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MDSCs drive the process of endometriosis by enhancing angiogenesis and are a new potential therapeutic target

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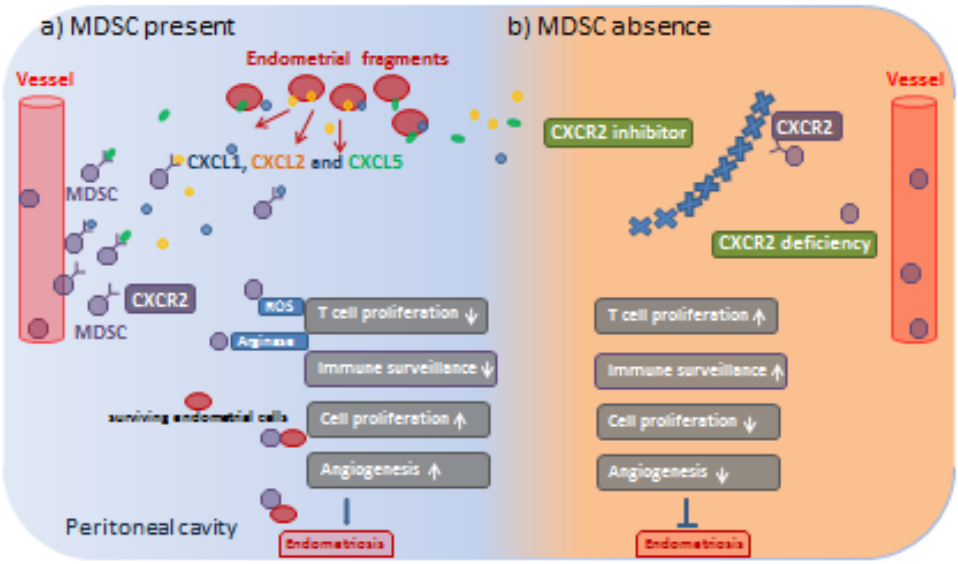
Abstract

Endometriosis affects women of reproductive age via unclear immunological mechanism(s). Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of myeloid cells with potent immunosuppressive and angiogenic properties. Here we found MDSCs significantly increased in the peripheral blood of patients with endometriosis and in the peritoneal cavity of a mouse model of surgically-induced endometriosis. Majority of MDSCs were granulocytic, produced ROS and arginase, and suppressed T cell proliferation. Depletion of MDSCs by antiGr-1 antibody dramatically suppressed development of endometrial lesions in mice. The chemokines CXCL1, 2 and 5 were expressed at sites of lesion while MDSCs expressed CXCR-2. These CXC-chemokines promoted MDSC migration towards endometriotic implants both *in vitro* and *in vivo*. Also, CXCR2-deficient mice show significantly decreased MDSC induction, endometrial lesions and angiogenesis. Importantly, adoptive transfer of MDSCs into CXCR2-KO mice restored endometriotic growth and angiogenesis. Together, this study demonstrates that MDSCs play a role in the pathogenesis of endometriosis and identifies a novel CXC-chemokine and receptor for the recruitment of MDSCs, thereby providing a potential target for endometriosis treatment.

MDSCs play an immunosuppressive and pro-angiogenic role in the development of endometriosis. A novel CXC-chemokine and receptor were identified for the recruitment of MDSCs, thereby providing a potential target for endometriosis treatment.



Keywords: endometriosis; myeloid-derived suppressor cells (MDSCs); angiogenesis; CXCR2; immunosuppression

Introduction

Endometriosis is an inflammatory gynecological disorder that is frequently seen in women during their reproductive age[1, 2]. Patients with endometriosis experience pain[3, 4], irregular bleeding[4] and often infertility[5]. While pain medications[6], hormonal therapies[7] and surgery[7, 8] are usually used in the treatment of patients with endometriosis, such treatment modalities exhibit substantial side effects with high recurrence rates.

The theory of retrograde menstruation which describes how viable endometrial tissue spreads into the peritoneal cavity through the fallopian tubes during menstruation is widely accepted [9, 10]. However, the incidence of endometriosis is small when compared to the frequency of the retrograde menstruation experienced by most women. To explain this discrepancy, it was suggested that the immune functions of women with endometriosis may be dysregulated in such ways that it is unable to prevent dissemination of endometrial fragments[11-14]. Moreover, new evidences suggest that the endometrial fragments may locally trigger sterile inflammation, which may in fact promote the growth of current and new endometrial lesions[1, 15]. Thus, while endometriosis is clearly associated with inflammation, the nature of the inflammatory process and how it may promote rather than prevent the lesions in women with endometriosis remains to be elucidated.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid cells that are highly immunosuppressive[16]. MDSCs are defined by their myeloid origin, immature state and ability to potently suppress T cell responses as well as those of NK cells and B cells. These cells are found in small numbers in a healthy state, and they rapidly expand in response to cancer, infections and inflammation[17, 18]. In human, MDSCs are HLA-DR-CD11b+CD33+, primarily enumerated in peripheral blood from cancer

patients[18]. In mice, MDSCs have been shown to express both Gr-1 and CD11b markers. The MDSCs have been divided into two major subpopulations: PMN-MDSC/G-MDSCs ($CD11b^{+}Ly6G^{+}Ly6C^{lo}$) and M-MDSC ($CD11b^{+}Ly6G^{-}Ly6C^{hi}$), of both subsets are elevated in tumor-bearing mice while G-MDSCs is the major proportion. MDSCs suppress the immune response by producing a number of immunosuppressive factors including NO, ROS, IDO, TGF-β and PGE_2[16]. Studies from our laboratory have shown that activation of Cannabinoid or Aryl hydrocarbon receptors on immune cells can also induce MDSCs that can suppress inflammation upon adoptive transfer in a variety of organs, thereby demonstrating that MDSCs may serve as a therapeutic modality against inflammatory diseases[19-21]. It is noteworthy that MDSCs, in the context of tumor microenvironment, have recently been found to promote angiogenesis[22, 23]. Angiogenesis represents a critical process for the establishment and survival of the endometriotic implants[24, 25], consequently, understanding the cellular and molecular pathways involved in this process is critical for developing better approaches to treat or prevent endometriosis.

While a wide range of immune cells have been characterized at sites of endometrial lesions, thus far there are no reports on the role of MDSCs in inflammatory lesions of endometriosis. In the current study, we tested the hypothesis that MDSCs may be responsible for the protection (prevents the rejection) and promotion (enhances angiogenesis and cell growth) of endometriotic implants. Therefore, the objective of this study was to understand the role of MDSCs in the early development of endometriosis. We report that both in women with endometriosis and in mouse model of surgically-induced endometriosis, there is a significant increase in MDSCs in peripheral blood and peritoneal fluid. Depletion of MDSCs significantly reduced the development of endometrial lesions. We also identified that specific CXCL1, 2 and 5 chemokines and their CXCR2 receptor are the mediators for the recruitment of MDSC in endometriosis. The current study demonstrates for the first time that MDSCs

play an important role in the pathogenesis of endometriosis and may be used as a therapeutic target for endometriosis treatment.

Results

Endometrial implants correlate with increased MDSCs numbers and abnormal chemokine profile

It is increasingly becoming clear that MDSCs not only suppress anti-tumor immunity but also promote tumor growth, differentiation and metastasis[26]. Because there are similarities between tumors and endometriosis[27, 28], in the current study, we first investigated the percentage of MDSCs, CD11b+CD33+HLA-DR-[29], in the peripheral blood mononuclear cells (PBMCs) of patients with endometriosis. Fig. 1A- C shows representative data of flow cytometric analysis in control and endometriosis patient, and panel D shows data from all subjects tested. Our data showed that patients with endometriosis had significantly increased percentages of MDSCs in the PBMCs when compared to normal female controls. Interestingly, removal of endometrial implants by laparoscopic surgery led to significant decrease in the percentage of MDSCs in the peripheral blood of these patients (Fig. 1E-G).

Next, we investigated if the endometriosis patients showed any alterations in circulating levels of cytokines or chemokines. Our analysis demonstrated that IL-6, IL-8, IL-10, GM-CSF and MCP-1 levels were significantly higher in the plasma from endometriosis patients when compared to controls (Fig. 1H). Furthermore, laparoscopic surgery of the peritoneal lesions also had an effect on the cytokine profile as demonstrated by a significant decrease of these cytokines and chemokines in the treated patients (Fig. 1I). These data shows a direct correlation between the elevated levels of MDSCs and specific cytokines/chemokines and the presence of endometriotic implants. However, we did not measure MDSCs and cytokines in peritoneal fluid.

Increase of circulating MDSCs in a mouse model of endometriosis

To validate the observed correlation between MDSCs and endometriotic implants in patients with endometriosis, we used a mouse model of endometriosis as previously described[24]. We noted dramatic induction of CD11b+Gr-1+ MDSCs in the recipient mice within 6 hours, peaking around 24 hours, and sustained for almost 7 days after transplantation (Fig. 2A-C; Supplementary Fig.2). The other peritoneal immune cells were very low and some of them decreased significantly in recipient mice when compared to controls (Fig. 2D; Supplementary Fig.3-8). These results suggested that MDSCs migrated into peritoneal cavity within short time after the transplantation of endometrial fragments, suggesting MDSCs may play a role in the early development of endometriosis.

MDSC in mice with endometrial implants have an immune suppression phenotype.

In mice, MDSCs are primarily divided into granulocytic (G-MDSC; $CD11b^{+}Ly6C^{low}Ly6G^{+)}$ and monocytic MDSC (M-MDSC; $CD11b^{+}Ly6C^{high}Ly6G^{-}$) subsets. G-MDSC and M-MDSC subsets may have different immunosuppressive properties [30]. In the recipient mice with endometrial transplants, we found both G-MDSC and M-MDSC cell fractions in the peritoneal cells although the proportion of G-MDSCs was significantly larger (Fig. 3A). The total number of G-MDSCs and M-MDSCs in the peritoneal cavity of recipient mice increased significantly in a time-dependent manner (Fig 3B).

In order to test the functional status of MDSCs induced by the endometrial implants, we tested first the most representative hall mark of MDSCs that is their effect on T cell proliferation. Our data showed that both G-MDSCs and M-MDSCs isolated from mice with endometrial implants significantly inhibited T cell proliferation in a dose-dependent manner, with M-MDSCs being more potent than G-MDSCs in inhibiting ConA induced T cell

proliferation (Fig. 3C, D). To further validate the inhibitory effect of MDSCs on T cell proliferation we evaluated the levels of Arginase and reactive oxygen species (ROS), two main mediators of MDSCs induced suppression of T cell proliferation. As shown in Fig. 3E, mice with endometrial implants expressed high levels of arginase compared to sham control group. Also, the MDSCs particularly G-MDSCs from recipient mice expressed higher levels of ROS (Fig. 3F).

MDSC depletion inhibits the process of endometriosis

In order to establish the role of MDSCs supporting the endometriosis process we depleted MDSCs using a previously reported system consisting of injection of anti-Gr-1 monoclonal antibody [31, 32]. MDSC depletion significantly inhibited the development of endometrial lesions (Fig. 4D) and decreased both the endometrial lesions in size and weight (Fig. 4E) while the endometriotic lesions in control IgG treated mice developed rapidly in the peritoneal cavity 3 days after endometrial implant transplantation and showed sustained growth on days 5 and 7 (Fig. 4E).

We then evaluated the pathological changes induced by depletion of MDSCs on the implants. First, we tested histological changes: while the control group was characterized for the presence of glands and a well-developed glandular epithelium, that was not the case for the implants obtained from the depletion group (Fig. 4F), suggesting that in the lack of MDSCs the glandular epithelium is not proliferating. This was confirmed when we evaluated the tissues with BrdU; as shown in Fig. 4G we noticed positive nuclei in the epithelium and stroma. Conversely, endometriotic lesions from the depletion group showed few BrdU positive cells, mainly localized in the stroma. Moreover, MDSC depletion was associated with a decrease in the formation on new blood vessel as demonstrated by staining the tissue with SMAα and CD31, markers for endothelial cells. As shown in figure Fig. 4H, the control

group showed positive SMAα and CD31 blood vessels within the stroma of the implants which were absent following MDSCs depletion. These results suggest that MDSCs are critical for glandular epithelial proliferation and neovascularization, two relevant processes in the formation of endometriotic lesions.

CXC chemokines mediates the chemotaxis of MDSCs in endometriosis

Our next objective was to determine whether the ectopic endometrium is responsible for the recruitment of MDSCs into the developing implants. We hypothesized that endometrial implants secrete into the peritoneal cavity chemokines and cytokines responsible for the recruitment of MDSCs. Thus we collected peritoneal fluid from mice within 24hours after transplantation of endometrial implants (Fig. 5A-B; Supplementary Data Table 1) and analyzed for the cytokine/chemokine profile using a protein array. Analysis of the cytokine and chemokine arrays revealed that C5/C5a, C10, CXCL1, CXCL2, CXCL5, G-CSF, MCP-1, MIP-1γ, VCAM-1 were up-regulated in the peritoneal fluid of mice with endometrial implants compared with sham control (Fig. 5A and B).

In order to identify which of the chemokines present in the peritoneal fluid are responsible for the recruitment of MDSCs, we used first an in vivo model consisting of mice injected with recombinant chemokines into the peritoneal cavity (without implants) and evaluated for the presence of MDSCs. Our data showed that CXCL1, CXCL2, CXCL5, promoted the recruitment of MDSCs into peritoneal cavity, similar as the recruitment observed with serum or peritoneal fluid obtained from mice with endometrial implants (Fig. 5C). We further validated these observations by using a trans-well migration assay where MDSCs localized in the upper chamber showed significant migration towards lower chambers containing CXCL1, CXCL2, CXCL5 or serum/peritoneal fluid from mice with endometrial implants (Fig 5C). These findings suggest that CXCL1, CXCL2 and CXCL5

may play an important role in the recruitment of MDSCs to the peritoneum in mice during the process of endometriosis. Estimation of CXCL1, CXCL2 and CXCL5 showed that their levels increased significantly within 6 hours of transplantation of endometrial implants and was sustained till 24 hours for CXCL1 and CXCL5 while for CXCL2, the levels were back to background levels within 24 hours (Fig. 5D-F).

In order to determine the specificity of CXCL1, CXCL2 and CXCL5 on their capacity to recruit MDSCs into the implants we evaluated the expression of their common receptor, CXCR2[33-35], in MDSCs. We found that MDSCs, particularly G-MDSCs expressed high levels of CXCR2 (Fig 5H) and the endometrial lesions expressed significant levels of CXCL1, 2 and 5, when compared to baseline control endometrium before transplantation (Fig. 5I).

We next investigated the effect of blocking CXCR2 on MDSCs recruitment by using the CXCR2 inhibitors SB265610 and SB225002 in the in vitro trans-wells chamber model. Our data revealed that the use of CXCR2 inhibitors SB265610 and SB225002 significantly blocked MDSC migration *in vitro* and *in vivo* induced by peritoneal fluid from mice transplanted with endometrial implants(Fig. 5G). These results demonstrated that CXCL1, CXCL2 and CXCL5 expressed on endometrial lesions are responsible of the recruitment of MDSCs that express CXCR2 receptors to the peritoneum at the sites of endometrial lesions in mice.

CXCR2 deficiency suppressed the development of endometriosis by inhibiting MDSC recruitment

To further corroborate the role of CXCR2 in the recruitment of MDSCs and their contribution for the establishment of the endometriotic implants, we used CXCR2 knockout (KO) mice (cannot respond to CXCL1, CXCL2 and CXCL5) as the host for endometrial implants obtained from WT mice (secrete chemokines) and found that in these mice, there was a

dramatic inhibition in peritoneal G-MDSCs accumulation (Fig. 6A) and the development of endometrial lesions in the peritoneal cavities at day 7 after the transfer of endometrial implants (Fig. 6B). CXCR2 deficiency in the host significantly suppressed the increase of endometrial lesions in both size and weight (Fig. 6C). Also, histopathological analysis showed that implants in CXCR2-KO mice the endometrial glands were smaller compared to WT hosts and angiogenesis was significantly decreased in endometriotic implants in CXCR2-KO mice when compared to WT mice (Fig. 6D). Importantly, adoptive transfer of exogenous MDSCs into CXCR2KO mice restored the growth of endometrial implants as well as angiogenesis in the peritoneal cavities of mice at day 7 after the transplantation of endometrial fragments (Fig. 6B-D). Together, these data clearly demonstrated that MDSCs play a critical role in the pathogenesis of endometriosis.

Discussion

In this study, we reported for the first time that MDSCs play a critical role in the regulation of pathogenesis of endometriosis. In humans as well as in a mouse model of endometriosis, we showed that MDSCs are recruited into the peripheral blood of patients with endometriosis as well as in the peritoneal cavity of mice following transplantation of endometrial tissue. In human patients, the number of MDSCs decreased following laparoscopic surgery to remove endometriotic lesions, thereby suggesting that during endometriosis there is an active process of MDSCs recruitment which may promote the survival and progression of the endometriotic lesions. Moreover, in the mouse model, we conclusively demonstrated the role of MDSCs in the pathogenesis of endometriosis as shown by the data that depletion of MDSCs leads to dramatic decrease in endometrial lesions while adoptive transfer of MDSCs restored the growth of the lesions.

Endometriosis is a chronic, estrogen-dependent disease with an inflammatory component. It affects 6-10% of reproductive-aged women and has an economic impact of $22 Billion/year in the USA[36]. Because of the lack of biomarkers and unknown etiology, endometriosis remains misdiagnosed, and treatment modalities are often ineffective. While the precise cause of endometriosis remains unclear, the theory of retrograde menstruation is widely accepted, in which the endometrial tissue sloughed off during menstruation, is refluxed into the fallopian tubes and peritoneal cavity. However, this theory alone does not explain the reasons because 76-90% of women have been shown to experience retrograde menstruation[37] while not developing endometriosis. This suggests that additional defects are existent in women with endometriosis. One of crucial factors that may play a role is the immune system of the host. Thus, the immune system in women with endometriosis may fail to control the establishment of lesions and furthermore, may facilitate tissue adhesion and angiogenesis thereby contributing towards endometrial pathogenesis. In the current study, we investigated if MDSCs that are known to be potent immunosuppressive cells may play a role in endometriosis.

In order to induce endometriosis, the endometrial fragments must survive the immune surveillance, attach to a surface, and promote angiogenesis and vasculogenesis to survive and grow[38, 39]. Thus, it is speculated that women who develop endometriosis may have certain immune defects or that the endometrial fragments may acquire the ability to suppress the immune system locally leading to the pathogenesis of endometriosis. It has been reported that the Th2 immune response, which plays an inhibitory role, is increased in endometriosis patients [40]. MDSCs were accumulated in placenta and cord blood to modulate polarization of Th2 cytokine response, thereby inducing and maintaining maternal-fetal immune tolerance[41, 42]. Additionally, several immune cell types, including neutrophils, macrophages, dendritic cells, natural killer cells, and B cells have been shown to be

dysregulated in women with endometriosis [43]. Our studies for the first time demonstrated that MDSCs are dramatically increased in both endometriosis patients and in a mice model of endometriosis thereby suggesting that these cells may promote suppression of NK cell and T cell activity against the transplanted endometrial cells thereby establishing these lesions. However, in this study we demonstrate that in addition to inhibit T cell proliferation, MDSCs have an "non-immunologic" role, that is to provide support for cell growth and angiogenesis, two biological processes critical for the survival of the endometrial implants. The role of MDSCs was conclusively demonstrated in the current study in which we noted that depletion of MDSCs led to dramatic decrease in endometrial lesions while adoptive transfer of MDSCs restored the lesions.

MDSCs are heterogeneous cell population[44]. There are two major subsets of MDSCs in mice based on their phenotypic and morphological features: polymorphonuclear or granulocytic (G)-MDSC and monocytic (M)-MDSC. Recent studies have suggested that these two subpopulations are not only phenotypically and morphologically distinct, but also have unique functional characteristics and biochemical traits, although both are highly immunosuppressive[16]. In the current study it was interesting to note that majority of the MDSCs seen in mice with endometriosis lesions were G-MDSCs. The ability of MDSCs to suppress T cell proliferation depends on the metabolic consumption and conversion of the amino acids such as L-arginine inducible enzymes such as arginase 1 (ARG1). MDSCs also produce high levels of soluble reactive species[16]. Consistent with these findings, we noted that the MDSCs induced during endometriosis expressed high levels of arginase and the G-MDSCs from recipient mice also expressed significant levels of ROS. The hallmark of MDSCs is their ability to suppress T cell proliferation. Thus, when MDSCs were tested for their ability to suppress T cell proliferation, we found that both G-MDSCs and M-MDSCs

were able to induce significant decrease in T cell proliferation, in a dose-associated manner, with M-MDSCs being more potent than G-MDSCs, in inhibiting T cell proliferation.

MDSCs associated with tumors have been well characterized for their ability to suppress anti-tumor immunity. Solid tumor microenvironment consisting of tumor cells and immune cells cross-talk leads to the release of proinflammatory cytokines (IL-1, IL-6, IL-17, TNF-α), chemokines (CCL2, CXCL5, CXCL12) and growth factors (TGF-β, GM-CSF, VEGF), and other molecules (S100A8/A9)[45]. These factors are believed to trigger a local immunosuppressive environment consisting of regulatory T cells, tumor-associated macrophages, and MDSC, which suppress anti-tumor immunity and promote tumor growth[45]. It is interesting to note that the endometriotic environment, as seen in the current study, had similar milieu of cytokines and chemokines. Several inflammatory cytokines have been shown to be increased in women with endometriosis, such as IL-1β, IL-6, and TNF-α[46]. These data are consistent with the current study in which we noted that cytokines such as IL-6, IL-8, GM-CSF and MCP-1 were significantly up-regulated in patients with endometriosis. In addition, there may also be an increase in anti-inflammatory cytokines such as IL-10[47], which may be produced as a regulatory mechanism to suppress the inflammation, which was also noted in the current study. Moreover, IL-10 is also produced by MDSCs[45] to suppress inflammation. While the serum cytokines may not accurately reflect the local milieu of mediators found at endometrial lesions, the mouse model that we tested provided us the opportunity to study cytokines/chemokines in the peritoneal cavity. We noted that C5/C5a, C10, CXCL1, CXCL2, CXCL5, G-CSF, MCP-1, MIP-1γ and VCAM-1 were significantly increased in the mouse model of endometriosis when compared to controls.

Because we noted the increase in several CXC chemokines in endometriosis, we tested the migration of MDSCs in the presence of various cytokines/chemokines or serum and peritoneal fluid obtained from endometriosis recipient mice, and found that CXCL1, CXCL2, CXCL5, as well as serum/peritoneal fluid promoted the migration of MDSCs *in vitro* as well as the accumulation of MDSCs in the peritoneal fluids of mice *in vivo.* Moreover, we found significant expression of CXC chemokines at endometrial lesions but not in normal endometrium, and expression of CXCR-2 chemokines on MDSCs. These data suggested that CXC chemokines may play an important role in the recruitment of MDSCs to the endometrial lesions in mice. Because CXCR2 is the common receptor for CXCL1, CXCL2 and CXCL5, we next investigated the effect of blocking CXCR2. Our data revealed that CXCR2 inhibitors significantly blocked MDSC migration. We further corroborated the role of CXCR2, using CXCR2 knockout (KO) mice and found that in these mice, there was a dramatic inhibition in the development of endometrial lesions in the peritoneal cavities both size and weight. Also, histopathological analysis showed that the development of endometrial lesions and angiogenesis were dysplastic in CXCR2KO mice when compared to WT mice. Importantly, adoptive transfer of exogenous MDSCs into the peritoneal cavities of CXCR2KO mice restored the growth and angiogenesis of endometrial lesions in the peritoneal cavities. It has been found that MDSCs promote tumor angiogenesis by secreting pro-angiogenic factors such as VEGF, FGF and CCL2 through activation of STAT3[48, 49], indicating that MDSCs may play a similar pathologically angiogenic role in endometriosis. Together, these data clearly demonstrated that CXC chemokines and CXCR2 receptors play a critical role in MDSC induction as well as the pathogenesis of endometriosis. It is interesting to note that recently, in a rhabdomyosarcoma model, it was shown that the tumor cells induced robust expansion CXCR2+ MDSCs and that the CXCR2 deficiency prevented

MDSC trafficking to the tumor[50]. Together, these data suggest that CXC chemokines may be play a key role in the migration of MDSC and consequent regulation of endometriosis.

In summary, the current study demonstrates conclusively for the first time that MDSCs play a critical role in the progression of endometrial lesions. The induction of MDSCs following endometriosis was seen in both humans and in the mouse model. Furthermore, we demonstrate that CXCL1, 2 and 5 are major mediators of the recruitment and function of MDSCs during endometriosis. Therefore, it opens a new venue for therapeutic approaches using CXCR2 inhibitors for the treatment of patients with endometriosis.

Materials and methods

Normal control and patient samples

All normal female controls and endometriosis patients agreed to participate in this study by signing the Institutional Review Board (IRB) approved consent forms. The recruited patients with endometriosis were diagnosed, evaluated and treated surgically by laparoscopy. The stage of endometriosis in each patient were evaluated according to the revised American Fertility Society (AFS) classification. 19 women were classified as either Stage I or II, 11 women were classified as either Stage III or IV. All patients did not received any GnRHa nor hormonal treatment within 6 months prior to the surgical operation. For normal controls, age matched healthy females, who did not have any symptoms of pelvic pain, active infection or have any history of immune compromise such as HIV, cancer, pregnancy or on chronic steroid therapy, or any gynecological disorders, such as myoma, adenomyosis, endometriosis, ovarian cysts were recruited. A total of 30 endometriosis patients and 15 normal controls were recruited in this study.

Preparation of PBMCs

Ten mL of peripheral blood was drawn from endometriosis patients and normal controls by hospital nurses using venipuncture and transferred into tubes coated with ethylenediaminetetraacetic acid (EDTA). One mL of peripheral blood from each sample was used to prepare plasma by centrifugation and the remaining 9 mL of blood was processed by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) centrifugation to isolate PBMCs. Viable PBMC were counted by trypan-blue exclusion assay.

Analysis of cell populations in PBMCs

PBMCs were stained with FITC-conjugated anti-human CD3, APC-conjugated anti-human CD4, PE-conjugated anti-human CD8, FITC-conjugated anti-human CD20 and PE-conjugated anti-human CD16 monoclonal antibodies. Then, flow cytometric analysis was carried out using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) to determine neutrophils, CD4 T cells, CD8 T cells, B cells, NK cells and NK T cells in PBMC samples, respectively. For MDSC analysis in human samples, PBMCs were stained with PE-conjugated anti-human CD11b, FITC-conjugated anti-human CD33 and APC-conjugated anti-human HLA-DR. The percentages and numbers of HLA-DR-CD11b+CD33+ MDSCs were calculated. All antibodies were purchased from BD Biosciences (San Jose, CA, USA).

Bio-Pex Cytokine assay in human plasma

Twenty seven cytokines (including IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, FGF-β, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF) in the plasma samples were analyzed using Bio-Plex Pro Human Cytokine 27-plex Assay kit (Bio-Rad, Hercules, CA) according to the instruction manual.

Mouse model of endometriosis

Endometriosis was studied in mice as described [24].BALB/c female mice, 7 weeks old, were provided by the Laboratory Animal Service Center of the Chinese University of Hong Kong and housed in Animal House of Prince of Wales Hospital. Female wild-type (WT) C57BL/6 and CXCR2 knockout (KO) mice on BALB/c background were obtained from the Jackson labs (Bar Harbor, ME, USA) and housed under specific pathogen-free conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC).For the establishment of animal model of endometriosis in BALB/c female mice, both donor and recipient mice were subjected to ovariectomy (OVX) and intramuscularly (i.m.) injected with 100 μg/kg estradiol (Sigma-Aldrich, St. Louis, MO, USA) at the time of OVX and 5 days after OVX. One week after OVX, uterine horns from the donor mice were removed into F12/DMEM medium. Endometrium was punched into 2 mm^2 endometrial fragments after peeling off the serosa and myometrium under microscope. Thirty five mg endometrial fragments suspended in 0.3 ml sterile phosphate-buffered saline (PBS) were injected into peritoneal cavity of recipient mice with an 18-gauge needle (Supplementary Fig.1).

Sample preparation from mice

At 0, 6, 12 and 24 h after the transplantation of endometrial fragments, recipient BALB/c female mice were sacrificed. Peritoneal fluid samples were collected by intraperitoneally (i.p.) injecting 3 ml phosphate buffered saline (PBS) at 0, 6, 12 and 24 h after the transplantation of endometrial implants, and centrifuged at 350 g for 10 min for peritoneal cell collection. The supernatants were concentrated to 250μl each sample by Amicon Ultra-4

centrifugal filter devices (EMD Millipore Corporation, Billerica, MA, USA) for cytokine and chemokine array.

The endometrial implants/lesion samples were collected from the peritoneal cavities of mice. The weight and size of endometrial implants/lesions were measured, and then the tissue was fixed in 4% paraformaldehyde and embedded in paraffin for histological analyses.

Flow cytometry analysis of mouse cells

Peritoneal cells were incubated with mouse Fc-block (anti-mouse CD16/CD32) and stained on ice for 30 min with anti-mouse monoclonal antibodies conjugated with different fluorochromes including CD45-APC, CD4-APC, CD8-PE, F4/80-PE, CD3-FITC, CD19-FITC, CD49b-FITC, CD11c-PE/Cy5, CD11b-PE/Cy7 or CD11b-APC or CD11b-Alexa-647, Gr-1-FITC or Gr-1-PE, Ly-6G-PE, Ly-6C-FITC and CXCR2-Alexa-647. After washing, the cells were analyzed by flow cytometer (FC500, Beckman Coulter, Brea, CA, USA; or FACSCalibur, BD Biosciences, San Jose, CA, USA). Flow cytometric data were analyzed by WinMDI 2.9 and Flow Jo software. Monoclonal antibodies including CD45-APC, CD4-APC, CD8-PE, CD11b-APC and F4/80-PE were purchased from R&D Systems (Minneapolis, MN, USA). Monoclonal antibodies including CD3-FITC, CD19-FITC, CD49b-FITC and Fc-block (anti-CD16/32) were purchased from BD Biosciences (San Jose, CA, USA). Monoclonal antibodies including CD11c-PE/Cy5, CD11b-PE/Cy7, CD11b-Alexa-647, Gr-1-FITC, Gr-1-PE, Ly-6G-PE, Ly-6C-FITC and CXCR2-Alexa-647 were purchased from BioLegend (San Diego, CA, USA).

Wright–Giemsa staining

The cell smears of isolated peritoneal G-MDSCs and M-MDSCs were prepared on a glass slide and dried completely for 30 min. The air-dried smears were fixed in methanol for 30 sec

and stained in a closed coplin jar containing Wright-Giemsa stain solution (Sigma-Aldrich, St. Louis, MO, USA) for 5 min. Then, the slides were rinsed in staining buffer for 3 min and air dried, mounted. The morphology was examined and recorded under a microscope.

Assay of arginase activity

G-MDSCs and M-MDSCs were isolated from peritoneal MDSCs by MDSC isolation kit (MiltenyiBiotec Inc., Auburn, CA, USA). The purity of G-MDSCs and M-MDSCs was > 95%, which was determined by flow cytometry. Peritoneal MDSC, G-MDSC or M-MDSC were lysed in 100 μL of Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO, USA) mixed with protease inhibitor cocktail (Roche, Nutley NJ, USA). Arginase activity in the cell lysates was determined by measuring urea production in the arginase reaction using Arginase Assay Kit (Abnova, Buckingham, MK18 1TF, UK) according to the manufacturer's protocol.

ROS production

Peritoneal MDSCs, G-MDSCs and M-MDSCs from sham control and mice with endometriosis were stained with 20 μ M DCFDA for 30 min. at 37℃ according to the protocol of DCFDA Cellular ROS Detection Assay kit (Abcam, Cambridge, MA, USA). The intensity of DCFDA was detected by flow cytometry.

Mouse T cell proliferation assay

MDSCs were isolated from the peritoneal cavities of BALB/c mice at 12 h after the transplantation of endometrial implants, and G-MDSCs and M-MDSCs were sorted from the peritoneal MDSC samples by MoFloAstrios EQ Cell Sorter (Beckman Coulter, Brea, CA, USA) (Supplementary Fig.9). The purity of sorted MDSC subsets was greater than 95%.

MDSCs, G-MDSCs and M-MDSCs were cultured in 96-well round bottom plates in the presence of different ratios with 5×10^5 purified T cells stimulated with 4 mg/mL Con A (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured in 200 μL of RPMI-1640 medium supplemented with 10% FBS, 10mM HEPES, 1mM penicillin–streptomycin and 50 mM β-mercaptoethanol (Invitrogen, Carlsbad, CA, USA) at 37°C in a 95% humidified incubator with 5% CO_2. After culture for 72 h, 10 μCi of [H3]-thymidine (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After culture for additional 12 h, the cells were collected by cell harvester and the incorporation of [H3]-thymidine was detected in MicroBeta^2 Plate Counter (Perkin Elmer, Waltham, MA, USA).

MDSC depletion in mice

BALB/c mice were divided into two groups. One group was injected with 250 μg of purified anti-mouse Gr-1 antibody by i.p. route while the second group received control antibody one day before transplantation and at day 0, 2 and 4 after transplantation. Mice were sacrificed, and then endometrial lesions and peritoneal cells were collected at days 3, 5 and 7 after transplantation for immunostaining and flow cytometry analysis to confirm the depletion of peritoneal G-MDSC and M-MDSC. For the assay of cell proliferation in endometrial lesions, mice were injected with BrdU (2mg/mouse) 2 h before sacrifice. The endometrial lesions were fixed and stained with BrdU antibody (Abcam, Cambridge, MA, USA) (Supplementary Fig.10).

Immunohistochemistry staining

Tissue sections were heat-fixed, deparaffinized, and rehydrated by standard methods. Tissues were blocked for endogenous peroxidase activity in 3% H_2O_2 in methanol for 20 min in dark. Antigen retrieval was performed by heating with microwave for 15 min at 98°C in 0.01

mol/L citrate buffer (pH=6.0). The slides were incubated with 5% rat or rabbit serum for 1 h at room temperature to reduce the non-specific signal. Then, the slides were incubated overnight with rat anti-mouse BrdU monoclonal antibody (Abcam, Cambridge, MA, USA) or rabbit anti-mouse CXCL1, CXCL2 and CXCL5 polyclonal antibody (Abcam, Cambridge, MA, USA). After washing, the slides were incubated with the secondary antibody (1:300) conjugated with HRP at room temperature for 1 h. Diaminobenzidinetetrahydrochloride (DAB) chromogen (Dako, Carpinteria, CA, USA) was finally incubated with the slides at room temperature for 1-5 min. The slides were counterstained with methyl green (Sigma-Aldrich, St. Louis, MO, USA). The slides were examined and photographed under a microscope.

Immunofluorescence staining

After fixation, deparaffinization and rehydration, the slides were incubated with donkey serum to reduce the non-specific antibody bindings, and then incubated with rabbit anti-mouse CD31 monoclonal antibody and mouse anti-mouse alpha-smooth muscle actin (α-SMA) monoclonal antibody (Abcam, Cambridge, MA, USA). Then, the slides were incubated with donkey anti-rabbit IgG antibody conjugated with Alexfluro 555 and donkey anti-mouse IgG antibody conjugated with Alexfluro 488 (Invitrogen, Carlsbad, CA, USA). The slides were mounted with mounting solution with DAPI (Vector Laboratories, Burlingame, CA, USA). The slides were examined and photographed under a fluorescence microscope.

Cytokine and chemokine antibody array

The cytokine and chemokine profiles in the peritoneal fluid samples from mice were determined by RayBio® C-Series Mouse Cytokine Antibody Array 6 Kit containing 97

cytokines (RayBiotech, Norcross, GA, USA) and Profiler™ Mouse Chemokine Array Kit containing 25 chemokines (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacture's instruction.

The intensities of cytokines and chemokines on the films were quantified using ImageQuant TL (GE Healthcare, Buckinghamshire, HP8 4SP, UK). The quantity of each spot was normalized according to positive controls. The trend of each cytokine and chemokine during the first 24 hours after the transplantation of endometrial fragments was analyzed by significance analysis of microarrays (SAM) software developed by Stanford University. The data analysis was permutation-based avoiding parametric assumptions about the distribution of individual cytokines or chemokines. Time course test was selected to detect the trend. The positive score represented increasing trend while the negative score represented decreasing trend.

Assay of MDSC migration in vitro

HTS Transwell®-24 well plates (3μm, Corning Inc., Corning, NY, USA) were used in the assay of MDSC migration *in vitro*. Control peritoneal fluid and control serum samples were obtained from naive mice, whereas endometriosis peritoneal fluid and endometriosis serum samples were obtained from mice at 12h after transplantation of endometrial implants. MDSCs were isolated from the peritoneal cells in mice at 12h after transplantation of endometrial implants. Control peritoneal fluid, endometriosis peritoneal fluid, control serum and endometriosis serum as well as 10 ng/mL of recombinant murine VCAM-1, MIP-1γ, CXCL-1, CXCL-2, CXCL-5, G-CSF, MCP-1, C10 and C5/C5a (PeproTech Inc., Rocky Hill, NJ, USA) were added in the bottom chamber of transwell plates. MDSCs (2×10^5) were seeded each well in the upper chamber of transwell plates. CXCR2 inhibitors, SB 265610 and SB225002 (R&D Systems Inc., Minneapolis, MN, USA) were also added in the upper

chamber for inhibition assay. After incubation for 4 h, migrated cells were harvested and counted under microscope using trypan blue staining. The percentages of migrated MDSCs were calculated.

Assay of MDSC migration in vivo

Peritoneal fluid and serum samples from naive mice and mice with endometriosis, recombinant murine VCAM-1, MIP-1 gamma, CXCL-1, CXCL-2, CXCL-5, G-CSF, MCP-1, C10 and C5/C5a (PeproTech Inc., Rocky Hill, NJ, USA) were intraperitoneally injected into naive mice. Peritoneal cells were harvested at 24 hours for flow cytometry analysis. The percentage and number of MDSCs in the peritoneal cavities of mice were calculated and compared among different treatment groups.

Chemokine ELISA

In order to confirm the result of cytokines and chemokines antibody arrays, selected cytokines and chemokines were quantified by ELISA. Specifically, CXCL1, CXCL2 and CXCL5 in the peritoneal fluid samples from mice were determined by ELISA kits from RayBiotech (Norcross, GA USA) according to the manufacture's protocols.

Adoptive transfer of exogenous MDSCs

G-MDSCs were purified from peritoneal cells of WT C57BL/6 mice with endometriosis by MDSC isolation kit (MiltenyiBiotec Inc., Auburn, CA, USA) according to the manufacture's instruction. Ly-6G+ cells were positively selected and the purity of $CD11b^{+}Ly\text{-}6G^{high}$ cells was determined by flow cytometry. Purified G-MDSCs (5×10^{6}) were intravenously injected into CXCR2KO mice at the day of endometrial implant transplantation and at days 1 and 3 after transplantation. Seven days after the transplantation of endometrial implants, the mice

were sacrificed, and endometriotic lesions and peritoneal cells were collected for further analysis.

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Author contributions statement

TZ, GCWM, and BL conducted the mouse experiments and molecular studies; KTL, JK and PCN provided facilities and assisted in flow cytometry. JZ, BX, XM and YZ performed the analysis of human samples from control women and patients with endometriosis. GWSK, AKWY, SH and HH recruited human subjects, collected samples and carried out patient treatment; VLH assisted with some animal experiments; JZ, PSN, MN and CCW conceived the project and interpreted the data; TZ and JZ summarized the data and discussed the results. JZ wrote the manuscript; and PSN, MN and CCW edited the manuscript. Data and materials availability: available on request.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure legends

Figure 1. Analysis of MDSCs and cytokines in endometriosis patients. (A) Gating strategy of human HLA-DR-CD11b+CD33+ MDSC and representative plots of flow cytometric analysis of MDSCs in PBMCs from (B) a normal female control and (C) a patient with endometriosis, respectively. Data in A-C are from a single experiment representative of 10 independent experiments each (B/C) with 1 sample per experiment. (D) Comparison of the percentage of MDSCs in PBMCs between normal female controls and endometriosis female patients, 10 subjects in each group. (E and F) Representative plots of flow cytometric analysis of MDSCs in PBMCs from the same patient with endometriosis before (pre-treatment) (E) and 3 months after (post-treatment) (F) laparoscopic removal of the endometrial lesions, respectively. Data in E-F are from a single experiment representative of 10 independent experiments each (E/F) with 1 sample per experiment. (G) Comparison of the percentage change of MDSCs in PBMCs from endometriosis patients before and after laparoscopic surgery; data shown are from 10 independent experiments (before and after) with 1 sample per experiment. (H) Comparison of the concentrations of cytokines in the plasma between normal controls (n=15) and endometriosis patients (n=30); data shown are from a single experiment with each sample in duplicate. (I) Comparison of the concentrations of cytokines in the plasma from endometriosis patients between pre-treatment and post-treatment (n=30); data shown are from 2 independent experiments with each sample assayed in duplicate. All bar graphs show mean+SEM. Mann-Whitney U test was used to determine all the significant difference. * represents $P < 0.05$ ** represents $P < 0.001$ compared with control or pre-treatment.

Fig 1

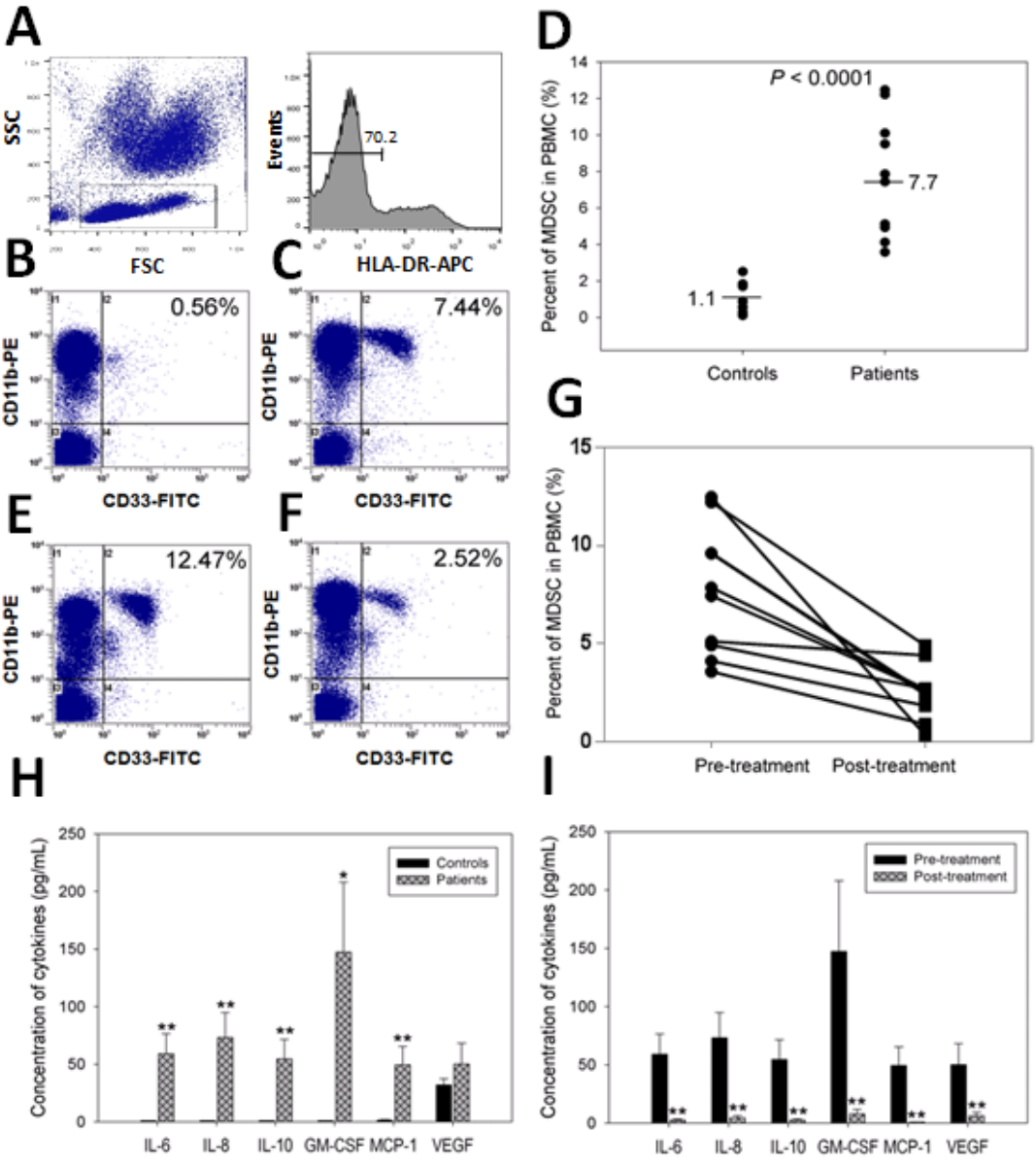

Figure 2. MDSCs in a mouse model of endometriosis. (A and B) Counts and exemplary FACS plot of peritoneal MDSCs within 24 hours after transplantation of endometrial fragments into recipent mice. Data in B are from a single experiment representative of 2 independent experiments at each time point with 5 samples per experiment. (C) Variation in counts of peritoneal MDSCs from mice with endometriosis and from sham control mice over 7 days post transplantation. (D) The profile of different peritoneal immune cells in the mouse model of endometriosis within 24 hours post transplantation; data shown are 2 independent experiments at each time point with 5 samples per experiment (A/C/D). All graphs and line chart show mean+SEM. Student's t test was used to determine the statistical changes. * represents P <0.001 compared with sham controls † represents P<0.001 compared with baseline 0 day.

Fig 2

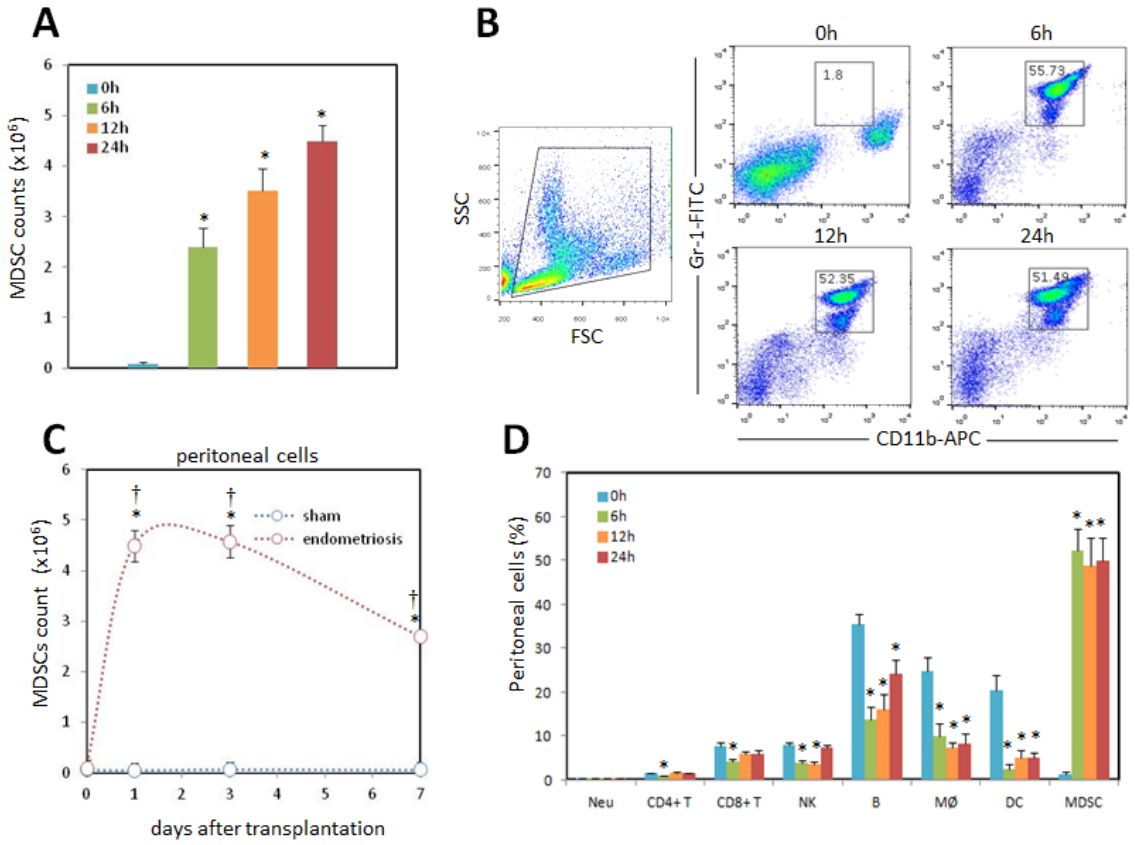

Figure 3. Characterization of MDSC subsets in mouse model of endometriosis. (A) Gating strategy and exemplary FACS plot and morphology of MDSC subsets in peritoneum. G-MDSCs are CD11b+Ly-6CloLy-6G+ and with polymorphonuclear morphology while M-MDSCs are CD11b+Ly-6ChiLy-6G- with monocytic morphology. Data in A are from a single experiment representative of 3 independent experiments with 1 sample per experiment. (B) Absolute cell number of peritoneal G-MDSCs and M-MDSCs measured by flow cytometry within 24 hours after transplantation of endometrial fragments into recipient mice; data shown are from a single experiment at each time point in each cell type with 5 samples per experiment. * represents P <0.001 compared with G-MDSCs, † represents P<0.001 compared with baseline 0 hour. (C and D) T cell proliferation assay. G-MDSCs and M-MDSCs were isolated from mice at 12 hours after transplantation, 5 mice were transplanted per experiment assayed for suppressive capacity in a T cell assay as described in the methods; data shown are from 3 independent experiments with 5 samples per experiment in triplicate. * represents P<0.001 compared with naive T cells. (E and F) Arginase activity (measures by an Arginase activity assay) and reactive oxygen species production (measured using flow cytometry) in MDSCs from sham mice (control), MCSCs, G-MDSCs and M-MDSCs from mice at 12 hours after transplantation, 3 sham mice and 2 mice with transplantation per experiment; data shown are from 3 independent experiments in each group with 5 samples per experiment. * represents P<0.001 compared with controls. All bar graphs show mean+SEM. Student's t test (B, E and F) and Mann-Whitney U test (C and D) was used to determine the significant differences.

Fig 3

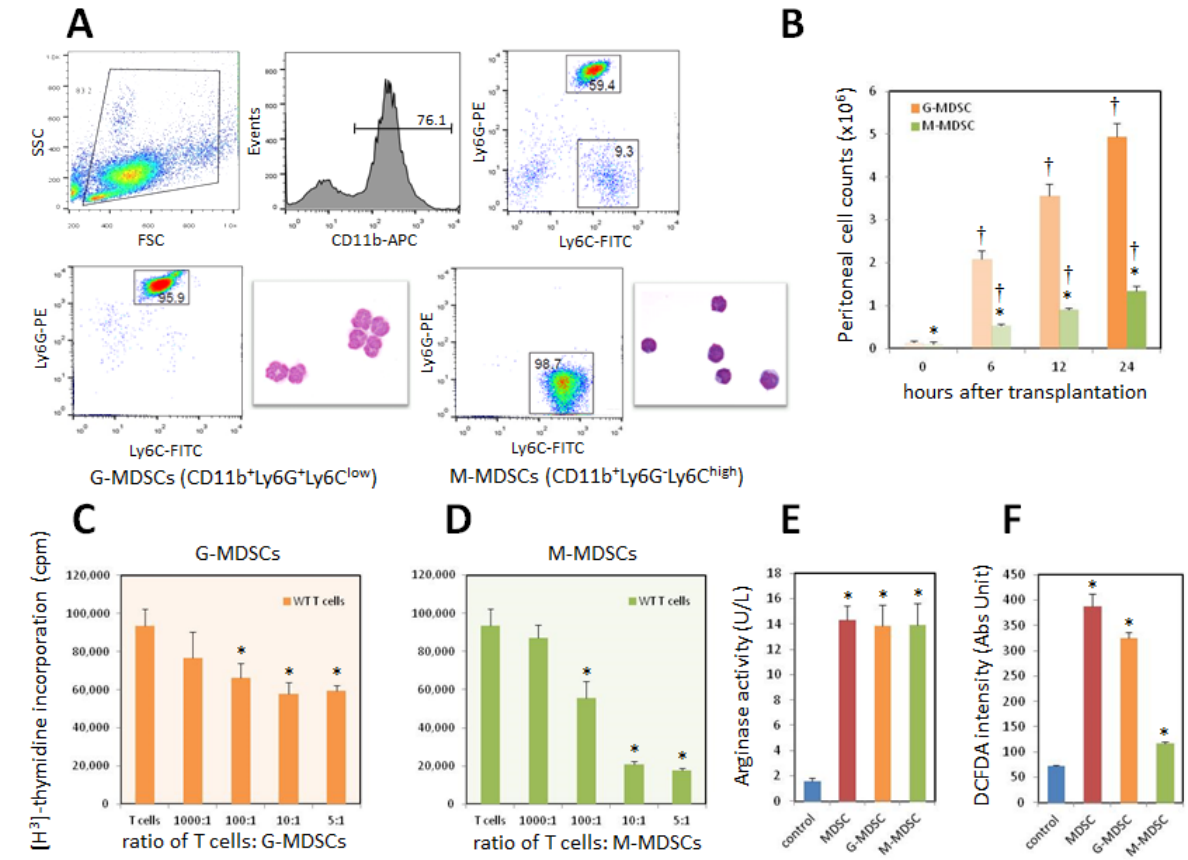

Figure 4. Effects of MDSCs depletion on the growth and development of endometriosis. (A) Changes in the number of peritoneal immune cells in mice was determined by flow cytometry at day 7 after transplantation of endometrial implants and antiGr-1 antibody treatment; data shown are from 5 independent experiments in each group with 5 samples per experiment. (B and C) Changes of peritoneal G-MDSCs and M-MDSCs at days 3, 5 and 7 after transplantation of endometrial fragments into recipient mice along with antiGr-1 antibody treatment; data shown are from 5 independent experiments in each time point and each group with 5 samples per experiment. (D and E) Comparison of the size and weight of endometrial lesions (yellow arrow) between IgG control and antiGr-1 antibody groups. All bar graphs show mean+SEM; data shown are from a single experiment representative of 5 independent experiments in each time point and each group. Student's t test, * represent $P < 0.001$ compared with control group † represents $P<0.001$ compared with baseline day 3. (F) The morphology of endometriotic lesions in the two groups following treatment with IgG or antiGr-1 antibody. The endometriotic glandular cells (g) and stromal cells (s) were marked. The magnification is 200X, scale bar: 100μm. (G) The endometrial lesions were stained with BrdU antibody for determination of proliferated cells as marked with black arrows. The magnification is 400X, scale bar: 50μm. (H) Micro-vessels in endometriotic lesions. The staining signals of DAPI were shown in blue, anti-CD31 mAb in red and anti-α-SMA in green. Premature vessels (white arrows) only express CD31. Mature vessels (yellow arrows) express both CD31 and α-SMA (magnification is 100X, scale bar: 200μm). Data in F-H are from a single experiment representative of 5 independent experiments in each staining at each time point and each group with 5 samples per experiment.

Fig 4

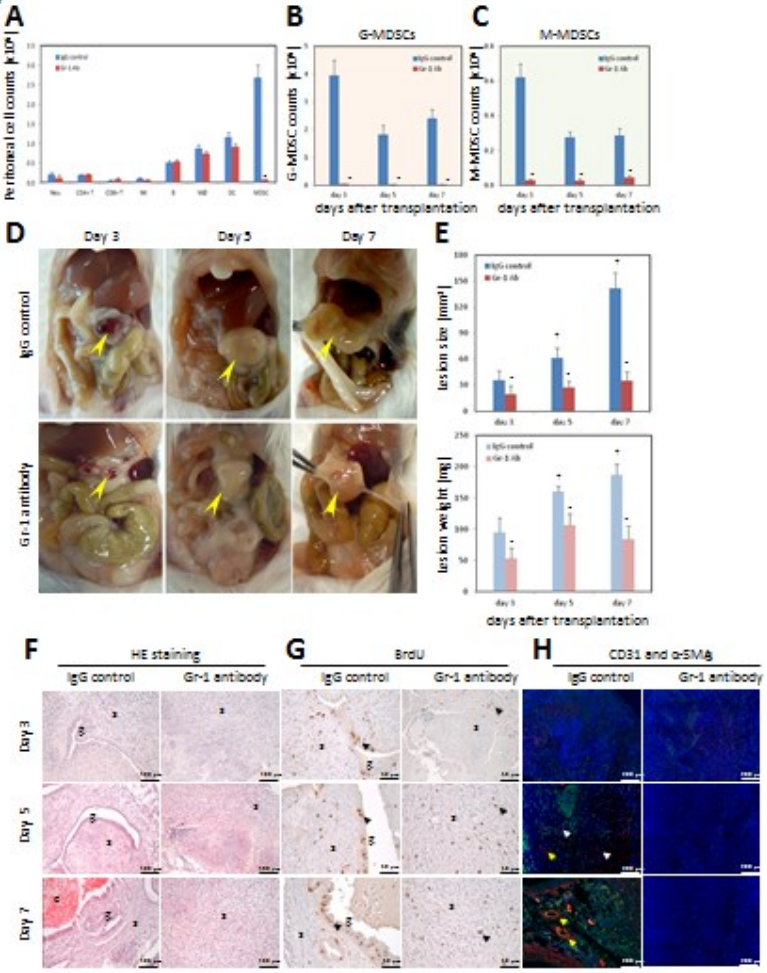

Figure 5. Effect of cytokines and chemokines on MDSCs. (A and B) Analysis of peritoneal cytokines and chemokines in the peritoneal fluids from mice within 24h after transplantation of endometrial implants by cytokine (left) and chemokine (right) arrays. The sample of peritoneal fluid at different time points were pooled together from three mice. The intensities of cytokines and chemokines on the films were quantified using ImageQuant TL. The data analysis was permutation-based avoiding parametric assumptions about the distribution of individual cytokines or chemokines. Time course test was selected to detect the trend. Data in A-B are from a single experiment each with 4 samples per experiment. (C) Migration of MDSCs in the presence of various cytokines/chemokines or serum and peritoneal fluid obtained from endometriosis recipient mice in vitro and in vivo; data shown are from 5 independent experiments in each group with 5 samples per experiment. * represents $P<0.001$ compared with control in vitro † represents $P<0.001$ compared with control in vivo. (D to F) Estimation of CXCL1, CXCL2 and CXCL5 by ELISA; data shown are from 7 independent experiments in each time point and each group with each sample assayed in duplicate. * represents $P< 0.001$ as compared with the control, † represents $P<0.001$ compared with baseline 0 hour. (G) Migration of MDSCs in the presence of peritoneal fluid obtained from endometriosis recipient mice and CXCR2 inhibitors including SB265610 and SB225002 in vitro and in vivo; data shown are from 5 independent experiments in each group with 5 samples per experiment. * represent $P < 0.001$ as compared with and without inhibitors in vitro † represents $P<0.001$ compared with and without inhibitors in vivo. All bar graphs show mean+SEM and data were analyzed by Student's t test. (H) Exemplary FACS plot of CXCR2 expression in G-MDSCs and M-MDSCs. (I) Expression of CXCL1, CXCL2 and CXCL5 in the endometriotic lesions and control (magnification is 400X, scale bar: 50μm). The endometriotic glandular cells (g) and stromal cells (s) were marked. Data in H-I are from a single experiment representative of 5 independent experiments in each marker with 5 samples per experiment.

Fig 5

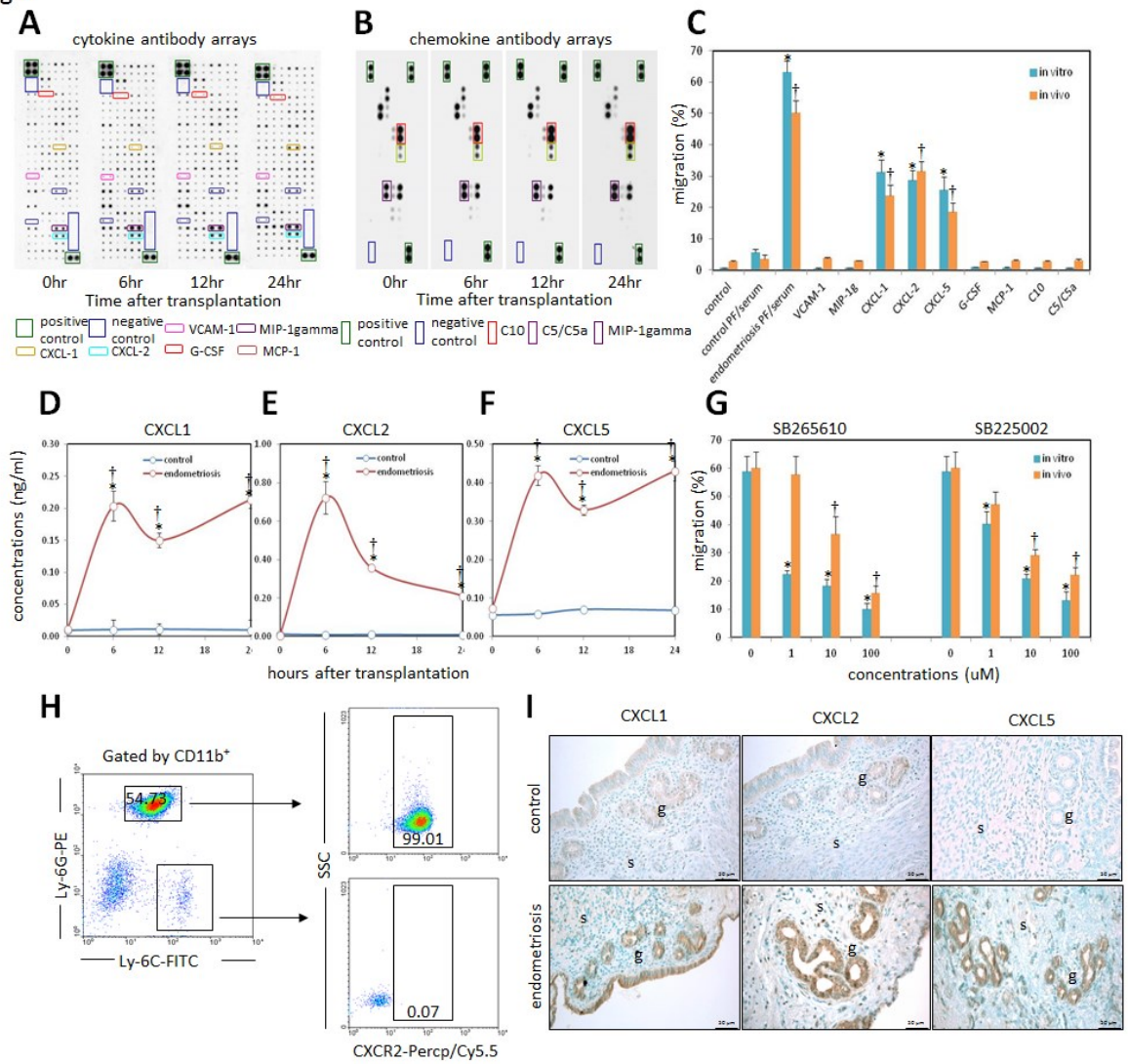

Figure 6. Role of CXCR2 in the development of endometriosis. (A) Exemplary FACS plot and frequency of G-MDSCs and M-MDSCs in CXCR2 KO mice. Data in A are from a single experiment representative of 5 independent experiments. (B) Development of endometrial lesions (yellow arrows) was investigated in CXCR2 KO mice and CXCR2 KO mice with exogenous G-MDSC transplantation. Endometrial lesions were collected at day7 after transplantation. Data in B are from a single experiment representative of 5 independent experiments with 5 samples per experiment. (C) Comparison of the size and weight of endometrial lesions in wide type (WT) mice, CXCR2 KO mice and CXCR2 KO mice with exogenous G-MDSC transplantation. Data are mean+SEM and are from 5 independent experiments in each group with 5 samples per experiment. * represents $P < 0.01$ as compared with wild-type mice and † represents $P<0.001$ compared with CXCR2KO mice by Student's t test. (D) The morphology of endometriotic lesions. The endometriotic glandular cells (g) and stromal cells (s) were marked. The magnification is 200X, scale bar: 100μm. Angiogenesis of endometriotic lesions was evaluated by IF staining of CD31 in red color and a-SMA in green color. Premature vessels (white arrows) only express CD31. Mature vessels (yellow arrows) express both CD31 and α-SMA. The magnification is 100X. Data shown are from a single experiment representative of 5 independent experiments in each group and staining with 5 samples per experiment.

Fig 6