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Eutopic endometrium from patients with endometriosis modulates the expression of CD36 and SIRP-α in peritoneal macrophages

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Abstract

Aim: This study aimed to investigate the *in vitro* alterations of the expression of signal regulatory protein-α (SIRP-α) and CD36 in macrophages in the endometriosis condition.

Methods: The expression of SIRP-α and CD36 was measured in peritoneal macrophages and peripheral blood mononuclear cells of endometriosis patients and control participants. The expressions of SIRP-α and CD36 were measured in human acute monocytic leukemia (THP-1) cell-derived macrophages that were treated with interleukin-6 (IL-6)-induced conditioned medium, eutopic versus normal endometrial homogenate, or lipopolysaccharide in the presence or absence of nuclear factor kappa-B (NF-κB) or transforming growth factor (TGF-β) inhibitors, respectively.

Results: Peritoneal macrophages that were isolated from women with endometriosis exhibited an enhanced expression of SIRP-α and a decreased expression of CD36 compared to control participants. Women with endometriosis had significantly higher levels of SIRP-α and CD36 in peripheral circulating mononuclear cells than in control participants. SIRP-α expression was significantly increased, whereas the CD36 expression was decreased in THP-1 cell-derived macrophages after treatment with eutopic endometrial homogenate. Intervention with IL-6-induced conditioned medium resulted in the downregulation of SIRP-α but the upregulation of CD36 in THP-1 cells. Incubation with the NF-κBp50 inhibitor decreased the expression of CD36 and SIRP-α in macrophages that were treated with normal endometrial homogenate, whereas the TGF-β inhibitor enhanced the CD36 expression of THP-1 cell-derived macrophages treated with eutopic endometrial homogenate.

Conclusion: The eutopic endometrium could reduce the phagocytic ability of peritoneal macrophages in women with endometriosis through the modulation of SIRP-α and CD36 expression. Inhibition of the TGF-β signal pathway may be a potential therapeutic target for the treatment of endometriosis.

Key words: CD36, endometriosis, macrophage, SIRP-α.

Introduction

Endometriosis (EM) is a chronic benign gynecological disease characterized by the presence of ectopic endometrial tissue outside of the uterus. It is considered to be an inflammatory, estrogen-dependent disorder and causes pelvic pain and infertility. EM affects an estimated 10–15% of reproductive-aged women, and approximately 20–40% of infertile women are found to have EM.[1,2] The etiology of EM is complex and multifactorial. The most widely accepted theory of EM is the retrograde menstruation theory.[3] However,

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this theory has been repudiated because retrograde menstruation occurs in approximately 90% of women while only a small subset of those women (10–15%) develop EM.[4] Thus, scholars have hypothesized that immunologic alterations may be a critical aspect involved in the pathogenesis of EM, which causes the inadequate removal of retrograde endometrial debris and an enhanced ability of endometrial lesions to implant on the peritoneal surface.[5,6] However, the exact mechanism is still unknown.

Macrophages are the predominant resident immune cells in the peritoneal cavity, functioning to eliminate aberrant cells or tissues. Macrophages are abundant in endometriotic lesions, where they are recruited and undergo alternative activation.[7,8] In women with EM, there is a dramatic increase in the number of peritoneal macrophages, accompanied by excessive production of cytokines, such as interleukin (IL), tumor necrosis factor (TNF)-α, prostaglandin (PGE2), and vascular endothelial growth factor.[9,10] These molecules have been subsequently found to facilitate the adhesion, angiogenesis and implantation of endometrial fragments, to reduce the phagocytic capacity of peritoneal macrophages and to increase the production of IL-6.[7,11] Macrophages with compromised function, especially reduced phagocytic capacity, further contribute to the impaired clearance of endometrial lesions and the occurrence of EM.

The signal regulatory protein-α (SIRP-α) is an immunoglobulin superfamily transmembrane glycoprotein, also known as Src homology regin 2 domin-containing protein tyrosine phosphatase (SHP) substrate-1, macrophage fusion receptor and p84 neural adhesion molecule. As a receptor, SIRP-α has an extracellular region containing three Ig-like domains, which bind to the broadly expressed CD47 on target cells, and a cytoplasmic region, comprising immunoreceptor tyrosine-based inhibitory motifs which allow the binding of SHP1 and SHP2. SIRP-α is selectively expressed in myeloid cells (e.g. macrophages and dendritic cells) and neuronal cells.[12] Recent studies have reported that SIRP-α, interacting with its ligand CD47, regulates neuronal networks, tumor development and immune homeostasis.[13–15] Through binding to CD47, SIRP-α initiates a cascade of events that lead to the inhibition of phagocytosis by macrophages, transmitting a '"don't-eat-me' signal. Thus, upregulation of SIRP-α/CD47 signaling may permit aberrant cells to escape from immunologic surveillance.

CD36 is an 88-kD membrane glycoprotein expressed on a variety of *cell* types, including macrophages, platelets and certain microvascular endothelium. It serves as a class B *scavenger receptor* that is involved in multiple physiological and pathologic processes, including the clearance of oxidized low-density lipoprotein and apoptotic neutrophils.[16,17] CD36 is required for early cardiac repair through phagocytosis of dying cardiomyocytes after myocardial infarction.[18] A previous study found that degradation of CD36 sabotages the phagocytic capacity of macrophages and prolongs neutrophil inflammation, which delays cardiac healing post-myocardial infarction.[19] Additionally, CD36 has been found to promote the degradation and clearance of ectopic cellular debris during EM.[20]

Despite an increased absolute number of peritoneal macrophages, they exhibit dysfunctional phagocytosis function, which is considered to be an essential contributor to the development of EM. We speculate that, in EM, macrophages show abnormal receptor expression, including the expression of SIRP-α and CD36, resulting in poor phagocytic capacity and reduced uptake of endometrial fragments that are shed during menstruation. However, the expression of SIRP-α/CD36 on macrophages during the development of EM is not yet known. In this study, we examined the expressions of SIRP-α and CD36 in peritoneal macrophages of patients with EM. We also investigated alterations in the expression of these two receptors in macrophages when patients were subjected to endometrial homogenates or serum from EM patients or control participants, as well as exploring the underlying signal pathways.

Methods and Materials

Participants

The experiment was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University. All patients signed informed consent forms prior to participation. The endometrial tissue samples were collected during laparoscopic surgery from 22 women who were admitted during the period between September 2015 and February 2016. Among these women, 15 women, aged 35.3 ± 2.6 years (ranging between 26 and 45 years), were histologically diagnosed with EM. In accordance with the American Fertility Society classification, 3 patients were in stages I and II and 12 patients were in stages III and IV. Another seven women, aged 31.3 ± 3.5 years (ranging between 18 and 43 years), who

underwent laparoscopic surgery for incision of uterine septum during the same period, were enrolled as control participants. The absence of EM was confirmed laproscopically. All of the enrolled women had a regular menstrual cycle, with no medical history of immune deficiency, malignancy or other major diseases or complications. None of the patients received hormone therapy treatment in the 3-month period before laparoscopic surgery.

Collection of peripheral blood, endometrial tissue and peritoneal fluid

Before laparoscopic surgery, 5 mL of peripheral blood sample was collected from each patient and placed in tubes with procoagulants.

The blood samples were stored at 4°C for further use. After anesthesia, the abdominal cavity was washed with 50 mL of normal saline. Peritoneal washings were then collected into a sterile centrifuge tube, and stored at 4°C.

During laparoscopic surgery, eutopic endometrium was obtained from women with EM and normal endometrium was obtained from control subjects. This tissue was then persevered in refrigerator at −70°C.

Mononuclear cell isolation in peripheral blood

The mononuclear cells were isolated as has been previously reported.[21] Briefly, the collected blood samples were centrifuged at 1200 rpm for 10 min at room temperature. The upper layer plasma was removed, and the samples were diluted with the same volume of phosphate-buffered saline (PBS). The mononuclear cells were isolated by Ficoll density gradient centrifugation at 1800 rpm for 25 min. The buffy coat layer was then harvested and washed three times with PBS. The final pellet was subjected to osmotic lysis to eliminate red cell contamination.

Macrophages isolation from peritoneal fluid

The peritoneal washings that were collected were centrifuged at 2000 rpm for 8 min at 4°C. Mononuclear cells in the peritoneal fluid were isolated by Ficoll density gradient centrifugation. The mononuclear cells were re-suspended and seeded with Roswell Park Memorial Institute (RPMI)-1640 medium in 6-well plates at a density of 1 × 106 cells/mL for 2 hours. The non-adherent cells were removed, and the adherent cells were collected as peritoneal fluid macrophages.

Preparation of endometrial homogenates

Endometrial tissue homogenates were prepared according to a previous study.[22] Each sample (totaling 100 mg), prepared by pooled frozen endometrial tissues from 1 to 3 women, was thoroughly washed with PBS, and homogenized in a liquid nitrogen-cooled homogenizer in 5 mL serum-free RPMI-1640 medium. The sample mixture was centrifuged at 1200 rpm for 10 min. Cell-free supernatant was collected and stored frozen at −70°C.

Preparation of conditioned media

Endometrial stromal cells (ESC) were a gift from Dr. Huang from the University of South Florida, United States. The ESC were cultured under a condition mimicking a normal menstrual cycle with physiological hormone levels mimicking the proliferative and secretory phase. The cells were seeded in 10-cm culture dishes at a density of 2×10^5/ml, and cultured in Dulbecco modified Eagle medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 10^{-8} mol/L estrogen (E_2) or 10^{-8} mol/L E_2 + 10^{-7} mol/L progestin (P) at final concentration. The medium was changed every 2 days, maintaining the concentrations of E_2 or E_2 + P. The cells were cultured for 7 days, following which the medium was removed and replaced with 10 mL of serum-free medium, with the addition of 10^{-8} mol/L E_2, 10^{-8} mol/L E_2 + 20 ng/mL IL-6, 10^{-8} mol/L E_2 + 10^{-7} mol/L P, or 10^{-8} mol/L E_2 + 10^{-7} mol/L P + 20 ng/mL IL-6 for 24 h. The supernatant was then collected as conditioned medium. For cells treated with IL-6, 2-μg IL-6 neutralizing antibody (Santa Cruz Biotechnology) and protein G (Sigma) were sequentially added to the supernatant to neutralize the remaining IL-6 and to precipitate the IL-6/antibody complex, followed by centrifugation at 8000 rpm for 10 min, to collect supernatant.

Cell culture and differentiation

Human acute monocytic leukemia (THP-1) cells were a gift from Dr. Xiang from the Cancer Research Institute of Central South University. THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% glutamine, 1% penicillin and 1% streptomycin at 37°C and 5% CO_2. The medium was changed every 2 days. When a high cell density was reached, cells were harvested in a tube and centrifuged at 1000 rpm for 5 min. The pellet was resuspended in RPMI-1640 medium with 10% FBS and seeded in

6-well plates at a density of 1×10^6 cells/mL. The cells were stimulated with 25-ng/mL phorbol 12-myristate 13-acetate (Sigma) for 3 days to differentiate. After the removal of nonadherent cells, the adherent cells were M0 macrophages.

Cell treatment protocol

THP-1 cell-derived macrophages were treated with or without 100 μL/mL of eutopic endometrial homogenate from EM patients, or endometrial homogenate from normal subjects for 72 h. The cells were then stimulated with 100 ng/mL lipopolysaccharide (LPS; Sigma) for 24 h, after which the cells were harvested.

THP-1 cell-derived macrophages were cultured in DMEM medium supplemented with conditioned medium, at a ratio of 2:1, for 5 days. The cells were then collected for analysis.

THP-1 cell-derived macrophages were subjected to 100-ng/mL LPS for 24 h. After resuspension, the cells were treated with 50-μg/mL NF-κBp50 inhibitor SN50 or 10 μM TGF-β inhibitor SB43154 for 1 h, followed by co-culture with endometrial homogenate at a final concentration of 100 μL/mL for an additional 72 h. The cells were then collected for molecular detection.

Quantitative reverse transcription polymerase chain reaction

The total RNA was isolated from cultured THP-1cells using Trizol reagent (1 mL; Invitrogen). Total RNA sample (1 ug) was reverse-transcribed into complementary DNA (cDNA) using SuperScript III First-Strand Synthesis System (Invitrogen). The sequences of the primers used are displayed in Table 1. The primers were synthesized by Make Research Easy Biotech. DNA amplification was performed using SYBR Green on a polymerase chain reaction thermal cycler (Thermo Fisher Scientific) with the following thermal conditions: an initial step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s, and a final phase at 60°C for 5 min. The relative amount of mRNA of each sample was calculated using the $2^{-\Delta\Delta CT}$ method, and corrected by referencing the expression of β-actin (the loading control).

Table 1 Primer sequences used for reverse transcription polymerase chain reaction

Genes	Primers	Length/bp
Signal regulatory protein-α	F: 5′- TGACCTGGTTGG AGAATGG-3′ R: 5′- TCTCAGCGGC GGTATTTG-3′	273
CD36	F: 5′- AAAATGTAACC CAGGAC-3′ R: 5′- GTGTCGATTA TGGCAACT-3′	205
β-actin	F: 5′- CATCCTGCGTCT GGACCTGG-3′ R: 5′-TAATGTCACGC ACGATTTCC-3′	116

Western blot

The following primary antibodies were used: SIRP-α, CD36 and β-actin (all from Proteintech). THP-1 cells were harvested and lysed in 50-ul Lysis Buffer (Thermo Fisher Scientific) for 30 min. The mixture was centrifuged at 12000 rpm for 15 min. The total proteins in the supernatant were quantified with a bicinchonic acid (BCA) kit. 30 ug of samples were then loaded onto each lane during electrophoresis, and then transferred onto 0.2 μm nitrocellulose blotting membranes. The membrane was blocked with 5% fat-free milk at 4°C overnight for 1 h, followed by incubation with the primary antibodies, CD36 (dilution 1:500), SIRP-α (dilution 1:200), or β-actin (dilution 1:1000), at 4°C overnight. The membranes were washed three times with Tris-buffered saline-Tween 20. The membranes were then incubated with goat antirabbit secondary antibody (dilution 1:4000–6000; Preteintech) at room temperature for 1 h. Immunoreactive bands on the membranes were visualized by enhanced chemiluminescence (Thermo Pierce) detection reagents. The density of each band was quantified by Quantity One software (Bio-Rad Hercules) and corrected by referencing the expression value for β-actin.

IL-6 concentration in endometrial tissue lysates

The endometrial tissue was lysed with a lysis buffer. Lysates were then centrifuged at 1500 rpm for 10 min, and the supernatant was collected and stored at −70°C for further determination. The concentration of IL-6 in the lysis supernatant was measured using an enzyme-linked immunosorbent assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The protein concentration in the lysis supernatant was quantified using a BCA kit. Thus, the IL-6 concentration per milligram of protein was calculated (pg/mL/mg protein). All assays were performed in triplicate in three independent experiments.

Statistical analysis

The SPSS for Windows version 19.0 software package (SPSS Inc.) was used for statistical data analysis. Quantitative data are presented as the mean ± standard deviation from independent experiments in triplicate. For normally distributed data, comparisons between the two groups were performed using independent-samples *t*-tests. One-way analysis of variance (ANOVA) with Bonferroni adjustment was used for multiple comparisons. For data that were not normally distributed, the significance of between-group differences was tested using the Mann–Whitney test and Kruskal-Walls test. $P < 0.05$ was regarded as statistically significant.

Results

The expression of SIRP-α and CD36 in peritoneal macrophages

The expression of SIRP-α in peritoneal macrophages that were isolated from women with EM was significantly higher, at both the mRNA and protein level, than in control participants ($P < 0.05$ or 0.01; Fig. 1).

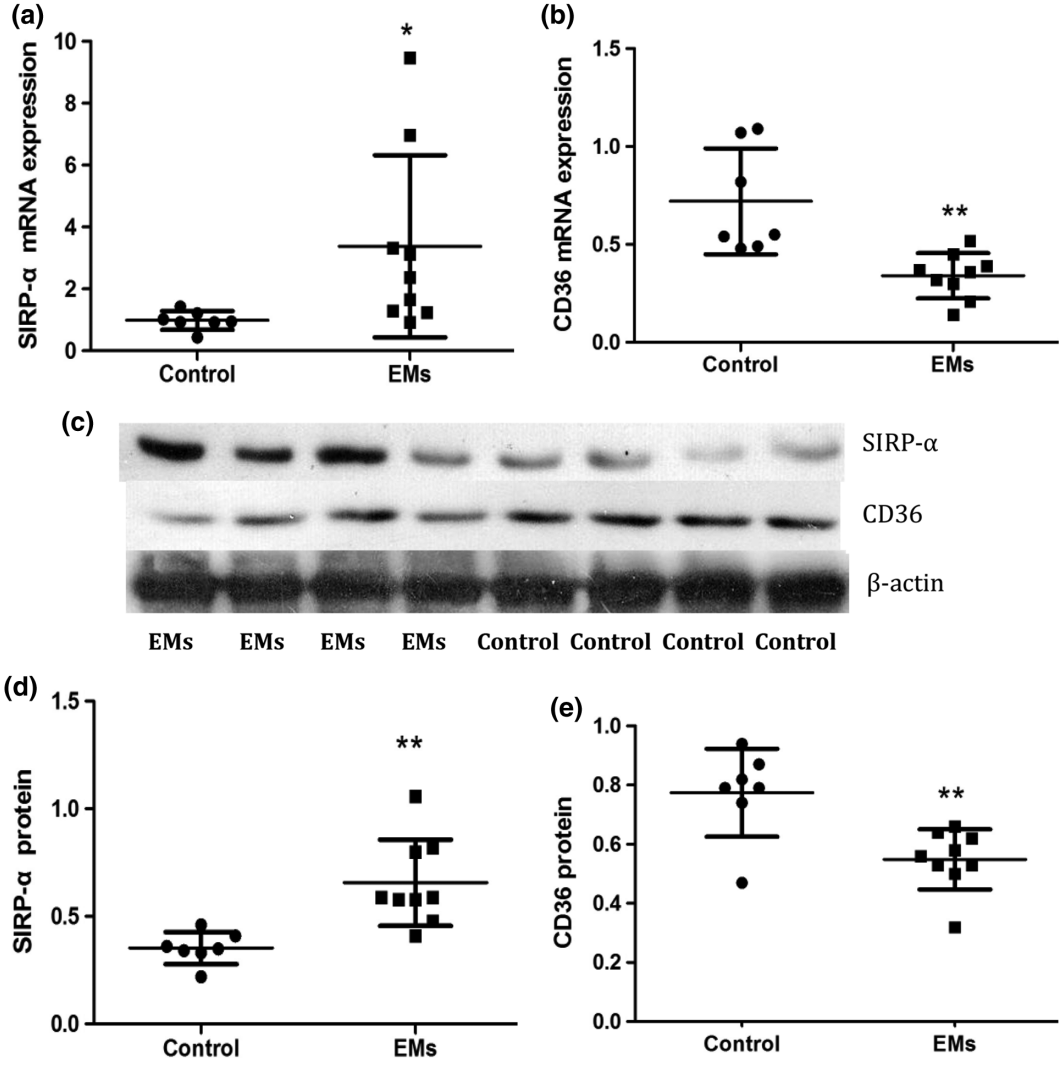

Figure 1 The expressions of signal regulatory protein-α (SIRP-α) and CD36 in peritoneal macrophages. Peritoneal macrophages were isolated from women with endometriosis (EM) or control participants. The mRNA expressions of SIRP-α (A) and CD36 (B) were detected by reverse transcription polymerase chain reaction. The band intensities (C) and quantitative analysis of SIRP-α (D) and CD36 (E) protein levels by Western blot are displayed. EM exhibit higher SIRP-α levels but lower CD36 levels than the controls. *$P < 0.05$, **$P < 0.01$. $n = 7$–9.

In contrast, the mRNA and protein expression of CD36 was lower in peritoneal macrophages that were isolated from EM patients, than in the control participants ($P < 0.01$).

The expression of SIRP-α and CD36 in peripheral blood mononuclear cells

The level of SIRP and CD36 was significantly higher in peripheral blood mononuclear cells isolated from women with EM, at both the mRNA and protein level, than in control participants ($P < 0.05$ or 0.01; Fig. 2).

Eutopic endometrial homogenate modulates the expression of CD36 and SIRP-α in THP-1 cells

THP-1 cell-derived macrophages were treated with either eutopic or normal endometrial homogenate for 72 h. The expression of SIRP-α and CD36 was then detected. The expression of SIRP-α at the mRNA and protein level was significantly higher in the THP-1 cell-derived macrophages that were treated with eutopic endometrial homogenate than those cells that were treated with the normal endometrial homogenate ($P < 0.01$ or 0.001). Conversely, the expression of

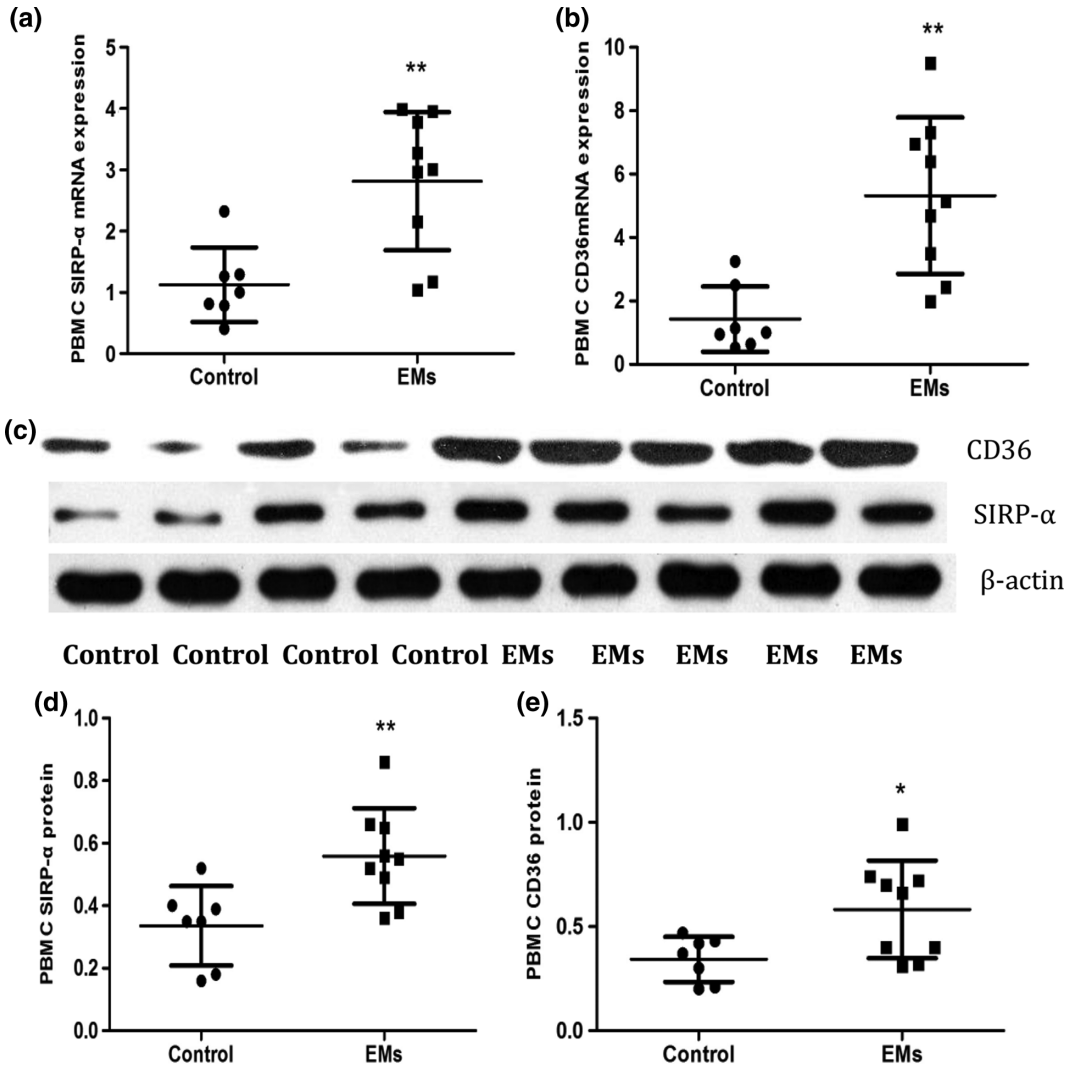

Figure 2 The expressions of signal regulatory protein-α (SIRP-α) and CD36 in peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were isolated from women with endometriosis (EM) or control participants. The mRNA expressions of SIRP-α (A) and CD36 (B) were detected by reverse transcription polymerase chain reaction. The band intensities (C) and quantitative analysis of SIRP-α (D) and CD36 (E) protein levels by Western blot are displayed. EM exhibited significantly higher SIRP-α and CD36 levels than the controls. *$P < 0.05$, **$P < 0.01$. $n = 7$–9.

CD36 was significantly lower in THP-1 cell-derived macrophages that were treated with eutopic endometrial homogenate than those cells that were treated with the normal endometrial homogenate ($P < 0.05$ or 0.01; Fig. 3).

IL-6-induced conditioned medium increases SIRP-α expression and decreases CD36 expression in THP-1 cells

The average concentration of IL-6 in the eutopic endometrial tissue lysates from women with EM was significantly higher than measured in the tissue of control subjects ($P < 0.01$; Fig. 4).

Conditioned medium was prepared by treating ESC in the presence or absence of IL-6, under a condition mimicking the normal menstrual cycle with physiological estrogen and/or progestin levels. The mRNA and protein expressions of SIRP-α and CD36 were detected in THP-1 cells after treatment with IL-6-induced conditioned medium. Intervention with IL-6 resulted in a downregulation of the SIRP-α expression and an upregulation of the CD36 expression in THP-1 cell-derived macrophages, as compared with the expression levels observed in the control condition ($P < 0.01$ or 0.001; Fig. 5).

The expressions of SIRP-α and CD36 in THP-1 cells under LPS stimulation

THP-1 cell-derived macrophages were treated with either eutopic or normal endometrial homogenate for 72 h, and then subjected to LPS stimulation for 24 h. LPS stimulation led to an higher expression of SIRP-α and CD36 at both the mRNA and protein level than in the control condition, in both the

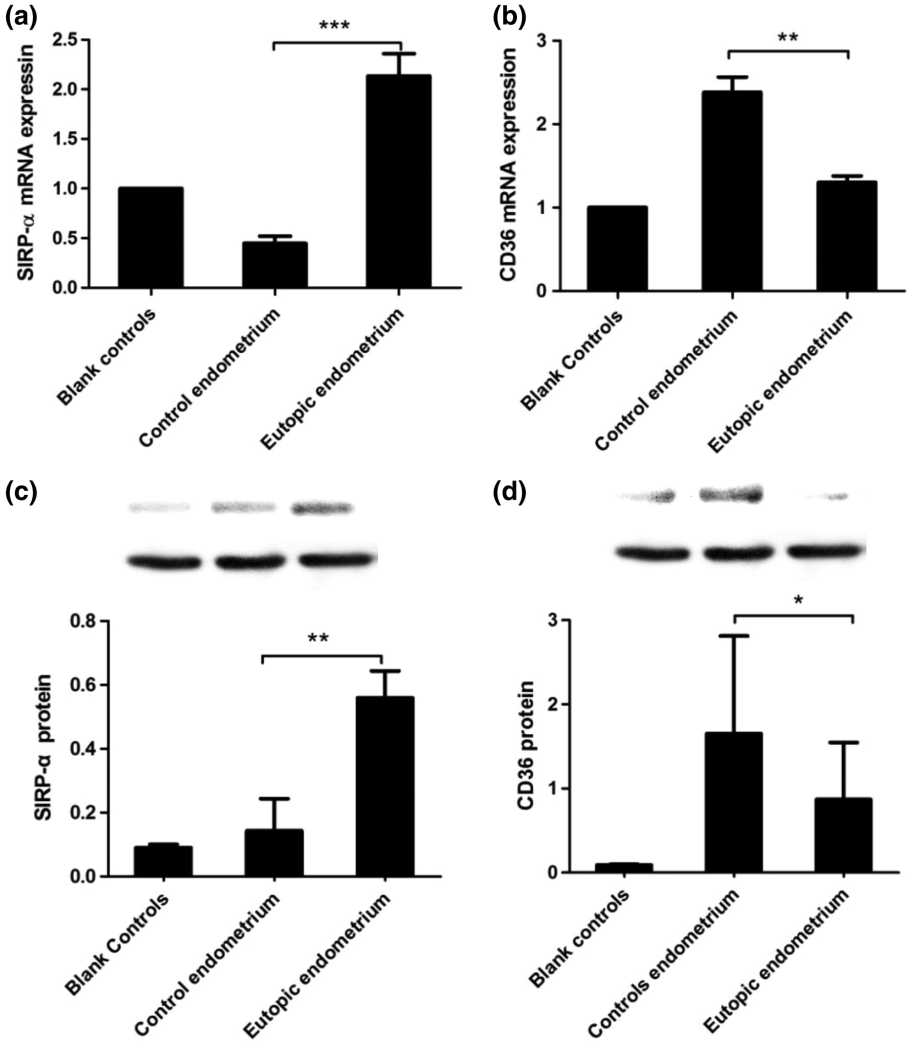

Figure 3 Eutopic endometrial homogenate induces increased signal regulatory protein-α (SIRP-α) but decreases CD36 expression in human acute monocytic leukemia (THP-1) cells. THP-1 cell-derived macrophages were treated with either eutopic endometrial homogenate from endometriosis (EM) patients, or normal endometrial homogenate from control participants for 72 h. The mRNA expressions of SIRP-α (A) and CD36 (B) were detected by reverse transcription polymerase chain reaction. The band intensities and quantitative analysis of SIRP-α (C) and CD36 (D) protein levels by Western blot are displayed. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. $n = 5$–8.

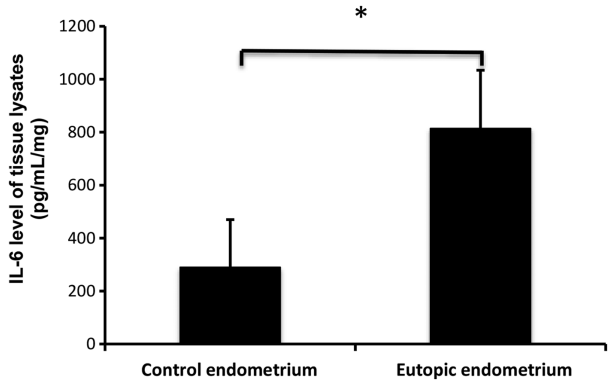

Figure 4 Interleukin-6 (IL-6) concentration in endometrial tissue lysates. The concentrations of IL-6 in the endometrial tissue lysates from women with endometriosis or control participants were measured by enzyme-linked immunosorbent assay assay, and presented as the concentration of IL-6 per milligram of protein (pg/mL/mg protein). n = 5–8.

eutopic and normal endometrial homogenate ($P < 0.01$ or 0.001; Fig. 6).

Inhibition of the NF-κB or TGF-β signaling pathway regulated the expression of SIRP-α and CD36 in THP-1 cells

THP-1 cell-derived macrophages were treated with 100 ng/mL LPS for 24 h, followed by treatment with SN50 or SB43154 for 1 h. The cells were then co-cultured with endometrial homogenate for 72 h. Incubation with the NF-κBp50 inhibitor, SN50, led to a decrease in both the mRNA and protein expression of CD36 and SIRP-α in THP-1 cell-derived macrophages that were treated with normal endometrial homogenate ($P < 0.01$). However, this decreased expression was not observed in cells treated with eutopic endometrial homogenate ($P > 0.05$; Fig. 7). Incubation with the TGF-β inhibitor, SB43154, led to an increase in both the mRNA and protein expression of CD36 in THP-1 cell-derived macrophages that were treated with normal and eutopic endometrial homogenate ($P < 0.01$ or 0.001). However, SB43154 only deceased the SIRP-α protein expression ($P < 0.01$), and not the mRNA expression ($P > 0.05$), in THP-1 cell-derived macrophages.

Discussion

Endometriosis is a complex disease that is associated with local and systemic aberrations in the immune response. Infiltration of macrophages with incompetent phagocytic capacity is a predominant feature of endometriotic lesions. It is not surprising that the significant changes that accompany EM induce immunological changes in macrophages, allowing a permissive environment where endometrial tissues implant via retrograde menstruation. We found that the expression of CD36 was reduced in peritoneal macrophages, coupled with an increased expression of SIRP-α in patients with EM, providing evidence to explain the loss of the phagocytic function of macrophages. Macrophages function via phagocytosis, which is, at least in part, regulated through the expression of the CD36 receptor. The CD36 receptor on infiltrating monocyte-derived macrophages has been reported to mediate phagocytosis during the recovery phase in poststroke brains.[23] Additionally, CD36-mediated phagocytosis of apoptotic cells serves as an important pathway in various pathogenic processes, such as chronic kidney fibrosis.[24] It has been reported that the peritoneal macrophages isolated from patients with EM are less active in phagocytosis.[7] Chuang *et al*.[25] found a reduction in CD36 expression in the peritoneal macrophages of women with EM. Blockage of CD36 impaired the phagocytic ability of normal macrophages; while forced expression of CD36 restored the phagocytic capacity of macrophages in women with EM. In another study, treatment with PGE2 inhibited the CD36-dependent phagocytosis of peritoneal macrophages and exacerbated endometriotic lesions in a murine model.[26] Consistent with previous studies, we found that the mRNA and protein expressions of CD36 were decreased in peritoneal macrophages of EM patients, as compared with the expression levels observed in the control condition, suggesting a reduced phagocytic ability of peritoneal macrophages.

SIRP-α interacts with its signaling molecules to regulate a variety of phagocyte effector functions, usually in a negative fashion, to maintain homeostasis. The expression of SIRP-α on macrophages prevents phagocytosis of Ig-opsonized red blood cells through the interaction with CD47; whereas depletion of SIRP-α promotes the FcR-mediated phagocytosis of red blood cells.[27] Yanagita *et al*. found that blockage of the interaction between SIRP-α and CD47 remarkably suppressed tumor formation through the enhancement of the cellular phagocytosis of tumor cells by macrophages.[28] In this study, we found that the SIRP expression in peritoneal macrophages from women with EM was significantly higher than the expression

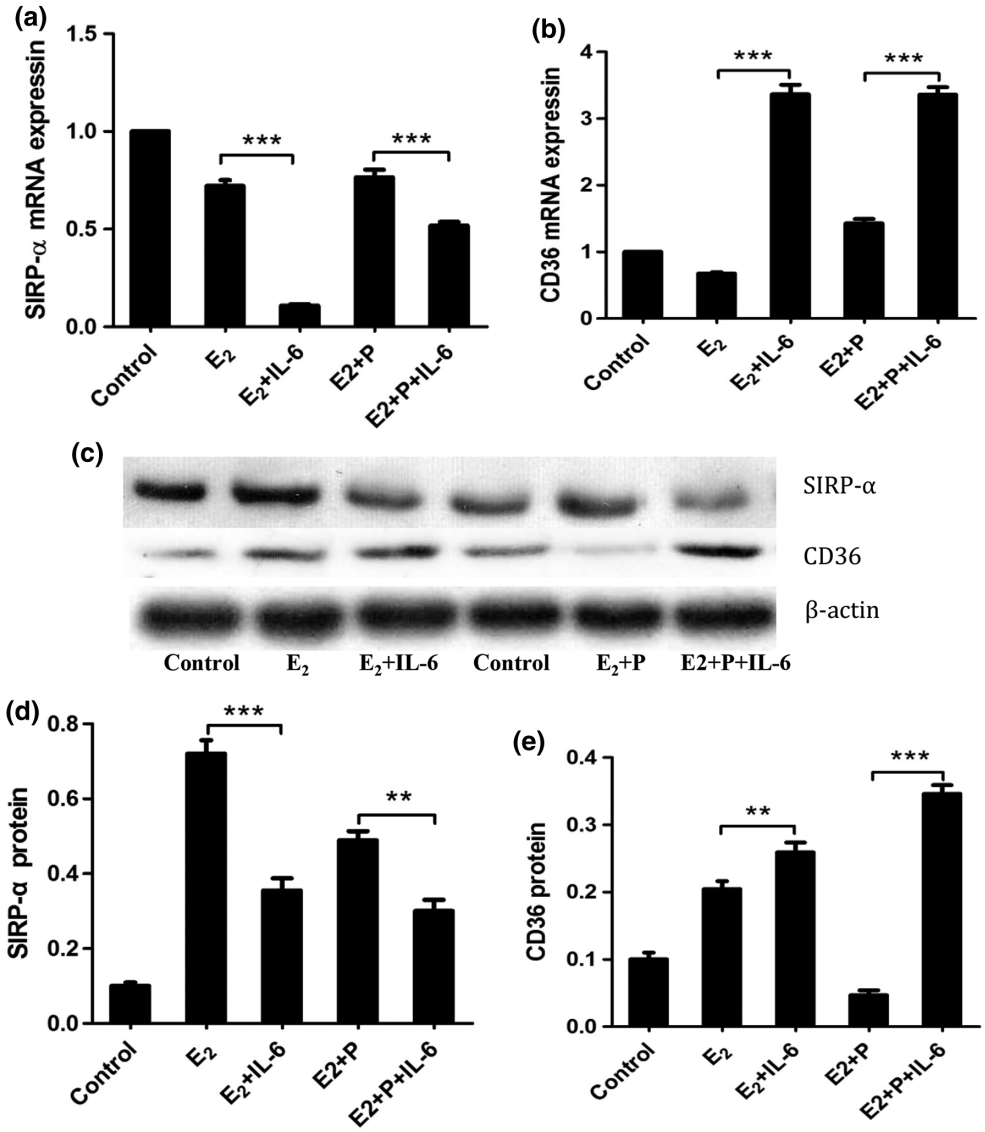

Figure 5 Intervention with interleukin-6 (IL-6) results in downregulation of signal regulatory protein-α (SIRP-α) but upregulation of CD36 in human acute monocytic leukemia (THP-1) cells. Conditioned medium was prepared by treating endometrial stromal cells in the presence or absence of IL-6 under a condition mimicking the normal menstrual cycle with physiological estrogen (E2) and/or progestin (P) levels. The mRNA expressions of SIRP-α (A) and CD36 (B) were detected by reverse transcription polymerase chain reaction in THP-1 cell-derived macrophages after treatment with IL-6-induced conditioned medium. The band intensities (C) and quantitative analysis of SIRP-α (D) and CD36 (E) protein levels by Western blot are displayed. $^{**}P < 0.01$, $^{***}P < 0.001$. $n = 5$.

observed in control subjects. We speculate that this increased SIRP expression combined with a decreased CD36 expression in peritoneal macrophages leads to impairment of phagocytic ability and insufficient clearance of endometrial lesions in the abdominal cavity, contributing to the development of EM. Interestingly, women with EM exhibited a significantly higher level of SIRP-α as well as CD36 expression in peripheral circulating mononuclear phagocytic cells, perhaps due to a compensatory mechanism to maintain the phagocytic activity of systematic macrophages. It is thus indicated that a defective phagocytic ability of macrophages may be a local immune dysfunction in patients with EM.

Previous evidence has demonstrated that macrophages that are present in the eutopic and ectopic

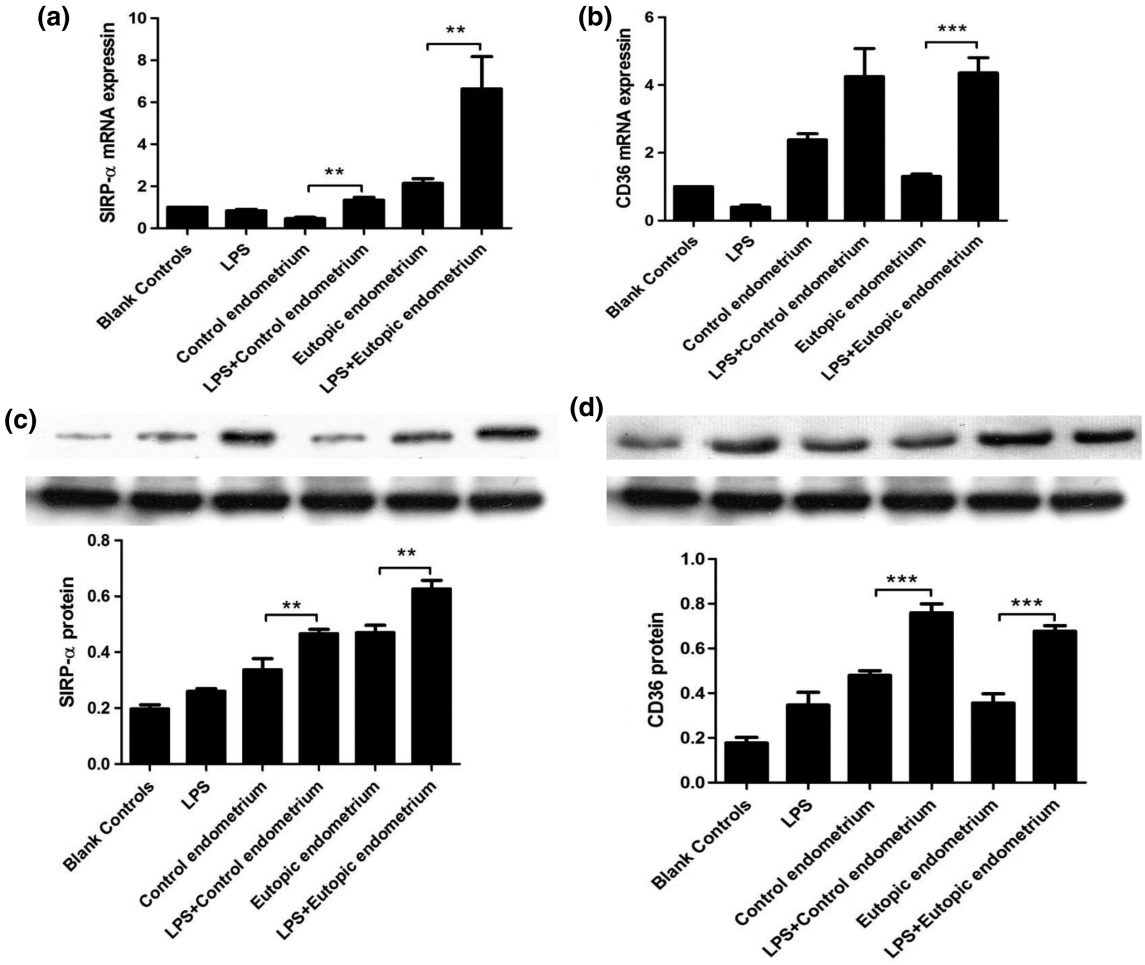

Figure 6 Lipopolysaccharide (LPS) stimulation leads to increased expressions of signal regulatory protein-α (SIRP-α) and CD36 in human acute monocytic leukemia (THP-1) cells. THP-1 cell-derived macrophages were treated with either eutopic or normal endometrial homogenate for 72 h, and then subjected to LPS stimulation for 24 h. The mRNA expressions of SIRP-α (A) and CD36 (B) were detected by reverse transcription polymerase chain reaction. The band intensities and quantitative analysis of SIRP-α (C) and CD36 (D) protein levels by Western blot are displayed. EM, women with endometriosis. $^{**}P < 0.01$, $^{***}P < 0.001$. $n = 5$–8.

endometrium can create an inflammatory microenvironment that contributes to the formation of endometriotic lesions.[29] On activation of macrophages, endometriotic implants produce a significant number of cytokines and chemokines, such as IL-1β, IL-6, and TNF-α, cyclooxygenase-2 (COX-2), PGE2, monocyte chemotactic protein-1, and Matrix metalloproteinases-9. The production of these cytokines and chemokines further facilitates the invasion of EM.[9,11,30–32] In this study, following treatment with eutopic endometrial homogenate, THP-1 cell-derived macrophages showed significantly increased SIRP-α expression and significantly decreased CD36 expression compared to control conditions. These data reasonably explain the defective macrophage phagocytosis that is caused by the retrograde shedding of the eutopic endometrium into the peritoneal cavity.

IL-6 is a multifunctional proinflammatory cytokine, which plays a prominent role in a variety of chronic inflammatory conditions. IL-6 is secreted by macrophages as well as ectopic and eutopic ESC.[33] Women with EM have an increased IL-6 concentration in their peritoneal fluid.[34] Consistent with this, we found an higher IL-6 concentration in the eutopic endometrial tissue of women with EM than in their controls. IL-6 is associated with the invasive, adhesive, and proliferative behaviors of eutopic and ectopic stromal cells from patients with EM, which has been implicated in

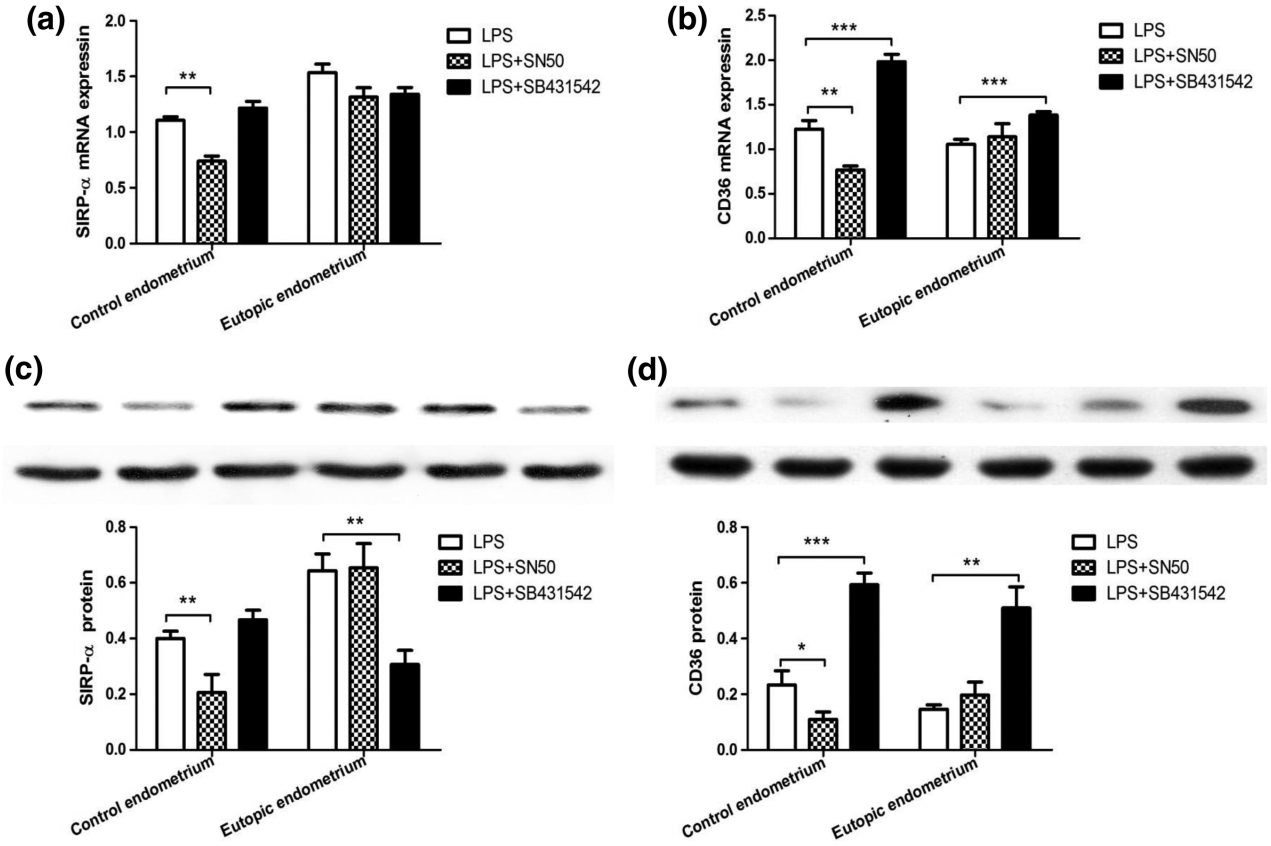

Figure 7 Inhibition of NF-κB or TGF-β/SMAD2/3 signaling pathway regulates signal regulatory protein-α (SIRP-α) and CD36 expressions in human acute monocytic leukemia (THP-1) cells. THP-1 cell-derived macrophages were sequentially treated with 100-ng/mL lipopolysaccharides (LPS) for 24 h, and then 50-μg/mL NF-κBp50 inhibitor (SN50) or 10-μM TGF-β inhibitor (SB43154) for 1 h, followed by 100-μL/mL endometrial homogenate for an additional 72 h co-culture. The mRNA expressions of SIRP-α (A) and CD36 (B) were detected by reverse transcription polymerase chain reaction. The protein expressions of SIRP-α (C) and CD36 (D) were detected by Western blot. EM, women with endometriosis. $^{**}P < 0.01$, $^{***}P < 0.001$. $n = 5–8$.

the growth of EM.[35–37] Additionally, secretion of IL-6 from macrophages has been reported to promote angiogenesis in the early stage of EM, resulting in a vicious cycle of new implants.[38] In this study, we developed an *in vitro* model that exhibits similarities to the human menstrual cycle with physiological estrogen and/or progestin levels. Intervention with IL-6 resulted in decreased SIRP-α expression and increased CD36 expression in THP-1 cell-derived macrophages, as compared with the expression levels observed in their controls, suggesting an enhanced phagocytic function of macrophages under IL-6 stimulation. This supposition appears to be contradicted by the indicated involvement of IL-6 in the pathogenesis of EM. We speculate that the expression of SIRP-α and CD36 in macrophages may be affected by various factors in the peritoneal fluid, such as other cytokines and EM lesions, which produce complex, inexplicable results. These findings need further investigation.

Bacterial LPS may have an initial role in the development of EM.[39] Takenaka *et al*. found that LPS promotes the growth and invasion of ESC through the upregulation of COX-2 and PGE2 expression, demonstrating an association between bacterial infection and endometriotic lesions.[40] In this study, LPS stimulation led to the increased expression of SIRP-α and CD36, in both the eutopic and normal endometrial homogenate. Dysregulation of NF-κB activation in the endometrium of EM patients modulates key cell processes that contribute to the initiation and progression of EM.[41,42] We found that the NF-κBp50 inhibitor, SN50, decreased the expression of CD36 and SIRP-α in macrophages that were treated with

normal but not eutopic endometrial homogenate, suggesting the involvement of different macrophage regulatory mechanisms between normal and eutopic endometrium. Previous evidence has demonstrated increased levels of TGF-β in eutopic and ectopic endometrial lesions, accompanied by decreased immune cell activity within the peritoneum, further fuelling EM lesion development.[43] Han *et al*. reported that TGF-β1/2 significantly decreases the expression of CD36 in macrophages through a mechanism that involves phosphorylation of mitogen-activated protein kinase.[44] Consistent with this, we found that inhibition of TGF-β-enhanced CD36 expression in THP-1 cell-derived macrophages that were treated with normal as well as eutopic endometrial homogenate, indicating that targeting this pathway may have therapeutic potential in the treatment of EM, through the restoration of the phagocytic function of macrophages. In this study, we also found that inhibition of TGF-β deceased the expression of SIRP-α in THP-1 cell-derived macrophages. However, this change was only observed at the protein level. The changes observed in the mRNA expression of SIRP-α were not exactly in accordance with the changes observed at the protein level. This result is probably due to some unknown factors, such as post-transcriptional gene regulation or modification and specific detection time. Further investigation is required to confirm and extend these results.

In conclusion, peritoneal macrophages that are isolated from women with EM exhibit enhanced expression of SIRP-α and decreased expression of CD36. Eutopic endometrium reduces the phagocytic ability of peritoneal macrophages in women with EM through the modulation of SIRP-α and CD36 expression. Inhibition of the TGF-β signal pathway enhances the CD36 expression of macrophages in the presence of eutopic endometrium, providing a potential therapeutic target for the treatment of EM.

Conflict of interest

The authors declare that they have no conflict of interests.

Disclosure

None declared.

References

1. Cramer DW, Missmer SA. The epidemiology of endometriosis. *Ann N Y Acad Sci* 2002; **955**: 11–22 discussion 34-16, 396–406.
2. Practice Committee of the American Society for Reproductive Medicine. Endometriosis and infertility: A committee opinion. *Fertil Steril* 2004; **81**: 1441–1446.
3. van der Linden PJ. Theories on the pathogenesis of endometriosis. *Hum Reprod* 1996; **11** (Suppl 3): 53–65.
4. Hogg S, Vyas S. Endometriosis. *Obstet Gynaecol Reprod Med* 2015; **25**: 133–141.
5. Kyama CM, Debrock S, Mwenda JM, D'Hooghe TM. Potential involvement of the immune system in the development of endometriosis. *Reprod Biol Endocrinol* 2003; **1**: 123.
6. Giudice LC. Clinical practice endometriosis. *N Engl J Med* 2010; **362**: 2389–2398.
7. Capobianco A, Rovere-Querini P. Endometriosis, a disease of the macrophage. *Front Immunol* 2013; **4**: 9.
8. Bacci M, Capobianco A, Monno A *et al*. Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. *Am J Pathol* 2009; **175**: 547–556.
9. Kralickova M, Vetvicka V. Immunological aspects of endometriosis: A review. *Ann Transl Med* 2015; **3**: 153.
10. Wu MH, Shoji Y, Wu MC *et al*. Suppression of matrix metalloproteinase-9 by prostaglandin E(2) in peritoneal macrophage is associated with severity of endometriosis. *Am J Pathol* 2005; **167**: 1061–1069.
11. Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. *Fertil Steril* 2012; **98**: 511–519.
12. Matozaki T, Murata Y, Okazawa H, Ohnishi H. Functions and molecular mechanisms of the CD47-SIRPalpha signalling pathway. *Trends Cell Biol* 2009; **19**: 72–80.
13. Barclay AN, Van den Berg TK. The interaction between signal regulatory protein alpha (SIRPalpha) and CD47: Structure, function, and therapeutic target. *Annu Rev Immunol* 2014; **32**: 25–50.
14. Zhao XW, van Beek EM, Schornagel K *et al*. CD47-signal regulatory protein-alpha (SIRPalpha) interactions form a barrier for antibody-mediated tumor cell destruction. *Proc Natl Acad Sci U S A* 2011; **108**: 18342–18347.
15. Zhang H, Li F, Yang Y, Chen J, Hu X. SIRP/CD47 signaling in neurological disorders. *Brain Res* 2015; **1623**: 74–80.
16. Febbraio M, Hajjar DP, Silverstein RL. CD36: A class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. *J Clin Invest* 2001; **108**: 785–791.
17. Park YM. CD36, a scavenger receptor implicated in atherosclerosis. *Exp Mol Med* 2014; **46**: e99.
18. Dehn S, Thorp EB. Myeloid receptor CD36 is required for early phagocytosis of myocardial infarcts and induction of Nr4a1-dependent mechanisms of cardiac repair. *FASEB J* 2018; **32**: 254–264.
19. DeLeon-Pennell KY, Tian Y, Zhang B *et al*. CD36 is a matrix metalloproteinase-9 substrate that stimulates neutrophil apoptosis and removal during cardiac remodeling. *Circ Cardiovasc Genet* 2016; **9**: 14–25.
20. Herington JL, Bruner-Tran KL, Lucas JA, Osteen KG. Immune interactions in endometriosis. *Expert Rev Clin Immunol* 2011; **7**: 611–626.

21. Higdon LE, Lee K, Tang Q, Maltzman JS. Virtual global transplant laboratory standard operating procedures for blood collection, PBMC isolation, and storage. *Transplant Direct* 2016; **2**: e101.
22. Dasgupta S, Bhattacharya-Chatterjee M, O'Malley BW Jr, Chatterjee SK. Inhibition of NK cell activity through TGF-beta 1 by down-regulation of NKG2D in a murine model of head and neck cancer. *J Immunol* 2005; **175**: 5541–5550.
23. Woo MS, Yang J, Beltran C, Cho S. Cell surface CD36 protein in monocyte/macrophage contributes to phagocytosis during the resolution phase of ischemic stroke in mice. *J Biol Chem* 2016; **291**: 23654–23661.
24. Pennathur S, Pasichnyk K, Bahrami NM *et al*. The macrophage phagocytic receptor CD36 promotes fibrogenic pathways on removal of apoptotic cells during chronic kidney injury. *Am J Pathol* 2015; **185**: 2232–2245.
25. Chuang PC, Wu MH, Shoji Y, Tsai SJ. Downregulation of CD36 results in reduced phagocytic ability of peritoneal macrophages of women with endometriosis. *J Pathol* 2009; **219**: 232–241.
26. Chuang PC, Lin YJ, Wu MH, Wing LY, Shoji Y, Tsai SJ. Inhibition of CD36-dependent phagocytosis by prostaglandin E2 contributes to the development of endometriosis. *Am J Pathol* 2010; **176**: 850–860.
27. Okazawa H, Motegi S, Ohyama N *et al*. Negative regulation of phagocytosis in macrophages by the CD47-SHPS-1 system. *J Immunol* 2005; **174**: 2004–2011.
28. Yanagita T, Murata Y, Tanaka D *et al*. Anti-SIRPalpha antibodies as a potential new tool for cancer immunotherapy. *JCI Insight* 2017; **2**: e89140.
29. Greaves E, Cousins FL, Murray A *et al*. A novel mouse model of endometriosis mimics human phenotype and reveals insights into the inflammatory contribution of shed endometrium. *Am J Pathol* 2014; **184**: 1930–1939.
30. Bergqvist A, Bruse C, Carlberg M, Carlstrom K. Interleukin 1beta, interleukin-6, and tumor necrosis factor-alpha in endometriotic tissue and in endometrium. *Fertil Steril* 2001; **75**: 489–495.
31. Collette T, Maheux R, Mailloux J, Akoum A. Increased expression of matrix metalloproteinase-9 in the eutopic endometrial tissue of women with endometriosis. *Hum Reprod* 2006; **21**: 3059–3067.
32. Ota H, Igarashi S, Sasaki M, Tanaka T. Distribution of cyclooxygenase-2 in eutopic and ectopic endometrium in endometriosis and adenomyosis. *Hum Reprod* 2001; **16**: 561–566.
33. Tseng JF, Ryan IP, Milam TD *et al*. Interleukin-6 secretion in vitro is up-regulated in ectopic and eutopic endometrial stromal cells from women with endometriosis. *J Clin Endocrinol Metab* 1996; **81**: 1118–1122.
34. Kalu E, Sumar N, Giannopoulos T *et al*. Cytokine profiles in serum and peritoneal fluid from infertile women with and without endometriosis. *J Obstet Gynaecol Res* 2007; **33**: 490–495.
35. Delbandi AA, Mahmoudi M, Shervin A *et al*. Eutopic and ectopic stromal cells from patients with endometriosis exhibit differential invasive, adhesive, and proliferative behavior. *Fertil Steril* 2013; **100**: 761–769.
36. Slater M, Cooper M, Murphy CR. Human growth hormone and interleukin-6 are upregulated in endometriosis and endometrioid adenocarcinoma. *Acta Histochem* 2006; **108**: 13–18.
37. Khan KN, Masuzaki H, Fujishita A *et al*. Interleukin-6- and tumour necrosis factor alpha-mediated expression of hepatocyte growth factor by stromal cells and its involvement in the growth of endometriosis. *Hum Reprod* 2005; **20**: 2715–2723.
38. Lin YJ, Lai MD, Lei HY, Wing LY. Neutrophils and macrophages promote angiogenesis in the early stage of endometriosis in a mouse model. *Endocrinology* 2006; **147**: 1278–1286.
39. Khan KN, Kitajima M, Hiraki K *et al*. *Escherichia coli* contamination of menstrual blood and effect of bacterial endotoxin on endometriosis. *Fertil Steril* 2010; **94**: 2860–2863.
40. Takenaka Y, Taniguchi F, Miyakoda H, Takai E, Terakawa N, Harada T. Lipopolysaccharide promoted proliferation and invasion of endometriotic stromal cells via induction of cyclooxygenase-2 expression. *Fertil Steril* 2010; **93**: 325–327.
41. Gonzalez-Ramos R, Defrere S, Devoto L. Nuclear factor-kappaB: A main regulator of inflammation and cell survival in endometriosis pathophysiology. *Fertil Steril* 2012; **98**: 520–528.
42. Gonzalez-Ramos R, Van Langendonckt A, Defrere S *et al*. Involvement of the nuclear factor-kappaB pathway in the pathogenesis of endometriosis. *Fertil Steril* 2010; **94**: 1985–1994.
43. Dela Cruz C, Reis FM. The role of TGFbeta superfamily members in the pathophysiology of endometriosis. *Gynecol Endocrinol* 2015; **31**: 511–515.
44. Han J, Hajjar DP, Tauras JM, Feng J, Gotto AM Jr, Nicholson AC. Transforming growth factor-beta1 (TGF-beta1) and TGF-beta2 decrease expression of CD36, the type B scavenger receptor, through mitogen-activated protein kinase phosphorylation of peroxisome proliferator-activated receptor-gamma. *J Biol Chem* 2000; **275**: 1241–1246.