune system control. *Nat Med* 2004;**10**:801–805.

Olkowska-Truchanowicz J, Bocian K, Maksym RB, Bialoszewska A, Wlodarczyk D, Baranowski W, Zabek J, Korczak-Kowalska G, Malejczyk J. CD4(+) CD25(+) FOXP3(+) regulatory T cells in peripheral blood and peritoneal fluid of patients with endometriosis. *Hum Reprod* 2013;**28**:119–124.

Podgaec S, Rizzo LV, Fernandes LFC, Baracat EC, Abrao MS. $CD4^{+}CD25^{high}Foxp3^{+}$ cells increased in the peritoneal fluid of patients with endometriosis. *Am J Reprod Immunol* 2012;**68**:301–308.

Qin SL, Sui YJ, Soloff AC, Junecko BAF, Kirschner DE, Murphey-Corb MA, Watkins SC, Tarwater PM, Pease JE, Barratt-Boyes SM. Chemokine and cytokine mediated loss of regulatory T cells in lymph nodes during pathogenic simian immunodeficiency virus infection. *J Immunol* 2008; **180**:5530–5536.

Sakamoto Y, Harada T, Horie S, Iba Y, Taniguchi F, Yoshida S, Iwabe T, Terakawa N. Tumor necrosis factor-alpha–induced interleukin-8(IL-8) expression in endometriotic stromal cells, probably through nuclear factor-kappa B activation: gonadotropin-releasing hormone agonist treatment reduced IL-8 expression. *J Clin Endocrinol Metab* 2003;**88**:730–735.

Santulli P, Marcellin L, Menard S, Thubert T, Khoshnood B, Gayet V, Goffinet F, Ancel PY, Chapron C. Increased rate of spontaneous miscarriages in endometriosis-affected women. *Hum Reprod* 2016;**31**:1014–1023.

Shi YL, Luo XZ, Zhu XY, Li DJ. Combination of 17 beta-estradiol with the environmental pollutant TCDD is involved in pathogenesis of endometriosis via up-regulating the chemokine I-309-CCR8. *Fertil Steril* 2007;**88**: 317–325.

Skrzypczak J, Szczepanska M, Puk E, Kamieniczna M, Kurpisz M. Peritoneal fluid cytokines and sICAM-1 in minimal endometriosis: search for discriminating factors between infertility and/or endometriosis. *Eur J Obstet Gynecol Reprod Biol* 2005;**122**:95–103.

Wang XQ, Yu J, Luo XZ, Shi YL, Wang Y, Wang L, Li DJ. The high level of RANTES in the ectopic milieu recruits macrophages and induces their tolerance in progression of endometriosis. *J Mol Endocrinol* 2010a;**45**:291–299.

Wang Y, Yu J, Luo XZ, Wang XQ, Li MQ, Wang L, Li DJ. Abnormal regulation of chemokine TECK and its receptor CCR9 in the endometriotic milieu is involved in pathogenesis of endometriosis by way of enhancing invasiveness of endometrial stromal cells. *Cell Mol Immunol* 2010b;**7**:51–60.

Yoshino O, Izumi G, Shi J, Osuga Y, Hirota Y, Hirata T, Harada M, Nishii O, Koga K, Taketani Y. Activin-A is induced by interleukin-1beta and tumor necrosis factor-alpha and enhances the mRNA expression of interleukin-6 and protease-activated receptor-2 and proliferation of stromal cells from endometrioma. *Fertil Steril* 2011;**96**:118–121.

Yu J, Wang Y, Zhou WH, Wang L, He YY, Li DJ. Combination of estrogen and dioxin is involved in the pathogenesis of endometriosis by promoting chemokine secretion and invasion of endometrial stromal cells. *Hum Reprod* 2008;**23**:1614–1626.