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Expansion of monocytic myeloid-derived suppressor cells in endometriosis patients: A pilot study

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ABSTRACT

Endometriosis is a chronic inflammation disease and is closely associated with immune dysregulation. Myeloid-derived suppressor cells (MDSCs) are a negative regulator of the immune system. The aim of this study was to evaluate the possible role of MDSCs in endometriosis patients. We collected the peripheral blood and peritoneal fluid from endometriosis patients and controls and analyzed M-MDSCs level using specific monoclonal antibodies recognizing HLA-DR, CD33, CD11b, CD14 markers by flow cytometry. We found that there existed abnormal expansion of monocytic MDSCs (M-MDSCs) (HLA-DR$^{-/low}$CD33^{+} CD11b^{+} CD14^{+}) in peripheral blood and peritoneal fluid of patients with endometriosis. Functional studies revealed that M-MDSCs from endometriosis patients significantly suppressed T-cell responses and produced high level of reactive oxygen species (ROS). The elevation of M-MDSCs from endometriosis patients may contribute to the disease progression.

1. Introduction

Endometriosis is a chronic disease in which endometrial tissue is found outside the uterus, mainly on the pelvic peritoneum and/or ovaries. The symptoms caused by endometriosis include chronic pelvic pain, menstrual disorders, pelvic inflammatory reactions and infertility [1–2].

Multiple lines of evidence have suggested that endometriosis is associated with the changes of local and systemic immunity. However, the underlying mechanisms are diverse, including dysfunction of T and B cells; increased levels of various proinflammatory cytokines and angioregulatory cytokines, such as IL-6, IL-1 and TNF-α; reduced NK cell activity and the production of auto-antibodies [1–5]. One recent study showed that the percentage of CD25^{high}Foxp3^{+} Treg cells was significantly increased in endometriosis patients [6]. These studies collectively suggest a close relationship between endometriosis and the impaired immune response. Other immune cells with immunosuppression function may participate in the pathogenesis of endometriosis.

Myeloid-derived suppressor cells (MDSCs), are a heterogeneous population of myeloid progenitor cells that cannot fully differentiate into mature myeloid cells under some pathological conditions, such as cancer, autoimmune disorders and inflammatory diseases [7–12]. The main feature of MDSCs is their immunosuppression function, especially towards T cells in a context-dependent manner [8]. Human MDSCs with the phenotype of HLA-DR$^{-/low}$CD11b^{+}CD33^{+}, can be further divided into two major subsets: monocytic MDSCs (M-MDSC), which are CD14^{+}CD15^{-} cells and granulocytic MDSCs (G-MDSC), which express CD15 but not CD14 [8–9]. These two subsets differ in many aspects, such as immunosuppressive activity, tissue distribution, morphology and surface markers [8–9].

To our knowledge, little is known about the role of MDSCs in the pathogenesis of endometriosis, though MDSCs were found to be important in maintaining materno–fetal tolerance [13–14]. Therefore, the aim of our present study was to investigate the level and function of MDSCs in the peripheral blood and peritoneal fluid of patients with endometriosis.

2. Materials and methods

2.1. Subjects

In this study, Endometriosis (Endo) patients were recruited at Affiliated Guangzhou Women and Children's Medical Center, Guangzhou, China. A number of 32 women (mean age 29 years, range 18–38)

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were laparoscopically and histopathologically confirmed endometriosis included stage I/II (n = 14) and stage III/IV (n = 18), which was staged according to the revised American Fertility Society scoring system [15]. Stage I/II of endometriosis patients were those who had a small lesion in ovarian and stage III/IV of endometriosis had ovarian endometriotic cysts. The control group consisted of 31 women (mean age 32 years, range 19–44) without pelvic inflammation, visible endometriosis foci or related pathology who underwent laparoscopic excision of ovarian dermoid cysts or diagnostic laparoscopy.

Samples from the patients and controls were collected on days 6–9 of the menstrual cycle. All women were screened to meet the criteria as follows: no autoimmune disorder, no other chronic inflammatory, no recent acute infection and no pharmacological treatment which would affect immune response for at least 3 months prior to the study. The study was approved by the Ethics Review Board of the Guangzhou Women and Children's Medical Center Hospital, as well as the Ethics Review Board of the Sun Yat-Sen University. Written informed consent was obtained from all participants and/or their legal guardians.

2.2. Mononuclear cells (MNCs) isolation

Peripheral blood (PB) was collected in the vein and peritoneal fluid (PF) was aspirated from the *cul de sac* at the beginning of the standard laparoscopic procedure under general anaesthesia. There were 63 samples of PB, including 32 samples from patient and 31 samples from healthy persons. The samples of PF with blood contamination were discarded. As a result, there were 15 cases, including stage I/II (n = 7) and stage III/IV (n = 8), and 11 cases of peritoneal fluid in patients and healthy subjects, respectively. When the peripheral blood and peritoneal fluid were collected, the sample was temporarily stored at 4 °C. Then, the peripheral blood and peritoneal fluid were centrifuged (400*g*, 8 min, 4 °C), and the supernatant was frozen at −80 °C as soon as possible. Mononuclear cells, including peripheral blood mononuclear cells (PBMCs) and peritoneal fluid mononuclear cells (PFMCs), were isolated from heparinized blood and peritoneal fluid of patients by Ficoll-Hypaque. The isolated PBMC and PFMC were washed with ice-cold PBS to remove the residual Ficoll-Hypaque solution and analyzed immediately, as described previously [12].

2.3. Flow cytometry

The cellular phenotypic analysis was carried out by indirect immunofluorescence. The following anti-human antibodies were purchased from eBioscience (San Diego, CA): CD11b-FITC, CD33-PE, HLA-DR-PE-Cy5, CD14-PE-Cy7, CD15-eFluor450, CD4-PE, CD8a-PE-Cy5, CD3-PE-Cy7 CD25-FITC and the corresponding isotype controls. For phenotype analyses, 1 million cells were analyzed by flow cytometry on a flow cytometer (BD LSRFortessa; BD Biosciences, San Jose, CA) and data were analyzed with FlowJo 7.6 (Tree Star Inc. Ashland, OR, USA). Data were acquired as the fraction of labeled cells within a live-cell gate set for 50,000 events. For functional experiments, M-MDSCs and T cells were isolated on the FACSAriaII cell sorter (BD Biosciences). The strategy for sorting M-MDSC was HLR-DR$^{-/low}$CD11b^{+} CD33^{+} CD14^{+} CD15^{-} cells from live PBMCs; T cells were sorted as CD3^{+} cells from live peripheral blood mononuclear cells (PBMCs). 4′,6-Diamidino-2-phenyindole (DAPI) (1 μg/ml; Roche, Switzerland) was used to distinguish live cells from dead cells. All the experiments were performed in the biosafety laboratory. To control for instrument and staining variability over time, all the sample treatment was done by two investigators. For flow assay, each time, the same number of cells was stained by using the same amount of flow antibodies. All the flow data were obtained by pre-set flow cytometry protocol.

2.4. T cell proliferation assay

T cell proliferation was evaluated by CFSE (5, 6-carboxyfluorescein diacetate, succinimidyl ester) dilution as previously described [12]. Briefly, enriched PBMCs from endometriosis patients and controls, with or without the depletion of M-MDSC, were labeled with CFSE (3 μM; Invitrogen) and stimulated with anti-human CD3 antibodies (5 μg/ml; eBioscience) coated plates and soluble anti-human CD28 antibodies (5 μg/ml; eBioscience) for 3 days. The cells were then stained for surface marker expression with CD3-PE-Cy7, CD4-PE, and CD8a-PE-Cy5 antibodies.

Similarly, sorted CD3^{+} T cells containing CD4^{+} T cells and CD8^{+} T cells were cultured alone or co-cultured with autologous M-MDSCs at different ratios for 3 days (T/M-MDSC: 1:0, 8:1 and 2:1). Cells were then stained with CD4-PE and CD8a-PE-Cy5 antibodies. T cell proliferation was analyzed by flow cytometry on BD LSRFortessa flow cytometer (BD Biosciences, San Jose, CA). A proliferation index was calculated by FlowJo 7.6 (Tree Star Inc. Ashland, OR, USA), as previously described [16–17].

2.5. Cytokine quantification

The content of IFN-γ in supernatant, which was collected from the T cells and M-MDSCs co-culture, was determined by enzyme-linked immunosorbent assay (ELISA). ELISA was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

2.6. Arginase activity assay

The activity of arginase was measured in cell lysates (about 0.5 million cells). We followed the same procedure as described earlier [10].

2.7. NO production

We followed the same procedure as described early [12]. Briefly, plasma samples from endometriosis patients and controls (120 μl) were first mixed with $ZnSO_4$ (6.4 μl) and vortexed, and then NaOH (6.4 μl) was added, followed by centrifugation for 10 min at 14000 rpm. One hundred microliters of the deproteinized supernatants were transferred to a clean tube, mixed with Griess reagent, and incubated for 10 min at 60 °C. The absorbance at 550 nm was measured using a microplate reader (Bio-Rad, Hercules, CA). Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of 0.25 mM sodium nitrite.

2.8. ROS measurement

Oxidation-sensitive dye Dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probes/Invitrogen) (Carlsbad, CA) was used to measure reactive oxygen species (ROS) production by M-MDSCs. We followed the same procedure as described earlier [10]. Sorted M-MDSCs (0.3 million cells) from endometriosis patients and controls were incubated in 96-well plates at 37 °C in the presence of 2.5 μM DCFDA for 30 min. Cells were washed with PBS two times and analysis was then conducted by flow cytometry (BD LSRFortessa; BD Biosciences, San Jose, CA).

2.9. Quantitative real-time PCR

Total RNA from sorted M-MDSCs derived from endometriosis patients and controls was extracted with an RNase Minikit, and cDNA was synthesized using a SuperScript III reverse transcriptase kit (Qiagen, Valencia, CA). PCR was performed as described earlier [18] and the primers for analysis of M-MDSCs cell–related gene expression