Elevated heme impairs macrophage phagocytosis in endometriosis

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Abstract

Endometriosis (EMS) is a chronic inflammatory disease characterized by the presence of extrauterine endometrial tissues. It has been previously reported that the refluxed blood containing viable endometrial tissues and the defective elimination of peritoneal macrophages in the pelvic cavity may involve in EMS pathogenesis. However, the mechanism by which macrophages exhibit attenuated phagocytic capability in EMS remains undetermined. Herein, we found that heme, the byproduct of lysed erythrocytes, accumulated abnormally in the peritoneal fluid (PF) of patients with EMS (14.22 $\mu\text{mol/L}$, 95% confidence interval (CI): 12.54–16.71), compared with the EMS-free group (9.517 $\mu\text{mol/L}$, 95% CI: 8.891–10.1053). This abnormal accumulation was not associated with the color of PF, phase of the menstrual cycle or severity of the disease. The reduced phagocytic ability of peritoneal macrophages (pM ϕ s) was observed in the EMS group. Consistently, a high-concentration (30 $\mu\text{mol/L}$) heme treatment impaired EMS-pM ϕ s phagocytosis more than a low-concentration (10 $\mu\text{mol/L}$) heme treatment. A similar phenomenon was observed in the EMS-free control pM ϕ s (Ctrl-pM ϕ s) and the CD14⁺ peripheral monocytes (CD14⁺ Mos). These results indicated that a high heme concentration exhibits a negative effect on macrophage phagocytosis, which supplements the mechanism of impaired scavenger function of pM ϕ s in EMS.

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Introduction

Endometriosis (EMS) is a chronic inflammatory disease that is characterized by the presence of extrauterine endometrial-like tissue. EMS is estimated to affect approximately 5–10% women of reproductive age who exhibit pelvic pain, dysmenorrhea, dyspareunia and infertility (Zondervan *et al.* 2018). To some extent, these symptoms can be alleviated via treatment including with hormonal medication or surgical lesion removal. However, patients have been revealed to exhibit undesirable side effects like contraception with progestin drug treatment and have a post-operative EMS recurrence of 30–50% after 2–5 years (Vercellini *et al.* 2010, Leeners *et al.* 2018). Hence, this disease not only causes enormous physical and psychological distress on patients (Gambadauro *et al.* 2019), but also imposes a heavy economic burden on their families as well as the national health care system (Soliman *et al.* 2018).

The most widely accepted hypothesis for the etiology of EMS is Sampson's theory of retrograde menstruation

(Sampson 1927). Menstrual blood from the uterine cavity is refluxed through the fallopian tubes and releases erythrocytes, endometrial tissue and cell debris into the peritoneal environment, and subsequently viable endometrial stromal cells (ESCs) implant and grow. Sampson's theory helps to explain the increased prevalence of EMS in women with congenital menstrual outflow tract obstruction (Dietrich *et al.* 2014) and asymmetrical distribution of endometriotic lesions in the abdominopelvic cavity (Vercellini *et al.* 2007). However, this theory fails to explain why retrograde menstruation occurs in 61–90% women (Halme *et al.* 1984, Symons *et al.* 2018) but only 10% develop EMS.

It has also been suggested that the impaired immune surveillance may result from the dysfunction of immune cells, including Foxp3⁺ regulatory T (Treg) cells, natural-killer (NK) cells and macrophages, and this may contribute to the formation and development of endometrial lesions (Wei *et al.* 2016, Yang *et al.* 2017, Symons *et al.* 2018). Macrophages, as the predominant immune cells in peritoneal cavity, serve a role in

exacerbating inflammation and sustaining EMS, and the mechanism by which this occurs has gained increased attention in recent years. An accumulation of studies have revealed that macrophage populations are significantly increased in the PF and eutopic endometrium, and pro-inflammatory cytokines secretion increases (Berbic *et al.* 2009, Zhang *et al.* 2018). However, macrophages have also presented decreased phagocytic ability and failure to remove endometriotic tissue from the peritoneal cavity (Riccio *et al.* 2018), facilitating tissue adhesion to extrauterine structures (Shao *et al.* 2016, Chan *et al.* 2017). In previous studies, Mei *et al.* have linked the impaired phagocytosis with the activation of IL-33 and overexpression of indoleamine 2,3-dioxygenase-1 (IDO1) in ectopic ESCs. Chuang and Wu focused on the suppressive effect of prostaglandin E₂, which was revealed to reduce scavenger receptor CD36 and matrix metalloproteinases expression (Wu *et al.* 2005, 2013, Chuang *et al.* 2009, 2010).

Heme is a complex of iron with protoporphyrin IX (PPIX) and is the prosthetic group of many essential biological proteins. Specially, it participates in the formation of hemoglobin (Hb) and erythrocytes (Immenschuh *et al.* 2017). During hemolysis, ruptured erythrocytes release Hb. After exhausting the corresponding scavenging proteins haptoglobin (Hp), cell-free Hb is rapidly oxidized and further releases free heme. Without being chelated, heme can cause oxidative damage and inflammation (Chiabrando *et al.* 2018). To prevent this harmful effect, heme scavenger hemopexin (Hx) binds to heme and forms a heme-Hx complex, which is subsequently taken up by cells through receptor-mediated endocytosis. Once incorporated into the cell, heme is degraded by heme oxygenase-1 (HO) into equimolar amounts of CO, iron and biliverdin (Vijayan *et al.* 2018). However, under pathological conditions, the capacity for the organism to scavenge heme may saturate and the increased heme can cause tissue damage via pro-oxidant and immunoregulatory effects (Martins & Knapp 2018).

Previous studies have demonstrated that women with EMS exhibit shorter menstrual cycles and higher volumes of refluxed menstrual blood compared with healthy women (Parazzini *et al.* 2017, Shafrir *et al.* 2018). In patients developing EMS, bleeding from red endometrial lesions may further increase the number of erythrocytes. Therefore, it is reasonable to assume that heme, as a byproduct of lysed erythrocytes, may accumulate in the peritoneal cavity of patients with EMS. This causes a high level of free heme that can exert its oxidative effect and immunoregulatory effect, which may help to build the defective peritoneal disposal system in EMS. Herein we aimed to verify the existence of abnormal heme accumulation in the pelvic cavity of patients with EMS and further assess the association between heme and macrophages. This study revealed the potential effect of heme on pMφs phagocytosis.

Materials and methods

Patients and samples collection

The current study was approved by The Human Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University. All donors provided written informed consents. The patients included in the current study were premenopausal women (aged 22–49 years) who attended the Obstetrics and Gynecology Hospital of Fudan University between January 2018 and April 2019. No patients received hormonal therapy within 6 months prior to surgery. Endometriosis stage was diagnosed according to the revised American Society for Reproductive Medicine Staging (1997). Endometriotic tissues and endometriotic PF were collected from women who had undergone laparoscopic surgery for ovarian endometriosis ($n=45$), which was confirmed by pathological assessment. Control endometrial tissues were obtained from premenopausal women who had undergone fractional curettage but exhibited no abnormalities after pathological examination ($n=35$). Control PF was collected from premenopausal women undergoing laparoscopic surgery but exhibited no evidence of endometriosis, including patients exhibiting uterine fibroid, mature teratoma or ovarian simple cyst ($n=45$). PF samples were divided into two groups by color and included a reddish and yellowish group. Peripheral blood samples were obtained from healthy volunteers ($n=20$). All samples were collected under sterile conditions.

ESCs isolation and culture

Human endometrial and endometriotic tissues were purified as previously described (Wei *et al.* 2016). Tissues were cut into 1 mm pieces and digested into single-cell suspensions using collagenase type IV (0.001 g/mL, Sigma-Aldrich) for 30 min at 37°C with a constant agitation. Nylon strainers (100, 300 and 400 µm) were subsequently used to filter the resulting suspension. The filtrate was collected and centrifuged at 288 g for 8 min at 4°C. Later the supernatant was discarded and the cell sedimentation was resuspended in Dulbecco's Modified Eagle Medium (DMEM)/F12 complete medium with 10% fetal bovine serum (FBS; Hyclone) and 100 U/mL of penicillin and 100 mg/mL of streptomycin. Finally, the suspended cells were plated into culture flask in 5% CO₂ at 37°C. The culture medium was replaced every 3 days. Flow cytometry (FCM) analysis was used to verify the purity of vimentin⁺ cytokeratin7⁺ ESCs (>95%). Additionally, the HESC cell line (human ESCs derived from a uterine myoma) was used in the experiments, which was a generous gift from Professor Zhao Dong (Shanghai First Maternity and Infant Hospital, Tongji University). This cell line was maintained in the same condition as the ESC isolated from tissues.

Treatment of PF and peripheral blood

PF was centrifuged at 450 g for 8 min at 4°C. The supernatant was collected and stored at –80°C. The cell pellet was treated with Red Blood Cell Lysis Buffer (BioLegend, San Diego, USA) for 10 min at 4°C and washed with PBS twice. Subsequently human anti-CD14 microbeads (Miltenyi Biotec,

Bergisch Gladbach, Germany) were used to select peritoneal macrophages (pMφs), according to the manufacturer's protocol. Peripheral blood mononuclear cells (PBMCs) were firstly isolated from peripheral blood samples using Ficoll-Hypaque density gradient centrifugation. CD14⁺ monocytes (CD14⁺ Mos) were subsequently purified from PBMCs using human anti-CD14 microbeads. The purity of pMφs or CD14⁺ Mos was >95% as determined by FCM.

Heme measurement

Peritoneal heme concentration was measured by using the Heme Assay kit (MAK316, Sigma-Aldrich). A total of 250 μL of water was added into wells in a 96-well plate to create a blank well, while 50 μL of heme calibrator and 200 μL of water was mixed to create a standard well. A total of 50 μL of samples were pipetted from each PF supernatant and subsequently mixed with 200 μL of reagent respectively. After 5-min incubation at room temperature, the absorbance of well contents was determined at 400 nm. All samples and standards were performed in duplicate. Total heme concentration of a sample was determined using the following equation: $(OD_{\text{sample}} - OD_{\text{blank}}) / (OD_{\text{calibrator}} - OD_{\text{blank}}) \times 62.5$ (μmol/L).

Heme

Heme (hemin, Sigma-Aldrich) was first dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to create a stock concentration of 20 mM, filtered through a 0.02 mm syringe filter unit to assure sterility and subsequently stored at -20°C. This was then diluted to different concentrations (10, 30 μmol/L) in complete RPMI 1640 medium with 10% FBS immediately prior to use. Heme concentrations of the ultimate solution were verified by a Heme Assay kit based on the potential FBS interference (which may contains heme per se or heme scavenging proteins, including hemopexin and albumin). The DMSO concentration of the ultimate solution was kept lower than 0.1% in all experiments.

Quantitative real-time PCR

Total RNA was extracted from cells using RNAiso Plus reagent (TaKaRa Biotechnology), according to the manufacturer's protocol. Total RNA (500 ng) was reverse transcribed into first-stand cDNA (RR036A, TaKaRa Biotechnology) following the

manufacturer protocol. cDNA was subsequently amplified with TB Green (RR820A, TaKaRa Biotechnology) and specific primers (detailed sequences listed in Table 1) (Sangon Biotech, Shanghai, China) using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems). PCR was performed for 40 cycles using the following conditions: denaturizing at 95°C for 30 s, annealing at 95°C for 5 s and elongation at 60°C for 34 s. The mRNA expression of the samples was normalized using the 2^{-ΔΔCT} method. All measurements were performed in triplicate.

Phagocytosis assay

To assess phagocytosis efficiency, 10⁵ macrophages were plated into 96-well low attachment plates and treated with different concentration of heme (10, 30 μmol/L) or DMSO for 20 h. CFSE-labeled (BioLegend) HESCs were then incubated with Mφs at a ratio of 1:1 at 37°C for 4 h. After a 4-h coculture, cells in the 96-well plates were transferred to 1.5 mL EP tubes, washed by PBS and stained with allophycocyanin (APC)-conjugated anti-CD45 (BioLegend) to mark Mφs. The phagocytosis ratio was determined using FCM and was calculated as follows: CD45⁺CFSE⁺ cells / CD45⁺ cells × 100%.

Flow cytometry (FCM)

Fluorochrome-conjugated antibodies of human antigens were used for flow cytometry analysis and included allophycocyanin (APC)-conjugated anti-CD45 (BioLegend), Percp/cy5.5-conjugated anti-CD45 (BioLegend), APC/Cy7-conjugated anti-CD14 (BioLegend). Cells were washed with PBS on ice and then incubated with the corresponding fluorescent-labeled antibody for 30 min at 4°C, according to the recommended dosage. Isotype controls were used. Following incubation, cells were washed in PBS and analyzed immediately. Sample data were collected in a CyAn ADP analyzer (Beckman Coulter) and analyzed using FlowJo version 10 software for Windows (Tree Star).

Statistical analyses

Data collected from at least three independent experiments were analyzed using the Graphpad Prism statistical package (Graphpad Software). Data normality was tested using the Shapiro-Wilk test. Comparisons were performed using an

Table 1 The sequences of primers used for qRT-PCR.

Gene	Size (bp)	Forward primer (5'–3')	Reverse primer (5'–3')
ACTB	281	CTACCTCATGAAGATCCTCACC	AGTTGAAGGTAGTTTCGTGGAT
CD163	127	GCCACAACAGGTGCTCATCC	CAAGCCGCTGTCTGTCTTTCG
CD91	154	TTGCTGCTGCTGCTGGTCTG	GCTCTCCGCCTTCGTACATCTTG
HCP1	106	GCCTTGCTGATAGCCATGACTCTC	CAATGGATCGGTGGTGACGGAAC
FLVCR1	116	GCTTCGTGGTGCTCCTGATCTTC	GTGCAGCAAGGTGACACCGTAG
FLVCR2	84	TGTGCTGCTTGTCATCACCTATGG	TGCCAGATCACCATGCCATTGAG
ABCG2	123	GCCATAGCAGCAGGTGACAGTG	ACTGAAGCCATGACAGCCAAGATG
HRG1	195	TTCCTCGTCTGGACGGTGGTC	GCAGAAGGCAGCGATGGAGAC
HO-1	116	TGCCAGTGCCACCAAGTTCAAG	TGTTGAGCAGGAACGCACTCTTG
BARC1	154	GTGGAGCGAGAAGTGGCAGAAC	TACCTAACACGGACACTCAGACC
NRF2	124	ACGGTATGCAACAGGACATTGAGC	TTGGCTTCTGGAACCATG

unpaired *t*-test or Mann–Whitney, one-way ANOVA with Turkey's multiple comparisons or Kruskal–Wallis test followed by Dunn's test as indicated in the corresponding figure legends. Data that were normally distributed are presented as the mean $\pm$ S.E.M., while non-normal data are presented as the median $\pm$ 95% confidence interval (CI). Statistical significance is indicated by $P < 0.05$.

Results

The concentration of heme is higher in PF in EMS patients

The concentration of heme was $14.22 \mu\text{mol/L}$ (95% CI: $12.54\text{--}16.71$) in the supernatant of the PF of patients with endometriosis. This was higher than $9.517 \mu\text{mol/L}$ (95% CI: $8.891\text{--}10.1053$) in the control group (Fig. 1A). The PF samples were divided into a reddish group and a yellowish group. After centrifugation, all the supernatant became yellow transparent liquid but reddish PF contained more erythrocytes in the pellets. Considering that the number of erythrocytes in PF may interfere with the concentration of heme, the heme level in the reddish PF and yellowish PF was assessed and no significant differences were exhibited (Fig. 1B). The heme level of the EMS and control group was also examined in relation to the PF color. This revealed that heme level in the EMS group was always higher than that in control group, irrespective of PF color (Fig. 1C). Samples were subsequently classified by menstrual cycle and disease

severity but no differences were exhibited between groups (Fig. 1D and E).

Heme metabolism molecules are elevated in both ectopic ESCs and the pMφs of EMS

To explore if heme exhibited an effect on ESCs, qRT-PCR was used to evaluate the expression of the molecules associated with heme metabolism. CD163 and CD91 are the receptors that mediate the Hb–Hp complex internalization and heme–Hx complex internalization respectively. This results in cellular heme uptake (Ponka *et al.* 2017). The cytoplasmic heme importer, named heme carrier protein1 (HCP1), is expressed in the cytoplasmic membrane of duodenal epithelial cells and is believed to participate in dietary heme absorption. Feline leukemia virus subgroup C receptor (FLVCR) 2 is an additional protein that has been revealed to mediate heme uptake (Fujiwara & Harigae 2015). HRG1 has been demonstrated to be a special heme transporter due to the fact that it localizes to the phagolysosomal membranes and transports heme from the phagolysosome to the cytoplasm during erythrophagocytosis (White *et al.* 2013). Once delivered inside cells, heme is used to generate new hemoproteins or is catabolized by HO-1. The activation of HO is regulated via a heme-dependent pathway that is associated with the downregulation of the nuclear transcriptional repressor BTB domain and CNC homolog 1 (BACH1) and the upregulation of

Figure 1 The concentration of heme is higher in the peritoneal fluid of endometriosis patients. (A) Peritoneal heme level in control group ($n=31$) and endometriosis group ($n=31$). (B) Heme level in yellowish peritoneal fluid ($n=17$) and reddish peritoneal fluid ($n=45$). (C) Heme level in yellowish peritoneal fluid of the control group ($n=10$) and endometriosis group ($n=7$), in reddish peritoneal fluid of the control group ($n=21$) and endometriosis group ($n=24$). (D) Peritoneal heme level of the control group in the proliferative phase ($n=14$) and in the secretory phase ($n=17$), of endometriosis group in the proliferative phase ($n=13$) and in the secretory phase ($n=18$). (E) Peritoneal heme level in the control ($n=31$) and the endometriosis group of I/II stages ($n=8$) and III/IV stages ($n=23$). In (A, B, C, D and E), data are presented as the median with 95% CI. Mann–Whitney test (A, B, C and D), Kruskal–Wallis test with Dunn's multiple comparison test (E); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, NS, no significance.

nuclear transcription erythroid 2-related factor (NRF2) (Chiang *et al.* 2018). Excess heme, produced subsequent to the degradation system being overwhelmed, is transported out of the cell via cell-surface heme exporter FLVCR1 and PPIX exporter ATP-binding cassette protein (ABCG2) (Immenschuh *et al.* 2017) (Model graph seen in Fig. 2A).

We found that compared with normal ESCs (NESCs), ectopic ESCs (EESCs) upregulated the expression of *CD91*, *FLVCR2*, *HO-1* and *NRF2* and downregulated *BACH1*, suggesting that an increased amount of heme was transported into cells and catabolized. Heme exporter *ABCG2* was also upregulated (Fig. 2B). The expression of these molecules in the peritoneal macrophages of endometriotic patients (EMS-pMφ) was also compared with patients who did not exhibit EMS (Ctrl-pMφ). Similar trends were demonstrated in the peritoneal macrophage. However, only *HO-1* and *HRG1* expression exhibited significant differences when compared with the control and the EMS group had significant differences (Fig. 2C).

Impaired phagocytosis of pMφs in patients with EMS

To further assess the phagocytosis of macrophage from EMS patients, firstly the purity of macrophages isolated from PF was investigated and the result revealed that macrophage purity was above 95% (Fig. 3A). FCM data revealed that the phagocytosis ratio of peritoneal macrophages in EMS was lower compared with the control group (Fig. 3B and C), which was consistent with results of previously published studies (Wu *et al.* 2013).

High level of heme impairs the phagocytic ability of macrophage

To investigate whether the effect of heme on macrophage phagocytosis was universal or not, three types of macrophages, including peritoneal macrophage from patients with EMS (EMS-pMφ) or without EMS (Ctrl-pMφ) and CD14⁺ peripheral monocyte (CD14⁺Mo) were used. Subsequent to the isolation from the PF or PBMC by anti-CD14 microbeads, three kinds of macrophages were treated with different concentration of heme or DMSO in 96 wells for 20h respectively, prior to coculture. Medium with a 10 μmol/L heme concentration was used to mimic the low heme peritoneal environment of the control group, while medium with 30 μmol/L heme was used to mimic the high heme peritoneal environment in the EMS group.

The results revealed that a higher heme concentration impaired the phagocytic ability of EMS-pMφ when compared with the low heme concentration treatment (Fig. 4A and B). A similar phenomenon has been observed in Ctrl-pMφ and CD14⁺Mo (Fig. 4C, D and E). However, when compared with the DMSO treatment, the effect of a low heme concentration was varied in

different macrophages. In the CD14⁺Mo group, the negative effect of heme on the phagocytic ratio was dose dependent (Fig. 4F). In relation to EMS-pMφ or Ctrl-pMφ, the dose-dependent phenomenon was not observed (Fig. 4B and D).

Discussion

This study has found that women with EMS had significantly higher heme levels in the PF compared with the control group. Heme level was not correlated with the differences in PF color, phase of menstrual cycle or disease severity. However, it should be noted that previous work performed by Wolfler revealed no significant differences in peritoneal heme concentration between an endometriosis group or control group (Wolfler *et al.* 2013). Possible explanations for the discrepancies between the studies may be due to different methods of centrifugation (not mentioned in Wolfler's article) and PF sample size. Wolfler verified lower Hx level in the PF of patients with EMS. Hx exhaustion, being the scavenger protein with highest binding affinity to heme, usually indicates the presence of increased heme (Hahl *et al.* 2017).

There is an abundance of indirect evidence in previous research implying a heme overload in the peritoneal environment of patients with EMS. The increased Hb concentration has been revealed in the PF (Van Langendonck *et al.* 2002), providing possibilities for the accumulation of its byproduct, heme. It is well known that HO-1, the inducible HO isoform, is highly upregulated by heme to protect cells against oxidative damage and apoptosis (Immenschuh *et al.* 2017). In the results gained in the present study, HO-1 overexpression was revealed in EESC and EMS-pMφ, indicating that cells may live in a high heme environment. Additionally, cells exhibiting FLVCR2 overexpression were demonstrated to exhibit a heightened sensitivity to heme overload (Duffy *et al.* 2010). The results of the current study identified the upregulation of *FLVCR2* and an additional heme importer *CD91*, in EESC. Notably, EESC also increased the expression of heme exporter *ABCG2*, which may indicate that the intracellular heme degradation system is overwhelmed and excess heme requires exportation out of cell to avoid heme toxicity. The differential gene expressions of heme metabolism molecules further sustains the assumption that heme is accumulated in the peritoneal cavity of EMS patients.

Many studies have concluded that heme serves a role as an oxidant in the pathogenesis of EMS (Van Langendonck *et al.* 2002, Iwabuchi *et al.* 2015, Vitale *et al.* 2018). Heme can trigger reactive oxygen species (ROS) production, exacerbating the oxidant/antioxidant imbalance in EMS (Donnez *et al.* 2016). Excess ROS can cause oxidative damage in the peritoneal mesothelium, inducing adhesion for ESC. On the other hand, the metabolism of heme by HO-1 can generate

Figure 2 Heme metabolism molecules are elevated in both the ectopic ESCs and the pMφs of EMS. (A) A schematic diagram shows the molecules that are associated with heme metabolism. '↑': the direction of heme transport. (B and C) Relative mRNA expression of heme metabolism molecules in (B) ESCs ($n=8$) and (C) pMφ ($n=6$). Data are presented as the mean $\pm$ s.e.m.; two-tailed, unpaired t -test; * $P < 0.05$, ** $P < 0.01$.

iron. Iron overload has been revealed in endometriotic endometrial tissues, endometriotic cyst fluid and the PF of patients with EMS (Asghari *et al.* 2018). Free iron

produces ROS via the Fenton reaction and also increases endometrial cells proliferation and angiogenesis in EMS (Scutiero *et al.* 2017). Until now, most previous

Figure 3 Phagocytosis of pMφs is reduced in patients with EMS. (A) Representative image of the purity of magnetically isolated pMφs using FCM. The number is the percentage of CD14⁺CD45⁺ cells. (B) Representative image of phagocytosis ratio of pMφs in women with or without EMS verified by flow cytometric analysis. Phagocytosis ratio = CFSE⁺CD45⁺ cells / CD45⁺ cells. (C) Quantification of phagocytosis ratio of pMφs of women with or without EMS; $n = 12$. Data are presented as the mean $\pm$ S.E.M.; two-tailed, unpaired t -test; *** $P < 0.001$.

studies have focused on the oxidative function of heme. However, these studies did not assess the immunological function which may facilitate the intense inflammatory response and may be involved in the impaired immune surveillance in EMS.

Emerging evidences has demonstrated how heme affects the function of various immune cells including macrophages (Martins & Knapp 2018, Zhong & Yazdanbakhsh 2018). Heme can activate macrophages and induce the pro-inflammatory cytokines or chemokines including tumor necrosis factor (TNF), leading to neutrophil migration. Heme has also been revealed to activate the nucleotide-binding domain, leucine-rich repeat and pyrin domain-containing protein 3 (NLRP3) inflammasome and upregulate TLR-dependent pro-inflammatory responses in murine macrophages (Vijayan *et al.* 2018). Additionally, heme can regulate the phagocytic potential of macrophages. In Martins research, human and murine macrophages undergoing heme treatments exhibited strongly impaired bacterial phagocytosis, allowing for unrestrained bacterial proliferation and an impaired outcome of *E. coli* sepsis. This process requires the activation of the GTP-binding Rho family protein Cdc42 using the guanine nucleotide exchange factor DOCK8 (Martins *et al.* 2016). Hualin also demonstrated that heme-activated murine macrophages appeared to enhance phagocytosis (Hualin *et al.* 2012), the opposite result to those recorded by Martins *et al.* The dose of heme for the former experiment was 3, 10 and 30 $\mu\text{mol/L}$ and subjects were human monocyte-derived and mice peritoneal macrophages. However, the latter experiment used 5 $\mu\text{mol/L}$ of heme to treat primary rat alveolar macrophages. This indicated that the effect of heme on macrophages may vary across a variety of cell types and concentrations of heme treatment.

In our research, the existence of heme accumulation was demonstrated in patients with endometriosis, and the efficiency of macrophage phagocytosis was assessed under different heme concentration, indicating the association between heme accumulation and decreased macrophage function in EMS. Despite the vague effect of low heme concentrations, high heme treatment, which mimics the peritoneal environment in EMS patients, impaired macrophage phagocytosis and thus provides a novel perspective in the explanation of the dysfunction of macrophage in EMS. Combined with the measurement of the concentration in the PF, the results suggest that in normal situations, viable ESCs reflux into the peritoneal cavity with retrograded blood, red blood cells rupture and a small amount of heme is released into the peritoneal cavity. Both peritoneal macrophages and recruited CD14⁺Mφs could eliminate excess heme and shed ESCs. In patients exhibiting EMS, a high level of heme accumulates due to the large amount of menstrual blood that is refluxed into the peritoneal cavity and the repeated hemorrhage from endometriotic lesions.

Figure 4 High level of heme impairs the phagocytic ability of macrophage. (A, C and E) Representative flow cytometric analysis of phagocytosis ratio of EMS-pMφ (A), Ctrl-pMφ (C) and CD14⁺Mo (E). (B, D and F) Flow cytometric quantification of phagocytosis ratio of EMS-pMφ (B), Ctrl-pMφ (D) and CD14⁺Mo (F), treated with DMSO or heme (10 μmol/L, 30 μmol/L); $n = 12$. Data are presented as the mean $\pm$ S.E.M.; one-way ANOVA with Turkey's multiple comparison test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

High concentrations of heme can significantly impair the phagocytic potency of the recruited CD14⁺Mos and resident pMφs and can lead to shed endometriotic cells remaining and finally causing endometriosis (Fig. 5).

According to previous studies, the increase of IDO1, PGE2 and IL-33, and the decrease of CD36, MMP9 and annexin A2 are associated with impaired phagocytosis of the peritoneal macrophage in EMS (Wu *et al.* 2005, 2013, Chuang *et al.* 2009, 2010, Mei *et al.* 2014). In particular, CD36 has been found to be regulated by heme in Alzheimer's disease, where it mediates the impaired astrocytic capacity of scavenging β -amyloid (Sankar *et al.* 2018). IDO1 is a heme-dioxygenase enzyme that catalyzes the metabolism of L-tryptophan. L-tryptophan is also stimulated by heme and the heme byproduct iron in *Mycobacterium leprae*

(de Mattos Barbosa *et al.* 2017). This study proposes the possibility that CD36 and IDO1 may be downstream molecules of heme that participates in the regulation of macrophage phagocytic function. The effects of heme on other molecules, including PGE2, IL-33 and MMP9, are quite ambiguous (Ma *et al.* 2017, Min *et al.* 2017, Lv *et al.* 2018) and cannot extend directly to EMS. Therefore, further work is required. Besides, the mechanisms by which EMS-pMφ and CD14⁺ Mo behave differently in low heme concentration remains to be established.

To summarize, our data revealed that an excess of heme exists in the pelvic cavity of patients with EMS and this causes impaired phagocytosis of the peritoneal macrophages. This finding not only provides support for the theory of immune dysfunction in the

Figure 5 Schematic diagram of the key findings. This study has shown that patients with and without EMS have a particular concentration of heme in the peritoneal fluid but the concentration was higher in the EMS group. Under normal conditions, both peritoneal macrophages and recruited peripheral monocytes will eliminate the ESCs, avoiding the presence of ectopic endometrial lesions. However, when the reflux of large amounts of menstrual blood and the hemorrhage from endometriotic lesions occurred repeatedly, heme is accumulated and a high level of heme impairs the phagocytosis of macrophages. Shed ESCs are able to survive and implant, leading to EMS.

pathogenesis of EMS, but also provides new perspective in the development of improved treatments in this refractory disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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