CSF-1-Induced DC-SIGN⁺ Macrophages Work In The Ovarian Endometriosis

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CSF-1-induced DC-SIGN⁺ macrophages work in the ovarian endometriosis

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Abstract

Background

CSF-1 was found to be accumulated in the lesions and peritoneal fluid of endometriosis patients, and CSF-1 induced THP-1-derived macrophages to polarize toward a suppressive phenotype. Researchers found that macrophages were the predominant cells in the peritoneal fluid (PF) of endometriosis patients, and the primary consensus is that the immune status in the PF of endometriosis patients exhibits a depressed state. Does the cytokine CSF-1 induce monocytes to differentiate into macrophages with a DC-SIGN⁺ suppressive phenotype in endometriosis?

Methods

The level of CSF-1 in control endometrium (N=11), eutopic endometrium (N=17), and ectopic (N=39) endometrium of endometriosis patients was

evaluated by real-time polymerase chain reaction and in the PF of control (N=25) and endometriosis (N=35) patients by enzyme-linked immunosorbent assay. CSF-1 was examined by a MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel in an in vivo study. DC-SIGN⁺ suppressive macrophages were detected by immunohistochemical staining of tissues and flow cytometric analysis of the PF of control (N=25) and endometriosis (N=35) patients. The phenotypes and biological activities of the resulting macrophages derived from THP-1 cells induced by CSF-1 were compared by an in vitro coculture system with peripheral blood lymphocytes from normal subjects.

Results

In this study, we found the proportion of DC-SIGN⁺ suppressive macrophages was larger in the abdominal immune microenvironment of endometriosis patients. CSF-1 was primarily secreted from the ectopic lesions and peritoneum of mice with endometriosis. And, CSF-1 induced the polarization of macrophages toward a DC-SIGN⁺ suppressive phenotype; this effect was abolished by the addition of anti-CSF-1R. CSF-1 induced DC-SIGN⁺ macrophages, leading to a depressed status of peripheral blood lymphocytes, including a high percentage of Treg cells and a low percentage of CD8⁺ T cells. Similarly, blockade with anti-CSF-1R abrogated this biological effect. This is the first study on the predominant role of DC-SIGN⁺ suppressive macrophages in the depressed

immune status of endometriosis patients.

Conclusions

This is the first study on the predominant role of DC-SIGN⁺ suppressive macrophages in the depressed immune status of endometriosis patients. Further study of the mechanism and biological activities of CSF-1-induced DC-SIGN⁺ suppressive macrophages will enhance our understanding of the physiology of endometriosis and indicate new directions for further study.

Key words: Endometriosis/DC-SIGN⁺ suppressive macrophages/CSF-1/Treg cells

Background

Endometriosis (EMS) is a common but complicated disease that causes 6–10% of women of reproductive age to suffer chronic pelvic pain, dyspareunia and infertility [1]. Although the retrograde menstruation hypothesis was widely accepted [2], in recent years, some studies have suggested that retrograde menstruation is a precondition of EMS formation and that the abnormal metabolism of hormones is secondary; however, immune dysfunction is a cause of EMS [3].

A number of studies were conducted on the peritoneal immunity of EMS, and many were limited to a description of immune cell dysfunction [4-6]. Some experiments proved the depressed state of peritoneal immunity in EMS patients [7-9]. With the widespread study of the immune system in

the abdominal microenvironment, the profound role of macrophages in the pathological physiology of EMS is becoming clear [10]. Crucial functions of macrophages, such as stabilising tissue structure, driving injury, and regulating the immune microenvironment, were observed [11]. Our previous studies suggested that macrophages are involved in the progression of EMS [12]. In addition, accompanied by the high number of macrophages, the proportion of Treg and Th1 cells in the peritoneal fluid of mice with EMS was increased in a previous in vivo study [13]. It was obvious that the microenvironment of the peritoneal fluid (PF) of mice with EMS was suppressive.

Peritoneal immunity is complex and powerful, and it is surprising that the ectopic endometrium survives during the menstrual cycle. This might be related to the depressed state of immunity in the microenvironment of the PF. We inferred that in EMS, the depressed state of immunity provides tolerance to the ectopic endometrium, which allows it to be maintained. Colony stimulating factor-1 (CSF-1), also known as macrophage colony-stimulating factor (M-CSF), is a key cytokine that induces monocytes to differentiate into CD169⁺ suppressive macrophages [14]. CSF-1 and its receptor CSF-1R (M-CSFR or CD115) are diffusely expressed and have been frequently reported in the context of EMS [15, 16]. A study showed that the expression of CSF-1 was increased in the PF of EMS patients [17]. According to the literature, CD169⁺ suppressive macrophages are

found in various diseases and indirectly affect Treg cells and CD8⁺ T cells [18, 19]. In a study of transplantation tolerance, CD169⁺ suppressive macrophages interacted with lymphocytes to increase the proportion of FOXP3⁺ Treg cells, decrease the proportion of CD8⁺ T cells, and then maintain transplant survival [20]. Many studies have proven FOXP3⁺ Treg cells play an important role in immunoregulation and immunosuppression [21, 22]. Treg cells interact with many other immune cells and influence their functions [21]. FOXP3⁺ Treg cells secrete cytokines, such as CCL22, CCL17, transforming growth factor-1 (TGF-1), interleukin (IL)-1, tumour necrosis factor (TNF), IL-8 and vascular endothelial growth factor (VEGF), while working with other immune cells to promote the progression of EMS [23].

Szylo K, et al determined the small percentage of CD8⁺ T cells related to EMS [24]. A study showed that CD169⁺ suppressive macrophages had a profound effect on the cellular immunologic response [25]. Moreover, CD169⁺ suppressive macrophages induced the polarization of Treg cells and regulated the immune response [26]. Therefore, the critical point was to identify the common targets through which macrophages communicate with Treg cells and CD8⁺ T cells.

DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) of CD169⁺ suppressive macrophages is a key target of the regulation of depressed immunity. This molecule was first described by Geijtenbeek, TBH, et al.,

in 2000 about a study which dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells [27]. And, these authors explained the mechanism of DC-SIGN as an important molecule that participates in the process by which DCs interact with CD4⁺ T cells [28]. Soilleux E. J, et al, proved that some special macrophages expressed DC-SIGN, such as those in the placenta and decidua [29]. Marie Larsson et al., observed that DC-SIGN⁺ DCs regulated CD8⁺ T cells [30]. Recently, some studies have suggested that DC-SIGN is a type of regulatory receptor on DCs and macrophages that varies the function of macrophages [31]. Our present results suggest that CD169⁺ DC-SIGN⁺ macrophages exist in the PF, accompanied by high CSF-1 expression. Supporting the idea that CSF-1 might play a role in these processes by promoting the depressed state of the immune microenvironment by CD169⁺ DC-SIGN⁺ macrophages via CSF-1R. We found that DC-SIGN⁺ macrophages induced by CSF-1 led to a depressed phenotype of peripheral blood lymphocytes, including a high percentage of Treg cells and a low percentage of CD8⁺ T cells, and blockade with anti-CSF-1R abrogated this biological effect. Overall, combining our studies, we assumed that CSF-1-induced DC-SIGN⁺ macrophages dominate the suppressive microenvironment of endometriosis.

Methods

Tissue and sample collection

For immunohistochemistry staining, this study recruited 39 women with EMS (American Fertility Society classification of endometriosis Stage III n=30 and Stage IV n = 9) who were diagnosed by clinical pathologists at Shanghai First Maternity and Infant Hospital, affiliated with the Tongji University School of Medicine, from June 2019 to May 2020. Eutopic endometrial samples were obtained from EMS women (n=17). For the controls, endometrial tissue samples were collected through curettage from 11 women who exhibited fallopian tube blockage but who did not have any clinical indication or history of adenomyosis or endometriosis. PF was collected, avoiding contamination with blood, from women with advanced-stage EMS (n=35) and from controls (n=25) during laparoscopic surgery. All the enrolled patients in the control group were confirmed to have no endometriotic lesions by laparoscopy, and they had histological diagnoses of ovarian mature teratoma (n=20) and benign cyst of mesosalpinx (n=5). The clinical characteristics of the patients and controls are summarized in Table I.

Cell lines, cytokines and animals

The acute monocytic leukaemia (THP-1) cell line was a gift from the Cryomedicine Lab, Qilu Hospital of Shandong University. Recombinant human M-CSF was purchased from R&D Systems (Minnesota, USA). C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. at 4 weeks of age.

Real-time polymerase chain reaction

Total RNA was extracted from 0.2*0.2*0.1 cm tissues using TRIzol Reagent (Invitrogen), according to the product manual. RNA (0.5 µg) was reverse transcribed into cDNA with the ReverTra Ace quantitative PCR (qPCR) RT Master Mix with gDNA Remover (Code No. FSQ-301, TOYOBO, Osaka Japan). Each 20 µl PCR mixture contained 1× SYBR Green PCR Master Mix (TOYOBO, Osaka Japan), 30 ng cDNA and 300 nM each specific primer from Sangon Biotech (Shanghai) Co., Ltd. Quantitative PCR was performed on an Applied Biosystems StepOnePlus RT-PCR System (Applied Biosystems, USA). Three separate experiments were performed on different cultures, and each sample was assayed in triplicate. The mean was obtained to determine the mRNA levels by quantitative real-time polymerase chain reaction (RT-PCR) analysis using the ABI StepOnePlus RT-PCR system (Applied Biosystems). The primers used for CSF-1 were 5'-GCTGCTTCACCAAGGATTATG-3' (sense) and 5'-GGGTCACCTGCTAGGGATG-3' (antisense), and the gene expression levels of each group were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was assessed with the following primers: 5'-AGCCACATCGCTCAGACA-3' (sense) and 5'-GCCCAATACGACCAAATCC-3' (antisense). The mean relative gene expression was determined, and the differences were calculated using the $2^{-\Delta\Delta C_t}$ method.

The establishment of the EMS model

All the mice were maintained under specific pathogen-free (SPF) conditions. All the procedures were approved by the Animal Care and Use Committee of Tongji University (Shanghai, China). The oestrous stage was monitored daily by a vaginal smear every morning for 2 weeks, and mice with normal oestrous cycles were used in the following experiments. Endometriosis was established by intraperitoneal injection of endometrial segments. Briefly, the donor mice were sacrificed, and the uteri were isolated and collected in a Petri dish containing phosphate-buffered saline (PBS). The isolated uterine horns were treated identically, including a longitudinal split with a pair of scissors, isolation of endometrial tissue and subtle disruption in small, uniform fragments of approximately 1 mm. Then, these fragments were intraperitoneally injected into recipient mice with a 1 ml syringe with a 25 g needle. Equal endometrial fragments from one donor mouse were injected into two recipient mice. Overall, 15 endometrial segments in 200 µl sterile PBS were injected into each recipient mouse. However, the control groups only received injections of 200 µL sterile PBS. Finally, ten mice from the endometriosis or control groups were sacrificed 42 days after the model was established.

Analysing CSF-1 by MILLIPLEX MAP Mouse Cytokine/Chemokine

Magnetic Bead Panel

The MILLIPLEX® MAP kit for analysis was used to evaluate tissue (uterus, peritoneum, and ectopic lesions) cytokine production (CSF-1) and release in the PF of the EMS mice, following the manufacturer's instructions. A mixture of beads with different fluorescence intensities coated with antibodies for the indicated cytokines, was preincubated in the dark for one hour on an orbital shaker. Concentration standards of 3.2, 16, 80, 400, 2000 and 10,000 pg/mL were used to generate the concentration curve of each analyte. In a 96 well plate, 200 µL of wash buffer was added, the plate was shaken for 10 min, and the supernatant was discarded. Twenty-five microlitres of the standards, 25 µL of running buffer, 25 µL of the stock solution, 25 µL of the supernatant from cells in the appropriate wells, and 25 µL of the bead mixture were added. The plate was incubated overnight with shaking at 4°C. The supernatant was then removed, and the samples were washed twice with 200 µL of wash buffer. Next, 25 µL of antibody was added for detection, and the samples were incubated for 1 h in the dark with shaking. Then, 25 µL of the streptavidin phycoerythrin binding protein was added and incubated for 30 min with shaking in the dark. The supernatant was discarded, and the wells were washed twice with 200 µL of wash buffer. Then, 150 µl of the sheath liquid was added, and the plate was analysed on a Luminex200 system. The sample results were analysed using xPONENTLX200 software (Madison, WI, USA).

Immunohistochemical staining

For each sample, 4 µm thick sections were dewaxed and rehydrated in ethanol and water. Antigen retrieval was performed in citrate buffer (pH 6.0, 15 min), and endogenous peroxidase activity was eliminated by incubation in 3% hydrogen peroxide. The tissue sections were incubated overnight at 4°C with rabbit primary antibodies against human CD169 (ab183356, Abcam, Cambridge, UK, 1:100), CSF-1R (ab183316, Abcam, Cambridge, UK, 1:100), and DC-SIGN (ab5715, Abcam, Cambridge, UK, 1:50), mouse monoclonal antibodies against human CD68 (ab955, Abcam, Cambridge, UK, 1:200) and FOXP3 (ab20034, Abcam, Cambridge, UK, 1:50). The secondary antibody kit (CWBIO, Beijing, China) was utilized to label the primary antibodies. The sections were counterstained with haematoxylin, dehydrated in ethanol and xylene, and mounted in PermountTM mounting medium. The images were obtained using a microscope (Olympus BX53, Olympus, Tokyo, Japan), which was fitted with a digital camera (Olympus cellSens Standard, Olympus). A series of five images was randomly selected from several sections per tissue sample. Positive cells in images taken at 400X magnification were counted manually by two independent investigators blinded to the treatment groups. The cells were counted in at least five different areas in ectopic lesions and plotted as percent positive cells relative to the total number of cells as previously described [32].

The macrophage induction and culture

THP-1 cells were induced to differentiate into macrophages in accordance with a previously reported method [33]. The cells cultured in 24-well plates were stimulated by adding 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma, USA) for 48 h and washed thrice with PBS to eliminate the effect of PMA.

Cell Immunofluorescence

We treated the differentiated macrophages with CSF-1 (50 ng/ml) and cultured them for 48 h. For confirmation, we cultured the differentiated macrophages with RPMI 1640 medium (Gibco, China) containing anti-CSF-1R (Abcam, UK, 1:20) for 2 h and then added CSF-1 (R&D Systems, USA, 50 ng/ml) to the medium for 48 h. Cell immunofluorescence staining was performed as previously described [34]. The target macrophages were washed twice with PBS, fixed in 4% paraformaldehyde (pH 7.0) for 30 min, permeabilized with 0.1% Triton X-100 (Sigma) for 10 min, blocked with 10% normal goat serum for 1 h, and incubated overnight with rabbit anti-human CD169 (ab183356, Abcam, UK, 1:100) or DC-SIGN (ab5715, Abcam, UK, 1:50) and mouse monoclonal antibodies against human CD68 (ab955, Abcam, UK, 1:200) at 4°C. The cells were then washed with PBS three times and incubated at room temperature with Dylight 488-conjugated donkey anti-rabbit secondary antibody (1:400, Abcam, UK) or Dylight 594-conjugated

donkey anti-goat secondary antibody (1:400, Abcam, UK) for 1 h. After the plates were washed, the cell nuclei were counterstained with fluorescent mounting medium with 4',6-diamidino-2-phenylindole (Abcam, UK).

Co-culturing peripheral blood mononuclear cells (PBMCs) with THP-1-derived macrophages

PBMCs were prepared as previously described [35]. Volunteers were recruited from healthy women without dysmenorrhea (n=7) with regular menstrual cycles. PBMCs were isolated from 15 ml of venous blood using Ficoll-Cardiografin (Tian Jin Hao Yang Biological Manufacture, China). After centrifugation, PBMCs were collected from the interphase layer and washed four times with PBS. The PBMCs were suspended in RPMI 1640 supplemented with FBS at a concentration of 1×10^6 cells/ml for coculture. The coculture system simulated the site of the lesion in the human body. Transwell membranes (Corning, USA) allow the exchange of secretions from macrophages with PBMCs. First, Transwell membranes were inserted into a 24-well plate, and the upper chamber was pre-seeded with macrophages derived from THP-1 cells. Moreover, 100,000 PBMCs cells were counted and seeded on the membrane. Second, two groups of macrophages derived from THP-1 cells were treated with rabbit anti-human DC-SIGN (Abcam, UK, 1:50) or CSF-1R (Abcam, UK, 1:20) and treated with CSF-1 (50 ng/ml) for 48 h. Third, we

co-cultured the differentiated macrophages and PBMCs, as shown in Fig. 6A. Then, the Transwell plates were placed in a 5% CO₂ incubator at 37°C for 2 days. After incubation, the cocultured PBMCs were collected.

Flow cytometry

The remaining peritoneal cells collected from the controls and the patients were equally divided into two parts for flow cytometry (FCM) analysis. To determine the percentages of the depressed macrophage subsets, cells in the PF at a density of 0.5×10^5 /tube were used and stained in duplicate in the dark at 4 °C for 30 min with the following antibodies: APC-anti-CD209, FITC-anti-CD45, PerCP-Cy5.5-anti-CSF-1R, and PE-anti-CD169 (Biolegend, USA); fluorescence- and isotype-matched antibodies served as the negative controls. The proportions of Treg cells and CD8⁺ T cells in the PBMCs collected from the coculture system were analysed using FCM. PBMCs at a density of 0.5×10^5 /tube were fixed, permeabilized, and stained in duplicate with the following antibodies: APC-anti-FoxP3, FITC-anti-CD45, PerCP-Cy5.5-anti-CD8, and PE-anti-CD4 (Biolegend, USA). The proportions of distinct subpopulations were eventually characterized on a BD FACSCalibur (BD, USA). The resulting data were examined using FlowJo software.

Enzyme-linked immunosorbent assay (ELISA)

First, an aliquot of each preparation was kept for protein estimation using the Bradford protein concentration assay kit (P0006C) from Beyotime.

The CSF-1 levels in the PF of EMS patients and controls were quantified using each ELISA kit (R&D Systems), according to the manufacturer's instructions. The absorption of the streptavidin-horseradish peroxidase colour reaction was measured by a microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm. The optical density was compared with serial dilutions of recombinant human CSF-1 as a standard. The relevant concentration was calculated, and standard curves were obtained.

Statistical analysis

All the statistical analyses were completed using Prism software, version 5 (Graph Pad, San Diego, CA, USA), and the measurement data were represented by the mean $\pm$ SD. Statistical significance was assessed using Student's t test for the univariate analysis of two sets of data and one-way or two-way analysis of variance (ANOVA) for multiple comparisons. $P < 0.05$ was significant.

Results

The proportion of DC-SIGN⁺ suppressive macrophages was larger in endometriosis patients.

To determine the immune macrophages affecting endometriotic lesion progression, we collected the peritoneal cells from the PF of patients for flow cytometry and performed a series of analyses (Fig. 1). Macrophages express higher levels of the CD45 antigen that is exclusively expressed

on all nucleated cells of the haematopoietic system, and the different frequencies of the monocyte/macrophage populations in the peritoneal cavity could be further confirmed in the morphology cytograms of the FSC (forward scatter) and SSC (side scatter) parameters obtained by flow cytometry [36] (Fig. 1A-B). In this gate, peritoneal CD169⁺ macrophages were gated as live CD45⁺ CD169⁺ cells (Fig. 1C-D), peritoneal CD115⁺ macrophages were gated as live CD45⁺ CD115⁺ cells (Fig. 1E-F), and peritoneal CD209⁺ macrophages were gated as live CD45⁺ CD209⁺ cells (Fig. 1G-H). Through data analysis, we clearly found that suppressive macrophages in the PF of endometriosis patients express CD169, CD115, and CD209 at high levels (Fig. 1 I). We also analysed the co-expression of DC-SIGN and CD169 by macrophages (Fig. 2C-D) and the co-expression of CSF-1R and CD169 by macrophages (Fig. 2E-F). From the data analysis, we found that in the PF of endometriosis patients, the co-expression of DC-SIGN and CD169 at macrophages was greater than that in the control group, and the co-expression of CSF-1R and CD169 at macrophages in the PF of endometriosis patients was greater than that in the control group (Fig. 2G).

We also conducted immunohistochemical staining and performed a series of analyses of the immune cells detected in all the tissues (Fig. 3A). In ovarian endometriotic lesions, we observed significantly more CD68⁺, CSF-1R⁺, and DC-SIGN⁺ macrophages than in the normal endometrium

and eutopic endometrium of the endometriosis patients. Additionally, in ovarian endometriotic lesions, we observed significantly higher amounts of FOXP3⁺ T cells than in the normal endometrium and the eutopic endometrium of the endometriosis patients. In contrast, concerning CD169⁺ macrophages, there was no significant difference in the ovarian endometriotic lesions, the normal endometrium and the eutopic endometrium of the endometriosis patients (Fig. 3B).

CSF-1 levels are elevated in peritoneal fluid and ectopic endometrium from women with endometriosis.

We observed a relative increase in CSF-1 gene expression in ectopic endometrial tissue compared with paired eutopic endometrium and normal endometrium (Fig. 4A). Our data demonstrated that peritoneal fluid from women with endometriosis had a significantly higher percentage of CSF-1 than that from control subjects (Fig. 4B). From the EMS model, we found that cytokine production and CSF-1 levels were elevated in the ectopic lesions and peritoneum of the mice with endometriosis, but there was no difference between the peritoneal fluid, peritoneal cells, and endometrium of the normal mice and mice with endometriosis (Fig. 4C-D). Although there were high levels of CSF-1 in the peritoneum of normal mice and mice with endometriosis, the level of CSF-1 in the peritoneum of mice with endometriosis was higher than that in normal mice; however, there was no statistically significant difference

(Fig. 4D). This finding suggests that the ectopic endometrium and peritoneum of endometriosis may be the main sources of CSF-1 in the peritoneal environment.

CSF-1 induced macrophages to a DC-SIGN⁺ suppressive phenotype, and it could be abolished by the addition of anti-CSF-1R.

The successful induction of macrophages from THP-1 cells was monitored by means of characteristic CD68-positive cells (Fig. 5). A significant increase in CD169⁺ macrophages was observed in the CSF-1-treated group, while in this model by immunofluorescence, but after the CSF-1 antagonist anti-CSF-1R was added, the expression of CD169 was minor (Fig. 5A). Similarly, CSF-1-treated macrophages demonstrated a significant upregulation of DC-SIGN, but after the anti-CSF-1R antibody was added, the expression of DC-SIGN was decreased (Fig. 5B).

CSF-1 induced DC-SIGN⁺ macrophages, leading to a suppressive phenotype of peripheral blood lymphocytes, while blockade with anti-CSF-1R abrogated this biological effect.

On the basis of the successful induction of macrophage differentiation from THP-1 cells, we conducted a coculture system with macrophages and peripheral blood lymphocytes (Fig. 6A). Lymphocytes express higher levels of the CD45 antigen that is exclusively expressed on all nucleated cells of the haematopoietic system, and the different frequencies of the monocyte/macrophage populations in the PBMC coculture system could

be further confirmed in the morphology cytograms of FSC (forward scatter) and SSC (side scatter) parameters obtained by flow cytometry (Fig. 6B). The number of CD8-positive T cells among the PBMCs of the CO2 group (CSF-1 treated only) was significantly lower than that of the NC group (no co-culture) and CO3 group (CSF-1 and anti-CSF-1R treated), while the number of Treg cells among the PBMCs of the CO2 group (CSF-1 treated only) was significantly higher than that of the NC group (no co-culture) and CO3 group (CSF-1 and anti-CSF-1R treated) (Fig. 6B). Interestingly, there were no differences in the numbers of CD8-positive T cells and Treg cells among the PBMCs of the CO4 group (CSF-1 and anti-DC-SIGN treated), CO2 group (CSF-1 treated only), NC group (no coculture) and CO3 group (CSF-1 and anti-CSF-1R treated) (Fig. 6B). Similarly, there were no differences in the numbers of CD8-positive T cells and Treg cells among the PBMCs of the CO1 group (coculture only), CO2 group (CSF-1 treated only), NC group (no coculture) and CO3 group (CSF-1 and anti-CSF-1R treated) (Fig. 6B). Through data analysis, we clearly found that the DC-SIGN⁺ suppressive macrophages induced by CSF-1 led to a depressed phenotype of peripheral blood lymphocytes, while the CSF-1 antagonist anti-CSF-1R abrogated this biological effect (Fig. 6C).

Discussion

We demonstrate DC-SIGN⁺ macrophages are required for the induction of a suppressive phenotype of peripheral blood lymphocytes. DC-SIGN is expressed in human DCs and macrophages [28, 37]. Importantly, DC-SIGN is involved in multiple aspects of the immunological response [38], but the role of DC-SIGN⁺ macrophages in the pathogenesis of endometriosis has not been studied. And, we found that the proportion of DC-SIGN⁺ macrophages was larger in the PF of endometriosis patients. Moreover, the proportion of CD169⁺ macrophages was larger in the PF of endometriosis patients. This phenomenon was not in agreement with observations from tissues. Although DC-SIGN⁺ macrophages were more abundant in the ectopic endometrium of the endometriosis group than in the ectopic endometrium of the endometriosis and normal endometrium groups, CD169⁺ macrophages were not different among the three groups. These differences may be due to the phenotypic differentiation diversity of macrophages in different tissues and regions.

As previously described, CD169⁺ macrophages, which were known as CD169⁺ suppressive macrophages [18], play a role in various diseases. Previously, Yvette van Kooyk's laboratory proposed that DC-SIGN could actively contribute to the maintenance of an immunosuppressive tissue environment [39]. In the in vivo study [13], the microenvironment of the PF of mice with EMS was suppressed. In our study, the percentage of FOXP3⁺ T cells was larger in the ectopic tissue of the endometriosis group

than in that of the other two groups. This proved that the ectopic tissue environment of endometriosis was immunosuppressive.

In the PF of endometriosis patients, we found that there were more CSF-1R⁺ macrophages than in that of the controls; the macrophages co-expressing CSF-1R and CD169 in the PF of endometriosis patients were more abundant than in the controls; and in the CD169⁺ macrophage subset, the expression of CSF-1R was significantly higher than that in the CD169⁻ macrophage subset. A previous study proved that CSF-1R inhibition altered macrophage polarization and blocked glioma progression [40]. CSF-1R and its ligand, CSF-1, were expressed diffusely and have been frequently reported in patients with EMS [15, 16]. Therefore, CSF-1R must be the key target for the differentiation of CD169⁺ DC-SIGN⁺ macrophages. However, in the tissue of ectopic lesions, CSF-1R expression did not differ from that of the normal endometrium and the eutopic endometrium of endometriosis patients. This result is not consistent with a previous study in which CSF-1R was found in nearly 70% of endometriosis patients compared with a statistically significantly lower percentage in normal endometrium [41]. This difference needs to be defined in more studies.

Although the expression of CSF-1R in the ectopic lesions of endometriosis patients was not different from that in the eutopic endometrium of endometriosis patients and normal endometrium, the gene expression level of CSF-1 was significantly higher. The level of CSF-1 cytokine in the PF

of endometriosis patients was higher than that in the controls. The source of the cytokine CSF-1 was not clear. Our in vivo experiment showed that the peritoneum of all mice had high levels of the cytokine CSF-1, while the level of the cytokine CSF-1 was significantly higher in the ectopic tissue of mice with endometriosis than in the eutopic endometrium of the mice with endometriosis and the normal endometrium of the control mice. These results proved that the sources of CSF-1 were peritoneal tissues and ectopic lesions of mice with endometriosis, and the ectopic lesion was the main initiator in endometriosis. This result was in agreement with a previous study that proved that ectopic endometriotic tissue exhibited a 3.5-fold increase in CSF-1 compared with eutopic tissue [15].

CSF-1 is a key cytokine that induces monocytes to differentiated into CD169⁺ suppressive macrophages [14], our study was consistent with this finding; and we found that the CSF-1 antagonist anti-CSF-1R could abolish this effect. Additionally, we found that CSF-1 could induce monocytes to differentiate into DC-SIGN⁺ suppressive macrophages; similarly, anti-CSF-1R could abolish this effect. Therefore, CSF-1 was also a key cytokine that induces monocytes to differentiate into suppressive macrophages.

We proved that there were many macrophages in the PF of mice with endometriosis, which were accompanied by an increased proportion of Treg and Th1 cells in the peritoneal fluid of mice with EMS observed in a

previous in vivo study [13]. In this study, we found that the percentage of FOXP3⁺ T cells was larger in the ectopic tissue of the endometriosis group than in the other two groups. This proved that the environment of endometriosis was immunosuppressive. CD169⁺ DC-SIGN⁺ suppressive macrophages may play a role in this immunosuppressive environment of endometriosis. The in vitro coculture system experiments proved that CSF-1 induced macrophages to express CD169 and DC-SIGN leading to a suppressive phenotype of peripheral blood lymphocytes, while blockade with anti-CSF-1R abrogated this biological effect. However, the important role and main mechanism of this phenomenon require further study.

Conclusions

Overall, our data suggest that CSF-1 is involved in the initiation of endometriosis. Interestingly, CSF-1 and its receptor, CSF-1R, are important for the induction of CD169⁺ DC-SIGN⁺ suppressive macrophages. We also demonstrate here that CSF-1-induced DC-SIGN⁺ macrophages inhibit the increase in CD8⁺ T cells and the decrease in Treg cells, and they are required for the induction of the immunosuppressive environment of endometriosis in vitro. We are studying the detailed mechanism by which DC-SIGN⁺ suppressive macrophages affect the proliferation and differentiation of T cells. This study provides a framework for developing CSF-1-based in vitro

protocols to induce therapeutic macrophages for clinical use
in order to prevent the immunosuppressive environment of endometriosis.

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Author contribution statement

An Min: design of the project, data analysis, interpretation of data,
writing of the manuscript, critical revision and final approval of the
manuscript. Hong Wei: sample collection. Zheng zhenzhen: sample
collection. Cai Yunlang: data acquisition, critical revision and final
approval of the manuscript. Li Xiaocui: design of the project,
interpretation of data, critical revision and final approval of the
manuscript.

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Availability of data and materials

The datasets used and analyzed during the present study are available
from the corresponding author on reasonable request.

Conflict of Interest

All the authors report no conflict of interest.

Ethical approval

The regional ethics committee of Shanghai First Maternity and Infant Hospital approved the study (KS1953). Written informed consent was obtained from all the enrolled participants. The animal ethics committee of School of medicine, Tongji University allow this study (TJLAC-019-125).

Consent for publication

Written informed consent for publication was obtained from all participants.

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Figure legend

Figure 1

Suppressive macrophages express high levels of CD169, CD115, and CD209 in the PF of endometriosis patients. (A) Morphological cytograms of FSC (forward scatter) and SSC (side scatter) parameters of peritoneal cells in the PF. (B) The monocyte/macrophage population gated by CD45 and SSC. (C) Gating CD169⁺ macrophages in the PF of the controls. (D) Gating CD169⁺ macrophages in the PF of the endometriosis patients. (E) Gating CD115⁺ macrophages in the PF of the controls. (F) Gating CD115⁺ macrophages in the PF of the endometriosis patients. (G) Gating CD209⁺ macrophages in the PF of the controls. (H) Gating CD209⁺ macrophages in the PF of the endometriosis patients. (I) Differences between groups were analysed by Student's t test (controls n=25; endometriosis patients n=35). The values are expressed as the mean $\pm$ SD. **P $\leq$ 0.01, ***P $\leq$ 0.001.

Figure 2

There were more CD169⁺ CD115⁺ macrophages and CD169⁺ CD209⁺ macrophages in the PF of endometriosis patients than in the control. (A) Morphological cytograms of FSC (forward scatter) and SSC (side scatter) parameters of peritoneal cells in PF. (B) The monocyte/macrophage

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Figure 3

Macrophages and Treg cells in the ectopic and eutopic endometrium of endometriosis patients and normal endometrium. (A) Macrophages expressed CD68, CD169, CSF-1R, and DC-SIGN, and Treg cells expressed FOXP3 in all the tissues of the normal endometrium, eutopic endometrium, and ectopic endometrium of endometriosis patients. Representative images are shown at 200 $\times$ magnification. (B) The comparison between the proportion of macrophages and Treg cells in tissues of the normal endometrium, eutopic endometrium, and ectopic endometrium of the endometriosis patients. The data are presented as the mean $\pm$ SD. Statistical analyses were performed by one-way analysis of variance (eutopic=17; ectopic=17; normal=11), and significant differences are indicated as *P $\leq$ 0.05, ***P $\leq$ 0.001.

Figure 4

CSF-1 levels are elevated in peritoneal fluid and ectopic endometrium from women with endometriosis and ectopic lesions and peritoneum of mice with endometriosis. (A) The gene expression of CSF-1 in the normal endometrium, eutopic endometrium and ectopic endometrium of endometriosis patients detected by RT-PCR. (B) The percentage of the cytokine CSF-1 in the total protein detected by ELISA in the PF of the control and endometriosis groups. (C) The model of EMS and ectopic lesions of the EMS model. (D) The cytokine CSF-1 in the PF, the peritoneal cells of PF, the peritoneum, the normal endometrium, the eutopic endometrium, and the ectopic endometrium of the control and the EMS models was analysed by the MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel. The data are presented as the mean $\pm$ SD. Statistical analyses were performed by Student's t test (control, n=25, endometriosis patients, n=35; normal mouse, n=9; EMS mouse, n=9) and one-way analysis of variance (eutopic=17; ectopic=17; normal=11). Significant differences are indicated as *P $\leq$ 0.05, **P $\leq$ 0.01.

Figure 5

CSF-1 induces macrophages to differentiated into a DC-SIGN⁺ suppressive phenotype, and this effect can be abolished by the addition of anti-CSF-1R. (A) CD169 expression in the original macrophages, macrophages induced by CSF-1 (MCSF) and macrophages induced by CSF-1 (MCSF) after blockade with anti-CSF-1R. (B) DC-SIGN

expression in the original macrophages, macrophages induced by CSF-1 (MCSF) and macrophages induced by CSF-1 (MCSF) after blockade with anti-CSF-1R. Representative images are shown at 200× magnification (n=5).

Figure 6

CSF-1-induced DC-SIGN⁺ macrophages, leading to a suppressive phenotype of peripheral blood lymphocytes, while anti-CSF-1R could abrogate this effect. (A) The coculture system of treated macrophages and PBMCs. (B) The percentages of CD8⁺ T cells and Treg cells in the PBMCs of the coculture system. (C) The experiment was repeated nine times, and the differences between the groups were analysed by two-way analysis of variance. The values are expressed as the mean±SD. *P≤0.05. NC: PBMC without coculture; CO1: PBMCs were cocultured with untreated macrophages; CO2: PBMCs were cocultured with CSF-1-induced macrophages; CO3: PBMCs were cocultured with CSF-1-induced macrophages, which were first treated with anti-CSF-1R; and CO4: PBMCs were cocultured with CSF-1-induced macrophages, which were first treated with anti-DC-SIGN.

Table I Demographic characteristics of patients with endometriosis and controls.

Item	Controls (n=25)	Endometriosis (n=35)	Statistical significance
Age (year)	Mean=31.9 (SD=7.7) Median=32 Range=15-41	Mean=35.4 (SD=6.3) Median=37 Range=25-48	NS
Menstrual phase			
Proliferative	12(48.0%)	20 (54.5%)	NS
Secretory	13(52.0%)	15 (45.5%)	
Gravidity			
0	9(36.0%)	14 (40.0%)	NS
1	6(24.0%)	5 (14.3%)	
≥2	9(36.0%)	16 (45.7%)	
Abortion			
0	16(64.0%)	21 (60.0%)	NS
1	3(12.0%)	10 (28.6%)	
≥2	6(24.0%)	4(11.4%)	
Visual analog scale on the severity of dysmenorrhea			
	Mean=2.5(SD=1.1)	Mean=2.9 (SD=1.3)	NS
Co-occurrence of adenomyosis			
No	25(100.0%)	32 (91.4%)	NS
Yes	0(0.0%)	3 (8.6%)	
Co-occurrence of endometrial polyps			
No	22(88.0%)	35 (100.0%)	NS
Yes	3(12.0%)	0 (0.0%)	

None of the patients had received steroid hormone therapy within the past 3 months, and none exhibited any obvious internal medicine or surgical comorbidities. Women with endometrial abnormalities, pelvic endometriosis, fibroids, ovarian cysts, or lesions were excluded. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS: $P > 0.05$. Wilcoxon's rank test was used for age and visual analog scale while for other data Fisher's exact test was used.

Figures

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