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Reduction of myeloid derived suppressor cells by inhibiting Notch pathway prevents the progression of endometriosis in mice model

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ABSTRACT

Growing evidence suggested that immune dysregulation is one of the crucial drivers to the development of endometriosis (EMS). Myeloid derived suppressor cells (MDSCs) represent a heterogeneous subset of immature myeloid cells, and have been reported to promote the onset and progression of EMS. Notch signaling pathway played a major role in immunological reactions. Studies have found Notch signaling pathway could regulate MDSCs. However, how the biological effects of Notch signaling pathway on MDSCs may work in EMS is still unknown. In our study, we first built an endometriosis induced mice model. Then we treated mice with DAPT, a Notch signaling pathway inhibitor, or saline. We found that the DAPT could prevent the progression of EMS. The ADAM17, Notch1, Jagged1 and Hes1 were overexpressed in EMS mice, however, when mice were treated with DAPT, the overexpression was reduced. Meanwhile, we found a lower level of MDSCs in the DAPT treated EMS mice as compared to EMS mice without DAPT, accompanied by an increase of T helper (TH) 17 cells and a decrease of regulatory T cells (Tregs). We also investigated the reactive oxygen species (ROS) in peritoneal and endometriotic cells. Our results showed that ROS level decreased in both peritoneal and endometriotic cells in the study group treated with DAPT. Overall, our study indicates for the first time that blockage of Notch signaling could lessen MDSCs and ROS, and therefore preventing the development of endometriosis.

1. Introduction

Endometriosis (EMS) is a common gynecological problem with heterogeneous symptoms such as dysmenorrhea, infertility, dyspareunia and pelvic inflammatory reactions [1,2]. It is characterized by the presence of endometrial tissue outside the uterus with complex pathogenesis [1]. 10% of all women with reproductive-age are affected by this disease, and symptoms caused by EMS have damaged patients' quality of life [3,4]. Studies about the etiology of endometriosis have been carried through for decades, however, explicit mechanisms are still absent.

Growing evidences support that immunologic and inflammatory imbalance could be related to the progression of EMS [5–10]. In recent years, studies have been focused on the aggrandized immunosuppressive microenvironment in EMS [11–14]. Excessive immunosuppressive regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) have been reported to foster the growth of endometriotic lesions [6,11–13]. Meanwhile, impaired immune response such as dysfunction of T and B cells, reduced cytotoxic NK were confirmed to exist in EMS microenvironment [5,6,9].

MDSCs represent a heterogeneous subset of immature myeloid cells which fail to reach final differentiation [15,16,17]. Researchers have identified MDSCs in mice as $CD11b^{+}Ly6G^{+}Ly6C^{low}$ polymorphonuclear MDSCs (PMN-MDSCs) and $CD11b^{+}Ly6G^{low}Ly6C^{+}$ monocytic MDSCs (Mo-MDSCs) subpopulations [16–18]. In human, MDSCs are divided into two subpopulations: $HLA\text{-}DR^{-/low}CD11b^{+}CD33^{+}CD14^{+}CD15^{-}$ Mo-

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MDSCs and HLA-DR$^{-/low}$CD11b^{+}CD33^{+}CD14^{-}CD15^{+} PMN-MDSCs [18]. The immunosuppressive mechanisms of MDSCs were reported to be their ability of generating nitric oxide (NO), arginase (ARG)-1 and reactive oxygen species (ROS) [19,20]. Our previous study indicated that NO produced by MDSCs might break TCR-ζ chain of effector T cells and therefore impair their ability to clear the endometriotic fragments [11]. Similarly, Chen and his group found that in vivo Mo-MDSCs from patients with EMS produced high level of ROS and remarkably suppressed T cells function [12]. However, which signaling pathways may function in the recruitment and modulation of MDSCs in EMS patients is still unclear.

One of the most well-known signaling pathways, the Notch pathway, has been found to take part in various important cellular fates, such as maintenance of stem cell, angiogenesis and metastasis of tumor cells, proliferation and development of immune cells [21,22]. Notch signaling is an evolutionarily conserved and highly cell-type dependent pathway [21]. The responses to this signaling involve two pathways: canonical and non-canonical pathways. In canonical pathway, the ligands such as Jagged1, Jagged2, DLL1, DLL3, or DLL4 interact with the Notch receptors (Notch 1–4), and then activate the Notch signaling [22]. The non-canonical pathway needs to be in league with other signaling such as Wnt, STAT3 and NFκB [22,23]. A variety of studies have proven that Notch signaling played a major role in many autoimmune disorders through regulation of immune cell differentiation and inflammatory reactions [24–28]. Researchers also implicated that Notch signaling pathway could promote the development of endometriosis through sprouting angiogenesis of endometriotic implantation, strengthening the invasion of endometrial lesions and formation of fibrosis [27]. However, till now there are no studies that have investigated the possible role of immune response regulated Notch signaling in the progression of endometriosis.

Numerous studies have demonstrated that Notch pathway is a key regulator for MDSCs [22,23,29]. Inhibition of Notch signaling in MDSCs and DCs were found to correlate with abnormal myeloid cell differentiation in cancer [22,23]. Notch pathway can support STAT3 pathway and activated IL-6-STAT3 pathway promote expansion of MDSCs in cancer [22]. Our study aims to find whether the notch signaling takes part in regulating the MDSCs in endometriosis microenvironment.

2. Materials and methods

2.1. Establishment of peritoneal endometriosis mouse model

A total of 54 seven weeks old BALB/c female mice (25–30 g in weight) were purchased from Animal Center of Anhui Medical University. Mice were kept under pathogen-free conditions in the animal center. Animal studies have been approved by the Institutional Animal Care and Use Committee (IACUC). Endometriosis was induced according the protocol from Z. Zhang's group [23]. 0.2 mg of estradiol valerate was injected subcutaneously to each mouse one week before the surgery. Then the uteruses from the donor mice were removed and the serosa and myometrium were peeled away. At the end, endometrium rinsed in phosphate buffered saline (PBS), and minced into 1.0 mm pieces. The processed endometrial pieces which were suspended in sterile saline were injected into the peritoneal cavities of recipient mice with a mount of 1 ml endometrial suspension per mouse, and endometrial fragments obtained from one donor mouse were injected to two recipient mice.

One day later, the mice were randomly divided into three groups: Control mice without endometriosis-induction (CM, n = 18), endometriosis mice group (saline-EMS, n = 18) and endometriosis mice treated with DAPT group (DAPT-EMS, n = 18). The mice in DAPT-EMS group were given intra-peritoneal dose of 1 ml 0.1% DAPT every 4 days for 4 weeks. The other two groups were given same amount of saline. After the 4 weeks of treatment, all mice were sacrificed. The peritoneal fluid (PF), the endometrium (EM) and endometriotic lesions (EL) were harvested from the mice. Every three mice were used separately for each experiment. And each experiment was repeated for one time.

2.2. Measurement and analysis of endometriotic lesions

After euthanasia, EM and EL from all mice were counted and scaled. Then, some lesions were grafted and formalin fixed, paraffin embedded. The paraffin blocks were sectioned. After deparaffination, the sections were stained with hematoxylin and eosin, then viewed by the Inverted Phase Contrast Microscope (Olympus, Japan).

2.3. Sampling mice

5 ml PBS was injected intraperitoneally to the mice, and then PF were collected from each mouse after the treatment. The fluid samples were centrifuged at 1500 rpm for 5 min. The cells and supernatants were preserved for later analysis. Single cell suspensions were prepared by collagenase I (Yusheng, China) according the protocol. Briefly, EM from CM groups and EL from saline-EMS and DAPT-EMS groups were cleaned and cut to pieces, then 2.5 mg/ml collagenase I was used, then a Ficoll (GE Healthcare, Sweden) gradient was performed. Mononuclear cells (MNCs) were collected for later experiments.

2.4. ROS measurement by fluorescence microplate

2, 7- dichloroluorescein diacetate (DCFH-DA) (Sigma, USA) was applied to detect ROS produced by peritoneal cells and endometrial and endometriotic cells. We followed the manufacturer's instruction (Fluorometric Intracellular ROS Kit, Sigma, USA). Fluorescence microplates (Thermo, USA) were used to value the samples.

2.5. Test of glutathione (GSH), hydrogen peroxide (H_2O_2) enzyme and superoxide dismutase (SOD)

GSH kits, CAT kits and SOD kits (Jiancheng, China) were applied according to the protocols from the manufacturer. In short, endometrial orendometriotic cells were homogenated. The homogenated fluid or supernatant from PF were added to each kit respectively.

2.6. RNA isolation and Real-Time PCR

Total RNA was extracted from the EM (CM group) and EL (saline-EMS and DAPT-EMS groups) under RNase-free condition. cDNA was synthesized using the SuperScript III cDNA synthesis kit (Thermo Fisher Scientific) according to the instruction from the producer. Real-time PCR was performed with SG Fast qPCR Master Mix (2X) using a LightCycler 480 II (Roche, Germany) using cycle conditions: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 sec, 60 °C for 1 min. The primers for analysis were: ADAM17-F – CCAAAAGCTTATTACAACCC, ADAM17-R – ATGTCCCAATTCATGAGTTG, Notch1-F – AGGGTGGTC AGGAAAATCAT, Notch1-R – CTCATTAACATCTTGCCTGC, Jagged1-F – GTCTACGCCTGTCATCGG, Jagged1-R – GTGTCATTACTGGAATCCCA, Hes1-F – TACCCCAGCCAGTGTCAA, Hes1- R – CCGGGAGCTATCTTTC TTAA.

2.7. Flow cytometry analysis

Cells from the EL (saline-EMS and DAPT-EMS groups) and EM (CM group) were incubated with FcR Blocking Reagent (Miltenyi Biotech, Germany) and stained accordingly with anti-mouse antibodies (mAbs). The antibodies used for MDSCs, T helper (TH) 17 cells and Tregs were listed in Table 1. For TH17 cells, we added 2ul of Leukocyte Activation Cocktail, with BD GolgiPlug™ (BD Biosciences, USA) for every 1 ml of cells culture and mixed thoroughly, then placed the culture in a 37 °C humidified 5% CO2 incubator for 4 h, and then antibodies were added. For intracellular staining, samples were pre-incubated with the FOXP3

Table 1
Antibodies used for MDSCs, TH17 cells and Tregs.

Antibodies	Company
Ly-6C Monoclonal Antibody, APC	eBioscience, USA
Ly-6C Monoclonal Antibody, PE	eBioscience, USA
CD11b Monoclonal Antibody, FITC	eBioscience, USA
Rat IgG2a kappa Isotype Control, APC	eBioscience, USA
Rat IgG2a kappa Isotype Control, PE	eBioscience, USA
Rat IgG2a kappa Isotype Control,FITC	eBioscience, USA
FcR Blocking Reagent (mouse)	Miltenyi Biotec
CD4 Monoclonal Antibody, FITC	eBioscience, USA
CD25 Monoclonal Antibody, PE	eBioscience, USA
IL-17A Monoclonal Antibody, PE	eBioscience, USA

fixation/permeabilization kit (eBioscience, USA) according to the manufacturer's instruction. Experiments were performed on FACS-Verse™ flow cytometry (BD Biosciences) with FACSuite software (BD Biosciences). The data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

2.8. Immunofluorescence and confocal imaging

The tissues sections were deparaffinated and were heated in microwave for 15 min at 92–98 °C. Then samples were blocked with 6% BSA for 60 min, followed by incubation with primary rabbit Anti-CD45 antibody (Abcam, USA) overnight at 4 °C. After washing by PBS for 3 times, anti-Alexa Fluor 488 (Abcam, USA) was added and incubated to 60 min at 37 °C. At the end, the stained sections were imaged using a Leica TCS SP5 Confocal Laser Scanning Microscope using the 63× objective magnification in oil medium, along with 2.4× zoom (151×).

2.9. Statistical analysis

Three independent mice were used per experiment, and analyzed with GraphPad Prism software (GraphPad Software, San Diego, USA). A one-way ANOVA test was performed for multiple groups' comparisons and an unpaired Student's *t* test was used for two groups' comparisons. A p value less than 0.05 was considered statistical difference.

3. Results

3.1. Notch inhibitor DAPT regressed endometriotic lesion growth

To determine whether Notch affect endometriotic implantation, we treated endometriosis-induced mice with DAPT. Other two groups were: mice without endometriosis and mice with endometriosis received saline only (Fig. 1A). We observed 42 endometriotic implantations in saline-EMS group, and lesions are 37 between 1 and 5 mm, 4 between 6 and 10 mm, and 1 between 11 and 15 mm. And we found 27 implantations in DAPT-EMS group, as well as lesions are 26 between 1 and 5 mm, 1 between 6 and 10 mm, and 0 between 11 and 15 mm. Mice treated with DAPT had less and smaller endometriotic implantations as compared to saline-EMS mice(Fig. 1B and C). The histopathological observations by HE staining displayed that lesions from saline-EMS group had more glandular and larger stromal areas as compared to lesions from mice treated with DAPT (Fig. 1D).

3.2. Notch inhibitor DAPT reduced oxidative stress (OS) in the lesions

Next, we aimed to assess the OS level in mice from all three groups: CM group, saline-EMS group and DAPT-EMS group. First, we measured the ROS level in the peritoneal cells by DCFH-DA, as shown in Fig. 2A, saline-EMS group had highest ROS level in these three groups. Then we isolated endometrial cells from CM group and endometriotic cells from saline-EMS group and DAPT-EMS group. We detected the ROS level from these cells. The results showed that DAPT-EMS group had a decreased level of ROS as compared to saline-EMS group, but still higher than that from CM group (Fig. 2B). We also analyzed SOD, CAT and GSH in the PF and EM/EL cells, DAPT-EMS group showed higher level of SOD, CAT and GSH both in PF and EL cells, as compared to those from saline-EMS group (Fig. 2C–H).

3.3. Effect of DAPT on ADAM17, Notch1, Jagged1 and Hes1 in endometriotic cells

We investigated changes in ADAM17, Notch1, Jagged1 and Hes1 mRNA levels in response to DAPT in ectopic (saline-EMS and DAPT-EMS) and eutopic (CM) endometrial tissues. Our data revealed that as compared to eutopic endometrial tissues in control group, endometriotic lesions showed increased mRNA levels of ADAM17, Notch1, Jagged1 and Hes1 in DAPT/saline-groups (Fig. 3A–D), while mice treated with DAPT had reduced level of these mRNA in the ectopic tissues.

3.4. Detection of leukocyte ($CD45^+$) in endometrial and endometriotic tissues

Immunofluorescence was used to detect leukocyte ($CD45^+$) in endometrial and endometriotic tissues from the three groups. Cells were fluorescently stained with EdU (green) and Nuclei were stained with DAPI (blue). Qualitative assessment revealed that leukocytes were diffusely distributed within the endometrium from control mice and ectopic lesions from both EMS mice (Fig. 4).

3.5. Blocking Notch pathway reduced MDSCs in mouse with endometriosis

To examine the role of Notch signaling on MDSCs in endometriosis, we prepared the single cells suspension from endometrium from CM group and endometriotic lesions from saline-EMS and DAPT-EMS groups, then we identified MDSCs as $CD11b^+Ly6G^+Ly6C^{low}$ PMN-MDSCs and $CD11b^+Ly6G^{low}Ly6C^+$ Mo-MDSCs by flow cytometry (Fig. 5A). We found that, compared to CM group, the saline-EMS group showed recruitment of MDSCs (Fig. 5B). Whereas, in the DAPT-EMS group, MDSCs got significantly reduced as compared to these from saline-EMS group (Fig. 5C). We also tested Tregs and TH17 cells in our animals. We used CD4, CD25 and FOXP3 to characterize Tregs (Fig. 6A), meanwhile, IL-17A and CD4 for TH17 cells (Fig. 6C). The results showed an elevated level of Tregs in the saline-EMS group as compared to these from CM group, while in DAPT-EMS group, mice showed decreased level of Tregs as compared to these from saline-EMS mice (Fig. 6B). Furthermore, we also found a deletion of TH17 cells in saline-EMS mice as compared to these from control mice, however, in the DAPT group, an increased level of TH17 cells was found as compared to these from saline-EMS group (Fig. 6D).

4. Discussion

As far as we know, our study is the first one to correlate MDSCs and ADAM17/Notch signal pathway in EMS. We aim to discover a pathway that associates immune disorder with the progression of endometriosis.

In the first step of our experiments, we confirmed that ADAM17/Notch pathway could be a potential driver of the implantation of endometriotic lesions, while its inhibitor DAPT could significantly reduce the size and amount of endometriotic implants [30]. Meanwhile, our real-time PCR displayed that the expression of ADAM17, Notch1, Jagged1 and Hes1 were decreased in endometriotic lesions after treatment of DAPT. Many studies revealed that ADAM17/Notch signal pathway was a key role in the development of endometriosis [24,26,27,31]. In women with endometriosis, it has been reported that hyperactivation of Notch pathway existed in their microenvironments, and this increased activity of Notch fostered the fibroblasts in EMS [26].

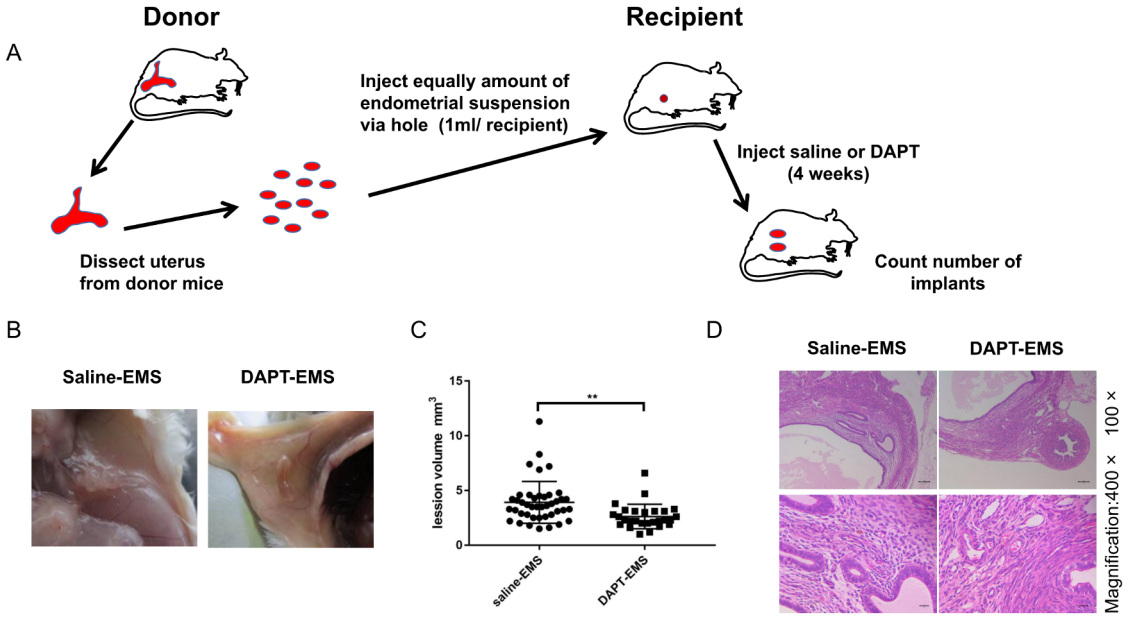

Fig. 1. Path for successful construction of mice models and differences of endometriotic lesion distribution and histopathological observation in saline-EMS group and DAPT-EMS group. (A) Showed the specific method of constructing mice model with endometriosis. (B) and (C) Exhibited the significant difference in the distribution and volume of ectopic lesions between saline-EMS and DAPT-EMS. **indicates $p < 0.01$. (D) Showed the histopathology observation of ectopic endometrial lesions by HE staining.

Laschke et al. found that Notch pathway also regulated angiogenesis in endometriosis [27]. To our best knowledge, till now there are no studies that have investigated the immunological dysregulation by Notch signal pathway in the endometriosis.

Nowadays, one well accepted hypothesis for the pathogenesis of EMS is the immunological disorder [5–10]. Our previous publications and other studies have shown that a strong immunosuppressive microenvironment was present in the EMS [11–12]. Immunosuppressive cells, such as MDSCs take a crucial part in the process of EMS [12–14]. This study is the first study which found an augment of MDSCs in the endometriotic lesions in mice. However, after mice were treated with DAPT, a significant reduction of MDSCs in the endometriotic lesions was found.

Numerous publications have pointed out that Notch pathway is a vital regulator of inflammatory reactions and immune cell development, and is involved in immunosuppressive microenvironment in different tumors and a variety of autoimmune diseases [25–28]. Myeloid cells play important roles in maintaining the homeostasis of immune reactions [22]. Many literatures have emphasized that Notch pathway is essential in myeloid cells differentiation [22,29,32]. Our results indicated that Notch pathway might take part in the inducement of MDSCs in endometriosis microenvironment, which is in line with previous studies of cancer [22,27,29,33].

As we mentioned before, one indispensable mechanism for the suppressive function of MDSCs is their ability to produce ROS [19,20]. Chen and his colleagues found that endometriosis-induced Mo-MDSCs

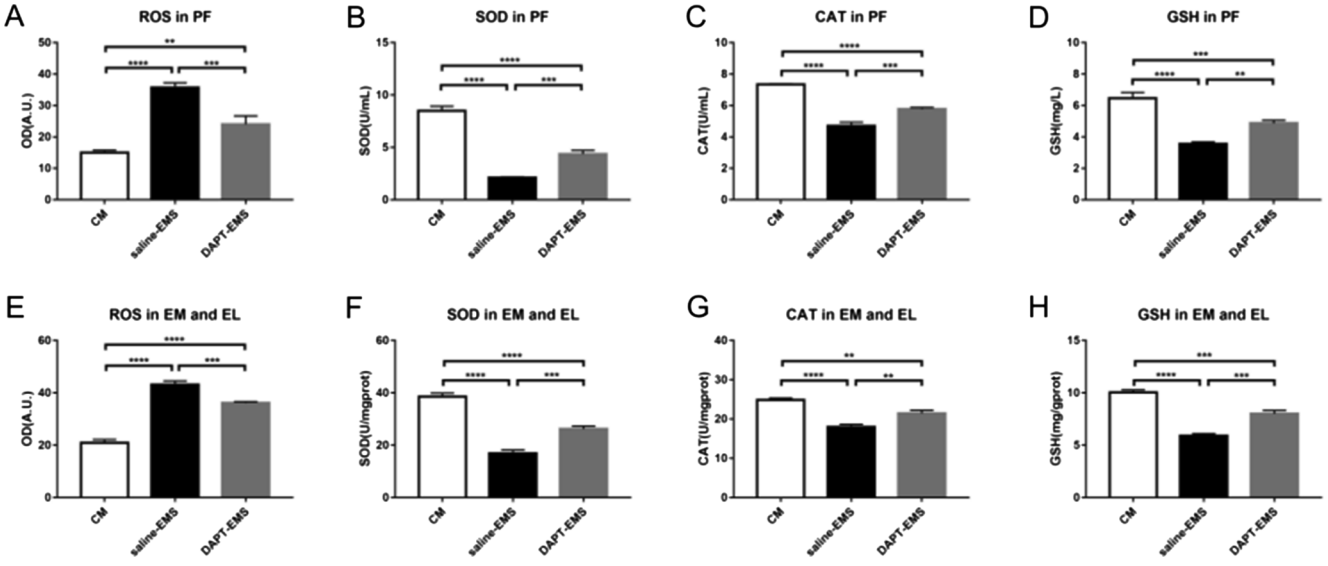

Fig. 2. Comparison of ROS, SOD, CAT and GSH levels in each group. (A) displayed the level of ROS in PF. (B)–(D) Showed the levels of SOD, CAT and GSH in PF. (E) Showed ROS levels in EM and EL. (F)–(H) displayed the levels of SOD, CAT and GSH in EM and EL. **indicates $p < 0.01$, ***indicates $p < 0.001$, ****indicates $p < 0.0001$. ROS = reactive oxygen species, CAT = catalase, SOD = superoxide dismutase, GSH = glutathione, CM = control mice, PF = peritoneal fluid, EM = endometrium, EL = endometrilotic lesion.

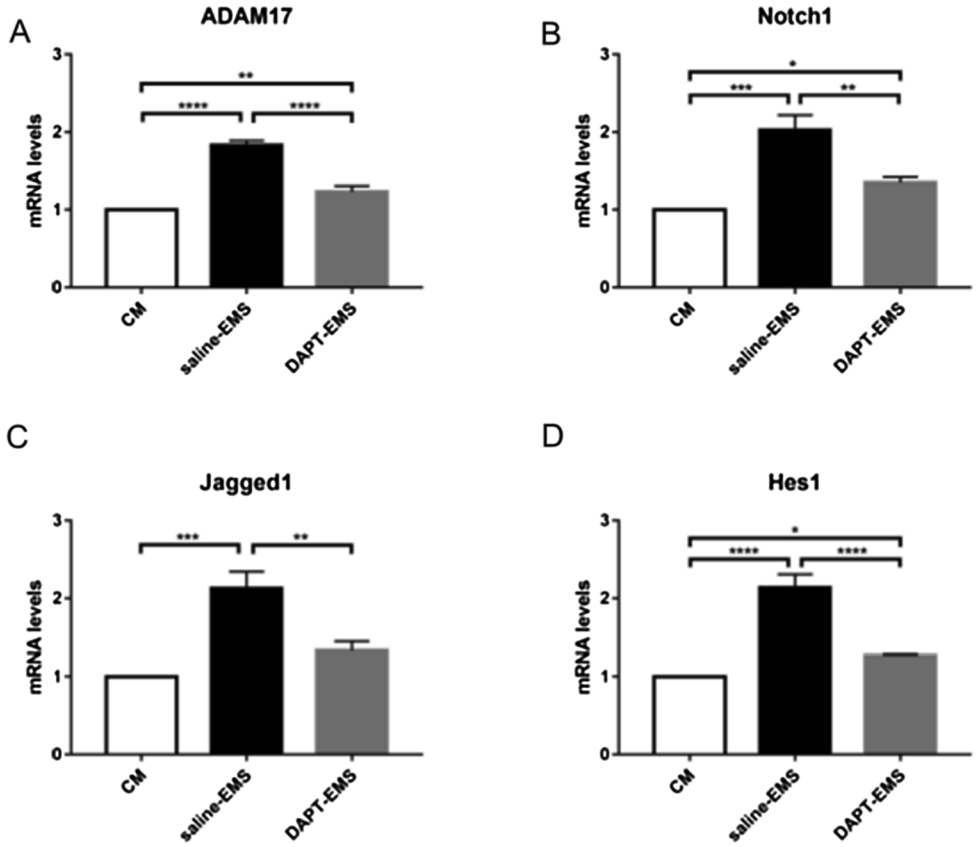

Fig. 3. Expressions of mRNA levels of ADAM17, Notch1, Jagged1, and Hes1 in EM and EL from each group. (A)- (D) showed the levels of ADAM17, Notch1, Jagged1, and Hes1 in three groups. *indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$, ****indicates $p < 0.0001$.

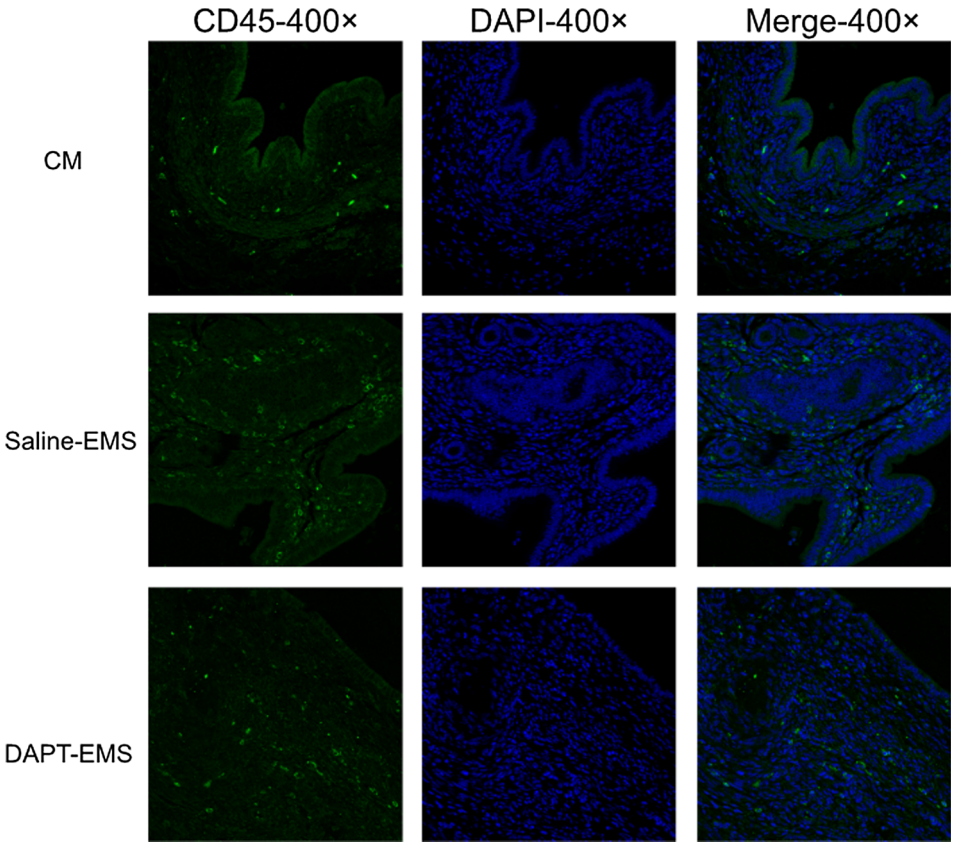

Fig. 4. Immunofluorescence detection of leukocyte (CD45+) in EM/EL and confocal microscope acquisition of images. Cells were fluorescently stained with EdU (green). Nuclei were stained with DAPI (blue). Magnification: 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

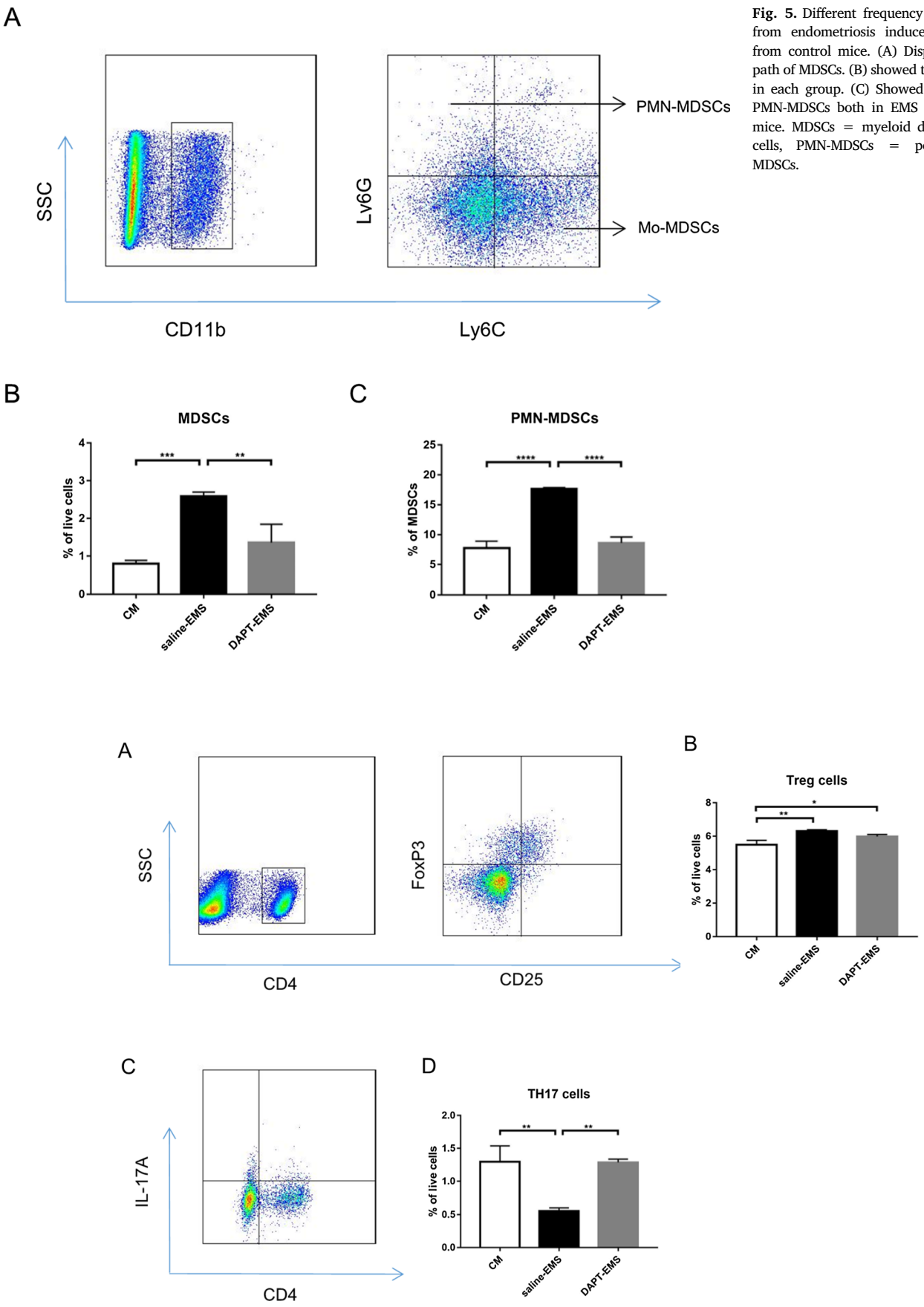

Fig. 5. Different frequency of MDSCs in EL from endometriosis induced mice and EM from control mice. (A) Displayed the gating path of MDSCs. (B) showed the level of MDSCs in each group. (C) Showed the frequency of PMN-MDSCs both in EMS mice and control mice. MDSCs = myeloid derived suppressor cells, PMN-MDSCs = polymorphonuclear MDSCs.

Fig. 6. Different levels of T cells in each group. (A) and (C) Showed the strategy to characterize Tregs and TH17 cells. (B) Displayed that both endometriosis mice groups had a significantly increased frequency of Tregs as compared to CM group. (D) Showed the TH17 cells had a reduction in endometriosis mice as compared to control mice. Tregs = regulatory T cells, TH17 = T helper 17.

could generate a high level of ROS in vivo, and therefore they deduced that Mo-MDSCs might perform their immunosuppression in a ROS-dependent manner in EMS microenvironment [12]. In the present study, we also detected the ROS level in peritoneal cells and cells isolated from endometriotic lesions [26,34]. Our results showed that mice with endometriosis had higher level of ROS in both cells as compared to mice without endometriosis, whilst in the endometriosis-induced mice treated with DAPT, these ROS levels were remarkably decreased. Further, we test SOD, CAT and GSH in the peritoneal fluid from our animals. We found a low level of SOD, CAT and GSH in the mice with endometriosis. DAPT could reverse these results and increase the level of these enzymes in endometriosis. Some study reported that blockage of Notch signaling could reduce the immunosuppressive function of MDSCs [29]. Taken together, we speculate that Notch signaling pathway may participate in the ROS production of MDSCs, however, further investigations are needed to confirm this hypothesis.

It has been well known that MDSCs and Tregs could activate each other [35]. Our results showed that Tregs also have been recruited into the endometriotic lesions in EMS mice, which is in accordance with other studies [36,37]. However, after treatment of DAPT the frequency of Tregs was reduced. Previous studies also indicated that MDSCs could suppress the differentiation and responses of TH17 cells [35,38]. Our results discovered an increased level of TH17 cells in the DAPT-EMS mice. Thus, we suggest that inhibiting Notch pathway might weaken the effect of MDSCs on Tregs and TH17 cells.

In conclusion, Notch signaling and MDSCs are two vital promoters for the initiation and progression of endometriosis. The present study indicates for the first time that blockage of Notch signaling could lessen the amount of MDSCs and weaken their immunosuppression, therefore preventing the development of endometriosis.

CRediT authorship contribution statement

Huanhuan Jiang: Funding acquisition, Writing - original draft, Methodology, Resources. **Kaihuan Bi:** Methodology, Formal analysis, Visualization. **Kangxia Wang:** Methodology, Formal analysis. **Zhimin Lu:** Data curation, Validation. **Yuping Xu:** Investigation. **Peipei Guo:** Investigation. **Caihua Li:** Validation. **Zhaolian Wei:** Writing - review & editing. **Ya Chen:** Software. **Yunxia Cao:** Conceptualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare no competing interests to this work.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2020.106352.

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