Acta Biochim Biophys Sin, 2015, 47(8), 620–629
doi: 10.1093/abbs/gmv053
Advance Access Publication Date: 11 June 2015
Original Article

Original Article

17β-estradiol contributes to the accumulation of myeloid-derived suppressor cells in blood by promoting TNF-α secretion

Guanjun Dong[1], Ming You[1], Hongye Fan[2], Jianjian Ji[1], Liang Ding[1], Pengfei Li[3], and Yayi Hou[1,4,*]

[1]State Key Laboratory of Pharmaceutical Biotechnology, Division of Immunology, Nanjing University Medical School, Nanjing 210093, China, [2]State Key Laboratory of Natural Medicines, School of Life Science and Technology, China Pharmaceutical University, Nanjing 210009, China, [3]Department of Laboratory Medicine, Jiangsu Province Hospital of Traditional Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210029, China, and [4]Jiangsu Key Laboratory of Molecular Medicine, Nanjing 210093, China

*Correspondence address. Tel/Fax: +86-25-83686441; E-mail: yayihou@nju.edu.cn

Received 9 December 2014; Accepted 7 April 2015

Abstract

Estrogens are strongly implicated in gender differences in immune responses by influencing the development and activation of immune cells. Recent studies have shown that myeloid-derived suppressor cells (MDSCs), derived from $CD11b^+Gr\text{-}1^+$ myeloid cells under pathological conditions, play vital roles in modulating immune responses. However, it is still unknown the effects of estrogens on MDSCs. In the present study, we investigated the effects and mechanisms of estrogens on regulating the accumulation of MDSCs. It was found that, compared with male patients with systemic lupus erythematosus (SLE), female patients with SLE showed a higher frequency of MDSCs in peripheral blood mononuclear cells and a higher level of tumor necrosis factor α (TNF-α) in serum. Notably, estradiol level in the serum of female patients with SLE was positively correlated with the frequency of MDSCs. Moreover, 17β-estradiol could promote TNF-α-induced accumulation of MDSCs *in vivo* by increasing the fundamental frequency of $CD11b^+Gr\text{-}1^+$ cells. Furthermore, 17β-estradiol promoted the secretion of TNF-α *in vivo*, which contributed to the increase of the frequency of $CD11b^+Gr\text{-}1^+$ cells. In addition, it was also found that female mice showed a higher frequency of $CD11b^+Gr\text{-}1^+$ cells and a higher TNF-α level in blood than the age-matched male mice. These data indicate that 17β-estradiol contributes to the accumulation of MDSCs in blood by promoting TNF-α secretion, which increases the fundamental frequency of $CD11b^+Gr\text{-}1^+$ cells. Our findings provide a new insight into the mechanism of gender difference in the prevalence of inflammation and autoimmune diseases.

Key words: gender difference, estrogen, myeloid-derived suppressor cells, $CD11b^+Gr\text{-}1^+$ cells, tumor necrosis factor α

Introduction

Gender differences are universal in health and in the progression of disease, as well as in medical and pharmacological research. Clinical and experimental researches have demonstrated naturally occurring gender differences in immune responses [1,2]. Interestingly, our and others' studies have shown that cells of males and females display several different features and behaviors [3–5]. For example, women had higher levels of $CD4^+$ and $CD8^+$ T cell activation and inflammation-associated gene expression [5]. Lymphocyte subset enumeration revealed higher B cells in females [6]. Although it has been

well known that immune responses are sexually dimorphic [7], the mechanisms responsible for the relationship of gender difference in immunity remain poorly understood.

Multiple studies have demonstrated significant gender differences in the prevalence of inflammatory and autoimmune diseases [8–10]. For example, systemic lupus erythematosus (SLE), an autoimmune disease characterized by the production of unusual antibodies and abnormal activation of B and T cells, develops at a female-to-male ratio of 9:1 [11]. Now, it has been well accepted that estrogens are strongly implicated in the gender differences in the prevalence of inflammatory and autoimmune diseases by mediating the development and activation of immune cells, such as B cells, T cells, and dendritic cells [12–14].

Myeloid-derived suppressor cells (MDSCs) are an abnormal accumulation of different stages of myeloid progenitor cells and immature myeloid cells (IMCs) under some pathological conditions, such as cancer, inflammatory disorders, and infections [15–17]. It can broadly be characterized as HLA-DR$^-$CD11b$^+$CD14$^-$CD33$^+$ in humans [18,19] and CD11b$^+$Gr-1$^+$ in mice [20,21]. In healthy mice, CD11b$^+$Gr-1$^+$ cells are composed of IMCs at the early stages of differentiation, including macrophages, granulocytes, and dendritic cells [22,23]. In pathological conditions, a partial block in the differentiation of IMCs into mature myeloid cells results in the expansion of this population. Importantly, these cells are collectively known as MDSCs because these activated cells possess immune suppressive activity [22]. Accumulating studies have shown that MDSCs possess broad and potent immune suppressive capacity by inhibiting both adaptive and innate immunity in a context-dependent manner [24,25]. The protective role of MDSCs against inflammation and autoimmunity has been widely observed in various pathophysiological conditions [26–28]. However, several recent studies have shown that MDSCs play a proinflammatory role in the pathogenesis of experimental allergic encephalitis (EAE) and rheumatoid arthritis (RA) by promoting Th17 cells differentiation [29–31], suggesting that the roles of MDSCs in inflammatory and autoimmune diseases remain controversial.

A recent study has shown that male (NZB × NZW) F1 mice harbor elevated levels of Gr-1^{high}CD11b$^+$ MDSCs in spleen and bone marrow when compared with age-matched female (NZB × NZW) F1 mice [32]. Interestingly, the elevated level of Gr-1^{high}CD11b$^+$ MDSCs in male mice was due to the male sex hormones, indicating that sex hormones are involved in the regulation of MDSC accumulation. However, there is no report about the effects of estrogens on MDSC accumulation.

Tumor necrosis factor-alpha (TNF-α) is a key inflammatory cytokine involved in the pathogenesis of SLE [33,34]. Much evidence has demonstrated that TNF-α can drive the accumulation of MDSCs [35,36]. Given that estrogens can alter the transcription of inflammatory genes by inhibiting NF-κB activity and recruiting steroid receptor cofactors that act as transcriptional repressors [37–39], we supposed that TNF-α may act as a link between estrogens and the accumulation of MDSCs.

To test our hypothesis, we investigated the possible involvement of estrogens in regulating the accumulation of MDSCs. Here, we reported for the first time that female patients with SLE showed a higher frequency of MDSCs in peripheral blood mononuclear cells (PBMCs) than male patients. Intriguingly, 17β-estradiol could promote TNF-α-induced accumulation of MDSCs *in vivo* by increasing the fundamental frequency of CD11b$^+$Gr-1$^+$ cells. Furthermore, 17β-estradiol could promote the secretion of TNF-α *in vivo*, which can increase the fundamental frequency of CD11b$^+$Gr-1$^+$ cells. Our results demonstrate a critical role of 17β-estradiol in regulating the accumulation of MDSCs. Our data provide a new insight into the pathogenic mechanism of estrogens in the pathogenesis of inflammation and autoimmune diseases.

Materials and Methods

Patients and healthy donors' information

The study protocol was approved by the Research Ethics Committee of Nanjing University (Nanjing, China). After obtaining written informed consent from each SLE patient and healthy donor, peripheral blood samples were obtained from SLE patients and healthy donors. All SLE patients were diagnosed according to the criteria set out by American College of Rheumatology revised criteria in 1997. Whole bloods of healthy subjects with the age of 28 ± 6 years and SLE patients with the age of 28 ± 8 years were recruited. All patients were on treatment, using standard protocols incorporating glucocorticoids and immunosuppressive agents (e.g. cyclophosphamide and azathioprine). Although patients were on a variety of disease-modifying agents, patients on high-dose immunocytotoxic therapies or steroids were excluded from the study. Patients with overlap syndrome were also excluded from the study. Disease activity was evaluated using the SLE Disease Activity Index (SLEDAI) and a cutoff of ≥8 was used to define active disease.

Isolation of human PBMCs

Human PBMCs were separated from plasma by Ficoll centrifugation (Lymphoprep; Nycomed, Oslo, Norway) according to the standard procedures.

Animals

Female and male C57BL/6 mice, 6–8 weeks old, were obtained from Model Animal Research Center at Nanjing University. Female C57BL/6 mice, ovariectomized at 4 weeks old, were also obtained from Model Animal Research Center at Nanjing University. All mice were maintained under specific pathogen-free conditions. All experiments were conducted in accordance with institutional guidelines for animal care and used based on the guide for the Animal Care Committee at Nanjing University.

Generation of mouse MDSCs *in vitro*

Bone marrow cells were planted into dishes using RPMI 1640 medium (Gibco, Carlsbad, USA) supplemented with 2 mM L-glutamine, 10 mM HEPES, 20 mM 2-ME, 150 U/ml streptomycin, 200 U/ml penicillin, and 10% fetal bovine serum (FBS), and stimulated with combinations of GM-CSF (40 ng/ml) and IL-6 (40 ng/ml). The cultures were maintained at 37°C in 5% CO_2-humidified atmosphere in 24-well plates. Medium was changed on the third day. The proportion of CD11b$^+$Gr-1$^+$ MDSCs was analyzed by flow cytometry on the sixth day.

Mouse ovariectomy and 17β-estradiol exposure

Thirty-six C57BL/6 female mice (4 weeks old) were randomly divided into three groups: sham mice (n = 13), ovariectomy (OVX) + placebo mice (n = 13), and OVX + 17β-estradiol mice (n = 13). Mice were surgically ovariectomized or sham-operated (n = 13) after anesthesia using sodium pentobarbital. All animals were well owed to recover for 2 weeks and then received daily s.c. injections of 17β-estradiol (100 μg/kg/day; Sigma, St Louis, USA) or phosphate-buffered saline

(PBS) every morning for 4 weeks. Mice were euthanized at 24 h after receiving their last injection.

TNF-α or TNF-α antagonist etanercept treatment

The TNF-α (0.01 mg/dose; PeproTech, Rocky Hill, USA) or TNF-α antagonist etanercept (0.5 mg/dose; Wyeth, New York, USA) was administered daily by systemic i.p. injection for 7 days before cell harvest. Control mice were injected with PBS.

Flow cytometry assay

For phenotype staining, cells were washed twice with PBS containing 1% FBS and 0.1% NaN_3. Then, the cells were incubated for 30 min at 4°C with anti-human CD14-PE-Cy5.5 antibody, anti-human CD33-PE antibody, anti-human CD11b-APC antibody, anti-mouse Ly6G-PE antibody, or anti-mouse CD11b-APC antibody (eBioscience, San Diego, USA) according to the standard procedure. After being washed twice with PBS, the cells were analyzed by FACS Calibur (Becton Dickinson, Pasadena, USA). An isotype control was used for each antibody. For the analysis of cell apoptosis, cells were washed twice with PBS containing 1% FBS and 0.1% NaN_3, and then re-suspended in 1× binding buffer (0.01 M Hepes/NaOH, pH 7.4, containing 0.14 M NaCl, and 2.5 mM $CaCl_2$). The cells were incubated for 3 min at room temperature in the dark with Annexin V and subsequently incubated with PI for 10 min at room temperature in the dark. Then, the cells were analyzed by FACS Calibur (Becton Dickinson). Data analysis was performed using the FlowJo (TreeStar, San Carlos, USA).

Proliferation analysis of MDSCs

For ^{3}H-thymidine uptake, MDSCs were cultured in 96-well plate at a density of 2×10^6 cells/ml. After pretreated with 17β-estradiol (1, 10, or 100 nM) or TNF-α (1, 10, or 100 ng/ml) for 48 h, MDSCs were pulse-labeled with ^{3}H-thymidine (0.2 μCi/well, Amersham Life Science, Little Chalfont, UK) for 6 h before harvest. Cells were harvested over glass fiber filters and radionuclide uptake was measured by scintillation counting. All experiments were performed in triplicate and were repeated three times.

Quantitative real-time PCR analysis

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) assays of mRNA were carried out on a StepOne Plus real-time PCR system or ABI Vii 7 detection system (Applied Biosystems, Foster City, USA) using SYBR Green PCR Master Mix. The reactions were incubated in a 96-well plate at 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ formula, with *GAPDH* as an internal control. All experiments were performed in triplicate. Primer oligonucleotides were synthesized by Invitrogen, and the sequence information is listed in Table 1.

Serum estradiol assay

Plasma samples collected from patients with SLE and healthy donors were kept at −80°C. Enzyme immunoassay was performed to determine the plasma concentration of estradiol using an Estradiol EIA kit (Cayman Chemical Co., Ann Arbor, USA). In brief, the sample of plasma (50 μl) from each sample was extracted three times using 200 μl of methylene chloride and dissolving the extract in 0.5 ml of EIA buffer. The sample (50 μl) and 200 μl of Ellman's reagent were added to each well, followed by covering the plate with plastic film. After 1 h, the plates were read at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, USA). All samples were assayed in duplicate.

Enzyme-linked immunosorbent assay

The concentrations of serum TNF-α in human and mice were determined by human TNF-α enzyme-linked immunosorbent assay (ELISA) kit and mouse TNF-α ELISA kit (Dakewe, Beijing, China) according to the standard procedure, respectively. In brief, a 100 μl sample of plasma was added to the well and incubated at room temperature (RT) for 2 h. After washed with 1× wash buffer for five times, 100 μl of biotinylated antibody was added to each well and incubated at RT for 1 h. After washed with 1× wash buffer for five times, 100 μl of streptavidin-horseradish peroxidase was added to each well and incubated at RT for 30 min. After washed with 1× wash buffer for five times, 100 μl of 3,3',5,5'-tetramethyl benzidine was added to each well and incubated at RT. After the appropriate time, 100 μl of stop solution was added to each well and the plates were read at 450 nm using a microplate reader (Molecular Devices). All samples were assayed in duplicate.

Statistical analysis

All data were presented as the mean ± standard error of the mean. Data were analyzed using one-way ANOVA and Student's *t*-test, and correlation coefficients were calculated using a linear regression analysis. A *P*-value of <0.05 was considered statistically significant.

Results

Gender difference in the accumulation of MDSCs in patients with SLE

Given that MDSCs play vital roles in regulating immune responses and that the accumulation of MDSCs has been reported in many inflammation and autoimmune diseases [24–28], we first investigated the accumulation of MDSCs in PBMCs from patients with SLE. Expectedly, the frequencies of MDSCs were remarkably elevated in PBMCs from both female and male patients with SLE (0.93% ± 0.077% and 0.55% ± 0.07%, respectively) compared with those from healthy female and male donors (0.18% ± 0.016% and 0.09% ± 0.12%, respectively) (**Fig. 1A,B**). Strikingly, female patients with SLE showed a significantly higher level of accumulation of MDSCs than male patients

Table 1. Primer sequences used in the qPCR

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)
HUMAN-*TNF-α*	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
HUMAN-*GAPDH*	*AGAAGGCTGGGGCTCATTTG*	*AGGGGCCATCCACAGTCTTC*
MOUSE-*TNF-α*	*TCGAGTGACAAGCCTGTAGC*	*CTCAGCCACTCCAGCTGCTC*
MOUSE-*GAPDH*	*AACGACCCCTTCATTGAC*	*TCCACGACATACTCAGCAC*

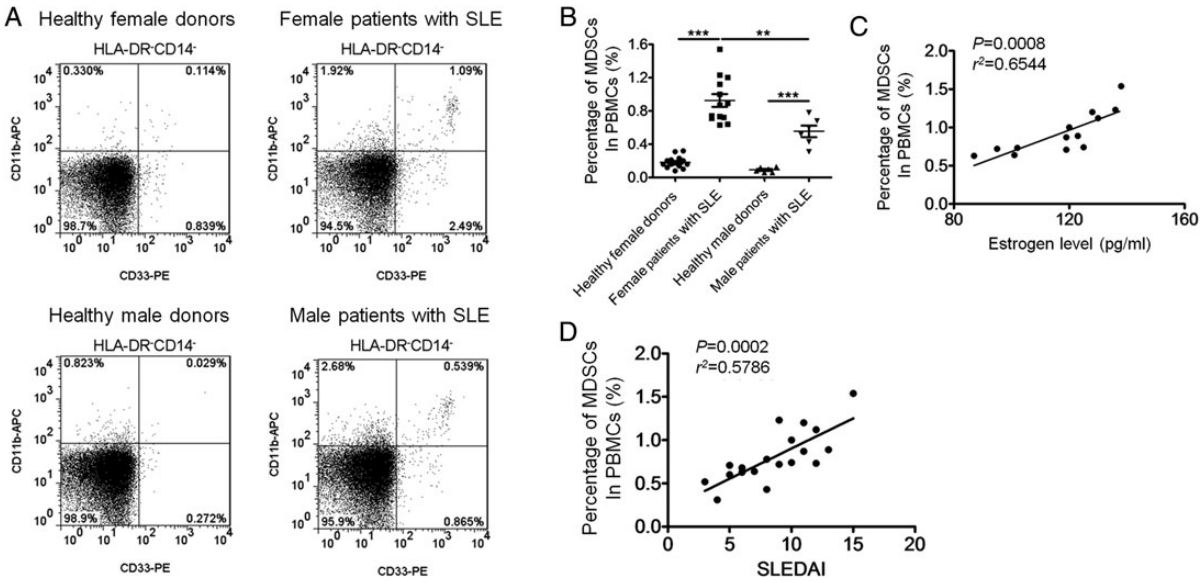

Figure 1. Gender difference in the accumulation of MDSCs in PBMCs from patients with SLE (A) FACS analysis of the percentages of HLA-DR$^-$CD11b$^+$CD14$^-$CD33$^+$ MDSCs in PBMCs from healthy female donors ($n = 16$), female patients with SLE ($n = 13$), healthy male donors ($n = 6$), and male patients with SLE ($n = 6$). Data are representative FACS analysis of MDSCs. (B) Statistical difference between these groups, $^{**}P < 0.01$, $^{***}P < 0.001$. (C) Estradiol levels in serum from female patients with SLE by EIA. The correlation between the percentage of MDSCs in PBMCs and estradiol level was quantified. (D) The correlation between the percentage of MDSCs in PBMCs from patients with SLE and SLEDAI of patients was quantified.

with SLE (0.93% ± 0.077% vs. 0.55% ± 0.07%, $P < 0.05$). Moreover, the percentage of MDSCs in PBMCs was positively correlated with estradiol level in serum from female patients with SLE (**Fig. 1C**) as well as the SLEDAI (**Fig. 1D**). These results demonstrate that female patients with SLE show a higher level of accumulation of MDSCs in blood than male patients with SLE.

Estrogen and TNF-α are related to the gender difference in accumulation of MDSCs in SLE

As TNF-α has been shown to play a critical role in driving the accumulation of MDSCs [35,36], we analyzed TNF-α levels in serum from patients with SLE and healthy donors. As shown in **Fig. 2A**, the levels of TNF-α in serum of female and male patients were 77.48 ± 7.23 pg/ml and 49.6 ± 4.47 pg/ml, respectively. The levels of TNF-α in serum of female and male healthy donors were 22.68 ± 2.25 pg/ml and 17.72 ± 1.56 pg/ml, respectively. These data indicated that the level of TNF-α was significantly higher in serum from patients with SLE than that in healthy donors. Intriguingly, female patients with SLE showed a significantly higher level of TNF-α in serum than male patients with SLE (77.48 ± 7.23 pg/ml vs. 49.6 ± 4.47 pg/ml, $P < 0.05$). Moreover, the level of TNF-α in serum from female patients with SLE was positively correlated with the percentage of MDSCs in PBMCs from female patients with SLE (**Fig. 2B**), as well as estradiol level in serum (**Fig. 2C**). All these results demonstrate that estradiol and TNF-α are related to the gender difference in the accumulation of MDSCs in PBMCs from patients with SLE.

17β-estradiol augments TNF-α-induced accumulation of MDSCs in mice

To further investigate whether estrogens can promote the accumulation of MDSCs, *in vivo* study was conducted in mice. Ovariotomy was performed on 4-week-old female mice. After recovered for 1 week, these ovariotomized mice were treated with 17β-estradiol or placebo for 2 weeks prior to treatment with TNF-α for 7 days. As shown in **Fig. 3A,B**, TNF-α could significantly induce the accumulation of MDSCs in blood in 17β-estradiol-treated ovariotomized mice (12.74% ± 1.45%), placebo-treated ovariotomized mice (5.48% ± 0.87%), and age-matched male mice (6.03% ± 1.52%). Interestingly, TNF-α induced a higher frequency of MDSCs in 17β-estradiol-treated ovariotomized mice than those in placebo-treated ovariotomized mice as well as age-matched male mice. These data indicate that 17β-estradiol augments the TNF-α-induced accumulation of MDSCs in blood *in vivo*.

In addition, we also investigated the effects of 17β-estradiol and TNF-α on proliferation and apoptosis of MDSCs. Murine bone marrow-derived MDSCs were stimulated with different concentrations of 17β-estradiol (1, 10, and 100 nM) or TNF-α (1, 10, and 100 ng/ml) for 48 h. As shown in **Fig. 3C,D**, 17β-estradiol and TNF-α had no effect on the proliferation of MDSCs. Although 17β-estradiol had no effect on apoptosis of MDSCs, TNF-α could significantly inhibit the apoptosis of MDSCs (**Fig. 3E,F**).

17β-estradiol increases the fundamental frequency of CD11b$^+$Gr-1$^+$ cells *in vivo*

We further investigated how 17β-estradiol augments TNF-α-induced accumulation of MDSCs in mice. Strikingly, we found an interesting phenomenon from the results of **Fig. 3A,B** that 17β-estradiol-treated ovariotomized mice showed a higher fundamental frequency of CD11b$^+$Gr-1$^+$ cells in PBMCs than placebo-treated ovariotomized mice and age-matched male mice. To further confirm it, ovariotomy or sham-operation was performed on 4-week-old female mice. The ovariotomized mice were treated with placebo ($n = 13$) or 17β-estradiol ($n = 13$). Two weeks later, these mice were euthanized, and the percentages of CD11b$^+$Gr-1$^+$ cells in PBMCs, spleen, and bone marrow were analyzed. Strikingly, ovariectomized mice showed a lower frequency of CD11b$^+$Gr-1$^+$ cells (1.52% ± 0.14%) in PBMCs

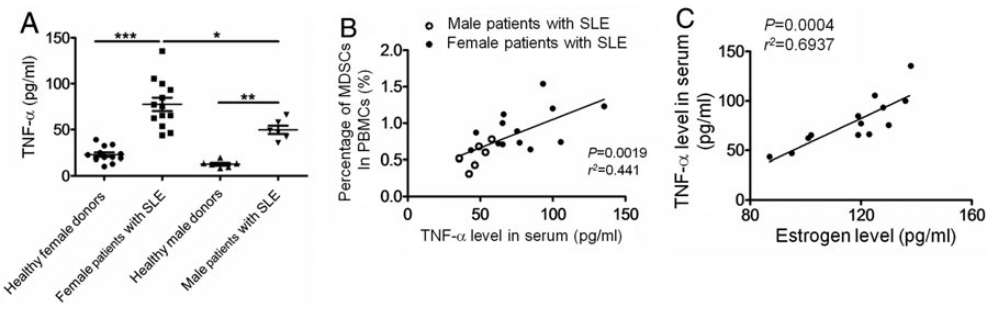

Figure 2. TNF-α is correlated with the accumulation of MDSCs in PBMCs from patients with SLE (A) TNF-α level in serum from healthy donors and patients with SLE by ELISA. $*P<0.05$, $**P<0.01$, $***P<0.001$. (B) The correlation between the percentage of MDSCs in PBMCs and TNF-α level in serum from patients with SLE. (C) The correlation between estradiol level and TNF-α level in serum from patients with SLE.

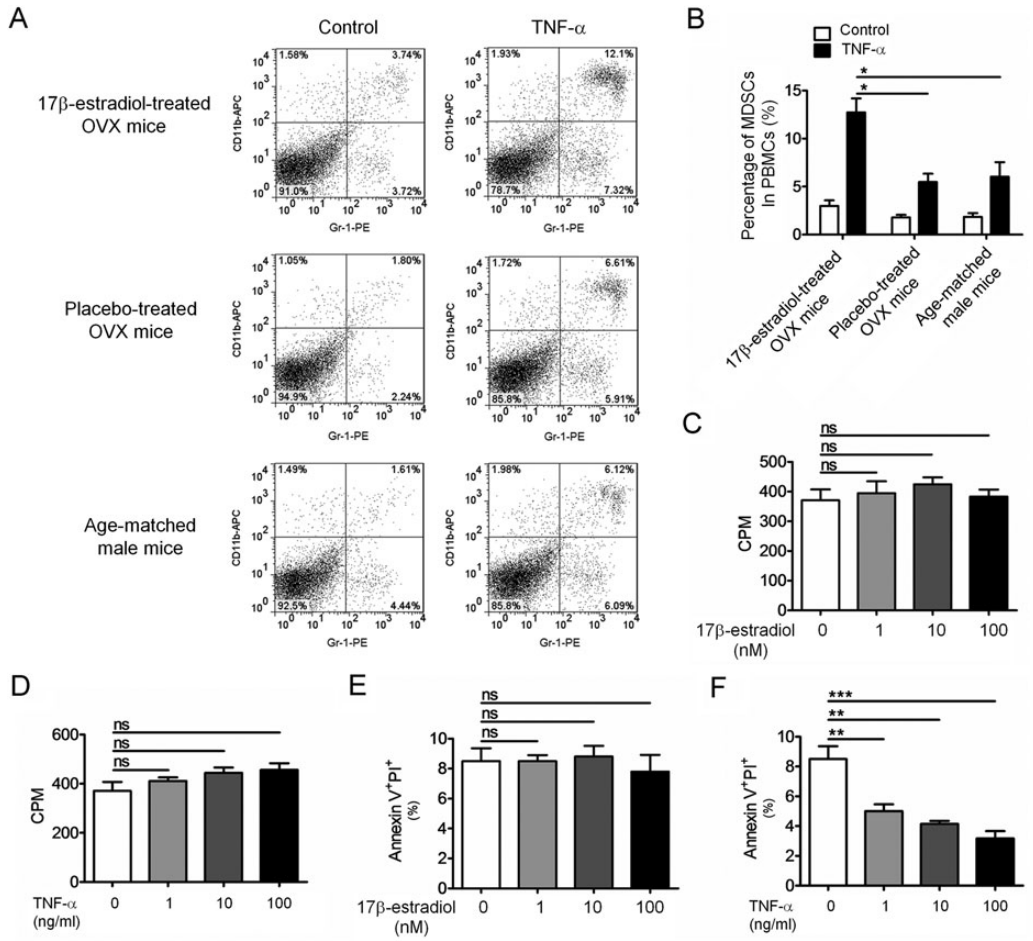

Figure 3. 17β-estradiol augments TNF-α-induced accumulation of MDSCs *in vivo* (A,B) FACS analysis of the percentages of $CD11b^+Gr\text{-}1^+$ MDSCs in PBMCs from 17β-estradiol-treated OVX mice ($n=6$), placebo-treated OVX mice ($n=6$), and age-matched male mice ($n=6$). (A) Data are representative FACS analysis of MDSCs. (B) The quantification data from three experiments. (C,D) The proliferation of MDSCs was detected by H^3-TdR incorporation assay. (E,F) FACS analysis of the apoptosis of MDSCs. $*P<0.05$, $**P<0.01$, $***P<0.001$, ns denotes $P>0.05$.

than sham female mice (3.38% ± 0.16%), whereas treatment with 17β-estradiol could elevate the frequency of $CD11b^+Gr\text{-}1^+$ cells in PBMCs from 17β-estradiol-treated ovariectomized mice (3.25% ± 0.14%) (**Fig. 4A,B**). Interestingly, the frequencies of $CD11b^+Gr\text{-}1^+$ cells in the spleen of 17β-estradiol-treated ovariotomized mice, placebo-treated ovariotomized mice, and age-matched male mice were 2.4% ± 0.11%, 2.15% ± 0.13%, and 2.25% ± 0.09%, respectively (**Fig. 4C,D**). The frequencies of $CD11b^+Gr1^+$ cells in bone marrow of 17β-estradiol-treated ovariotomized mice, placebo-treated ovariotomized mice, and age-matched male mice were 47.73% ± 0.59%, 47.94% ± 0.71%, and 48.27% ± 0.8%, respectively (**Fig. 4E,F**). OVX and treatment with 17β-estradiol have no

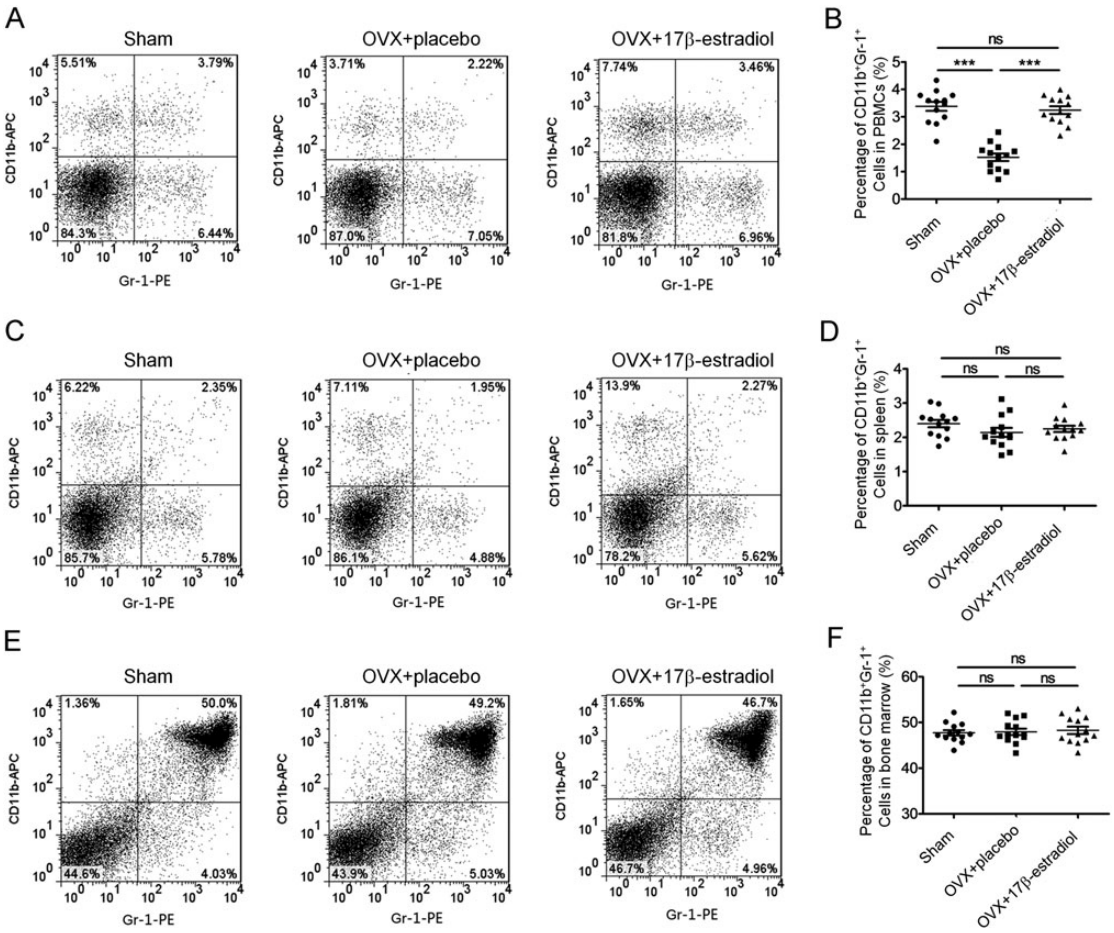

Figure 4. 17β-estradiol increases the fundamental frequency of CD11b$^+$Gr-1$^+$ cells in blood FACS analysis of the percentages of CD11b$^+$Gr-1$^+$ cells in PBMCs (A,B), spleen (C,D), and bone marrow (E,F) from sham female mice, placebo-treated OVX mice and 17β-estradiol-treated OVX mice. (A,C,E) Data are representative FACS analysis of MDSCs. ***$P < 0.001$, ns denotes $P > 0.05$.

effect on the frequencies of CD11b$^+$Gr-1$^+$ cells in spleen and bone marrow. These data demonstrate that 17β-estradiol contributes to the increase of the frequency of CD11b$^+$Gr-1$^+$ cells in blood.

Gender difference in the fundamental frequency of CD11b$^+$Gr-1$^+$ cells in mice

Then, we investigated the fundamental frequency of CD11b$^+$Gr-1$^+$ cells in PBMCs, spleen, and bone marrow from female mice and age-matched male mice. As shown in **Fig. 5A,B**, the percentage of CD11b$^+$Gr-1$^+$ cells was significantly higher in PBMCs from female mice than that in PBMCs from age-matched male mice (2.97% ± 0.23% vs. 2.13% ± 0.13%, $P < 0.01$). Interestingly, female mice showed significantly reduced percentages of CD11b$^+$Gr-1$^+$ cells in spleen (2.2% ± 0.11%) (**Fig. 5C,D**) and bone marrow (46.42% ± 1.21%) (**Fig. 5E,F**) compared with age-matched male mice (3.55% ± 0.29% and 56.77% ± 0.83%, respectively). These data demonstrate a remarkable gender difference in the frequency of CD11b$^+$Gr-1$^+$ cells in PBMCs, spleen, and bone marrow from mice.

17β-estradiol increases the frequency of CD11b$^+$Gr-1$^+$ cells in blood by promoting TNF-α secretion

TNF-α is a proinflammatory, multifunctional, and immunomodulating cytokine, which has been shown to drive MDSC accumulation [35,36]. To investigate whether TNF-α can affect the frequency of CD11b$^+$Gr-1$^+$ cells in blood from mice, *in vivo* study was conducted. TNF-α antagonist etanercept or PBS was injected into sham female mice, 17β-estradiol-treated OVX mice, placebo-treated OVX mice, and age-matched male mice for a week. Then, the frequencies of CD11b$^+$Gr-1$^+$ cells in PBMCs from these mice were detected. As shown in **Fig. 6A** and **Supplementary Fig. S1**, etanercept could significantly reduce the fundamental frequency of CD11b$^+$Gr-1$^+$ cells in PBMCs in sham female mice (from 3.06% ± 0.17% to 1.13% ± 0.19%, $P < 0.001$), placebo-treated OVX mice (from 1.72% ± 0.24% to 0.88% ± 0.22%, $P < 0.05$), 17β-estradiol-treated OVX mice (from 3.13% ± 0.41% to 1.1% ± 0.15%, $P < 0.001$), and age-matched male mice (from 1.79% ± 0.27% to 0.89% ± 0.25%, $P < 0.05$). Intriguingly, 17β-estradiol-treated OVX mice showed a higher frequency of CD11b$^+$Gr-1$^+$ cells than placebo-treated OVX mice, whereas etanercept could eliminate 17β-estradiol-induced increase of the frequency of CD11b$^+$Gr1$^+$ cells. These data suggest that TNF-α plays a critical role in 17β-estradiol-induced increase of the frequency of CD11b$^+$Gr1$^+$ cells in blood *in vivo*.

Then, we detected the influence of 17β-estradiol on TNF-α secretion. As shown in **Fig. 6B**, TNF-α level in serum from female mice was higher than that in age-matched male mice (34.63 ± 1.67 pg/ml vs. 27.53 ± 1.16 pg/ml, $P < 0.01$). Moreover, the mRNA level of *TNF-α* in PBMCs from female mice was also higher than that in age-matched

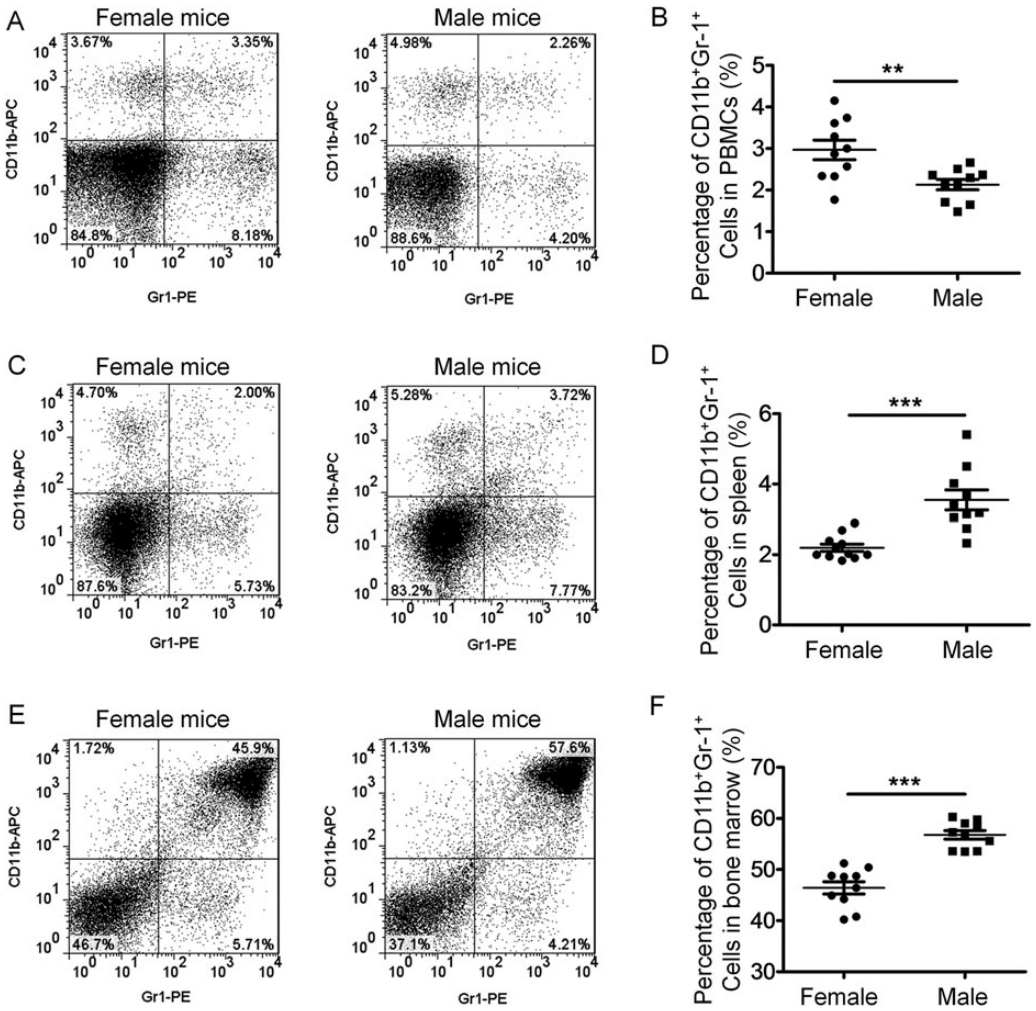

Figure 5. Gender difference in the fundamental frequency of CD11b$^+$Gr-1$^+$ cells in PBMCs from mice FACS analysis of the percentages of CD11b$^+$Gr-1$^+$ cells in PBMCs (A,B), spleen (C,D), and bone marrow (E,F) from female ($n = 10$) and age-matched male mice ($n = 10$). (A,C,E) Data are representative FACS analysis of MDSCs. $^{**}P < 0.01$, $^{***}P < 0.001$, ns denotes $P > 0.05$.

male mice ($P < 0.01$) (**Fig. 6C**). Notably, the level of TNF-α in serum was positively correlated with the percentage of CD11b$^+$Gr-1$^+$ cells in PBMCs from female and age-matched male mice (**Fig. 6D,E**).

We further analyzed the levels of TNF-α in serum and PBMCs from sham female mice, placebo-treated ovariectomized mice, and 17β-estradiol-treated ovariectomized mice. As shown in **Fig. 6F**, placebo-treated ovariectomized mice showed reduced TNF-α level in serum (25.48 ± 1.24 pg/ml) compared with sham female mice (36.26 ± 1.23 pg/ml), and 17β-estradiol treatment could elevate the TNF-α level in serum from 17β-estradiol-treated ovariectomized mice (34.82 ± 0.85 pg/ml). Moreover, placebo-treated ovariectomized mice showed reduced mRNA level of *TNF-α* in PBMCs compared with sham female mice, and hormone reconstitution could elevate the mRNA level of *TNF-α* in serum from 17β-estradiol-treated ovariectomized mice (**Fig. 6G**). Notably, the level of TNF-α in serum was positively correlated with the percentage of CD11b$^+$Gr-1$^+$ cells in PBMCs from placebo-treated and 17β-estradiol-treated ovariectomized mice (**Fig. 6H,I**).

In addition, we also investigated the levels of TNF-α in serum and PBMCs from pre-menopausal women, age-matched men, and post-menopausal women. As shown in **Fig. 6J**, the level of TNF-α in serum from pre-menopausal women (18.16 ± 1.42 pg/ml) was higher than those from age-matched men (10.94 ± 0.92 pg/ml) and post-menopausal women (11.89 ± 0.94 pg/ml). Moreover, the mRNA level of *TNF-α* in PBMCs from pre-menopausal women was also higher than those from age-matched men as well as post-menopausal women (**Fig. 6K**). Altogether, these data indicate that 17β-estradiol contributes to accumulation of MDSCs in PBMCs by promoting the secretion of TNF-α.

Discussion

SLE predominantly affects female population. It has been known that the estrogens have a promoting function in the pathogenesis of SLE [40]. However, the mechanism by which estrogens exert pathogenic effects on the immune system is still not well understood. In the present study, we investigated the effects of estrogens on MDSC accumulation. Manipulation of estrogen levels through ovariectomy significantly influenced TNF-α-induced accumulation of MDSCs *in vivo*, suggesting that estrogens may exert pathogenic effects on the pathogenesis of autoimmune diseases through influencing the accumulation of MDSCs. Our findings provide a new insight into the

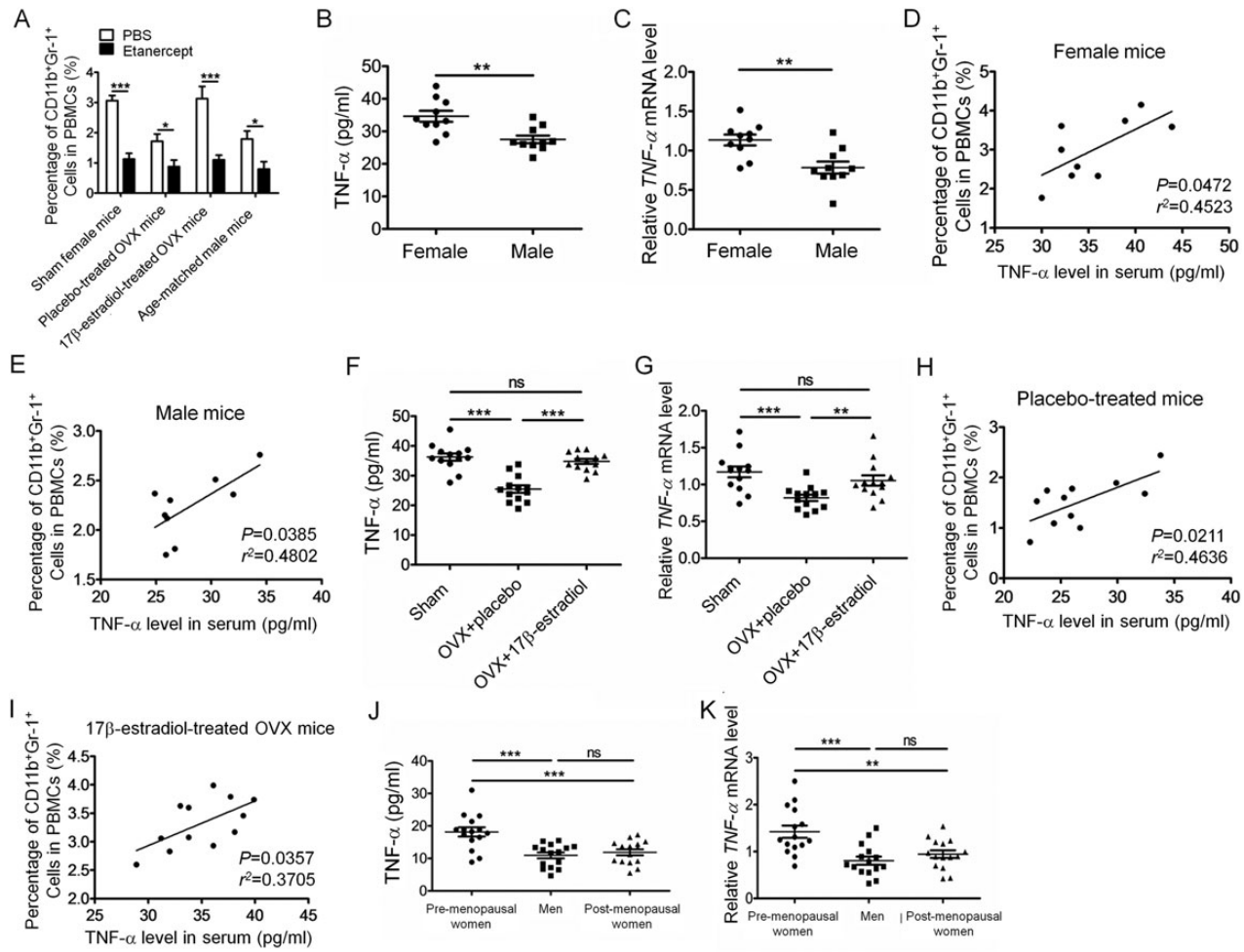

Figure 6. 17β-estradiol increases the fundamental frequency of CD11b$^+$Gr-1$^+$ cells through promoting the secretion of TNF-α *in vivo* (A) FACS analysis of the frequency of CD11b$^+$Gr-1$^+$ cells in PBMCs from mice. (B,C) ELISA analysis of TNF-α levels in serum (B) and qPCR analysis of *TNF-α* expression in PBMCs (C) from female ($n = 10$) and male mice ($n = 10$). (D,E) The correlations between the percentage of CD11b$^+$Gr-1$^+$ cells in PBMCs and TNF-α level in serum from female and male mice. (F,G) ELISA analysis of TNF-α levels in serum (F) and qPCR analysis of *TNF-α* expression in PBMCs (G) from sham mice ($n = 13$), placebo-treated OVX mice ($n = 13$), and 17β-estradiol-treated OVX mice ($n = 13$). (H,I) The correlations between the percentage of CD11b$^+$Gr-1$^+$ cells in PBMCs and the TNF-α level in serum. (J,K) ELISA analysis of TNF-α levels in serum (J) and qPCR analysis of *TNF-α* expression in PBMCs (K) from pre-menopausal women ($n = 15$), age-matched men ($n = 15$), and post-menopausal women ($n = 15$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns denotes $P > 0.05$.

pathogenic function of estrogens in the pathogenesis of inflammation and autoimmune diseases.

Although accumulation of MDSCs has also been reported in many inflammatory conditions [26–28], it remains unknown whether accumulation of MDSCs occurs in patients with SLE. Here, we reported for the first time that the frequency of MDSCs was significantly higher in PBMCs from patients with SLE than that from healthy donors. Strikingly, female patients with SLE showed a higher frequency of MDSCs in PBMCs than male patients. Although transplantation of MDSCs can improve the condition of several autoimmune diseases in animal models, several recently published studies have shown that MDSCs play a proinflammatory role in the pathogenesis of EAE and RA by promoting T helper 17 cell differentiation [29–31]. Notably, CD11b$^+$Gr-1$^+$ MDSCs are a complex mixture of myeloid phenotypes. Moreover, MDSCs morphologically and functionally differ in various tissues under different inflammatory conditions [9]. Even within the same inflammatory process, phenotypic differences in MDSCs occur over time [10]. Taken together, the phenotypes within MDSCs change in the process of inflammatory progresses [10]. This might account for their diverse immunological activities under different inflammatory conditions. Therefore, more studies are needed to assess the role of MDSCs in the pathogenesis of autoimmune diseases.

Notably, we found that 17β-estradiol promotes TNF-α-induced accumulation of MDSCs in mice. As is known, the severity and incidence of innate immune conditions, such as sepsis and post-surgery infections, are profoundly less in women than in age-matched men [40,41]. Given that MDSCs play a vital role in regulating immune responses and pathogenesis of many inflammatory and autoimmune diseases [15–17], we propose that estrogen may participate in the sex-based difference in the pathogenesis of infectious diseases through promoting accumulation of MDSCs under inflammatory conditions. Thus, more studies are needed to reveal the role of MDSCs in the sex-based difference in the pathogenesis of infectious diseases.

TNF-α is implicated in the pathogenesis and development of autoimmune diseases [33,34,42]. Several FDA-approved TNF-α antagonists have been used for the treatment of various chronic pathologies, such as RA, psoriasis, type II diabetes, Crohn's disease, and cancer [43]. Notably, etanercept, one of these antagonists, has

been shown to be effective in treating patients with several diseases [44,45]. However, the mechanism by which TNF-α and its corresponding antagonists regulate the host's immune system remains unclear. Interestingly, it was found that etanercept could significantly reduce the fundamental frequency of $CD11b^+Gr\text{-}1^+$ cells in PBMCs from C57BL/6 mice, suggesting that etanercept might relieve disease conditions of autoimmune diseases through reducing the fundamental frequency of $CD11b^+Gr\text{-}1^+$ cells *in vivo*.

In addition, we investigated the effect of 17β-estradiol and TNF-α on the proliferation and apoptosis of MDSCs. Murine bone marrow-derived MDSCs were stimulated with different concentrations of 17β-estradiol or TNF-α for 48 h. As shown in **Fig. 3C,D**, 17β-estradiol and TNF-α had no effect on the proliferation of MDSCs. Although 17β-estradiol had no effect on apoptosis of MDSCs, TNF-α could significantly inhibit the apoptosis of MDSCs. Given that TNF-α plays a critical role in driving the accumulation of MDSCs [35,36] and enhanced apoptosis is responsible for reduced MDSC numbers in $TNFR^{-/-}$ mice [36], more studies are needed to reveal the mechanism through which TNF-α inhibits apoptosis of MDSCs.

Although it is well accepted that estrogens contribute to the pathogenesis of SLE, the target of regulation and the specific mechanism of immune response mediated by estrogens remain unidentified. Notably, we found that 17β-estradiol could increase the frequency of $CD11b^+Gr\text{-}1^+$ cells in PBMCs from normal mice. We also found that the percentages of $CD11b^+Gr\text{-}1^+$ cells were significantly lower in spleen and bone marrow from female mice than that in age-matched male mice. These data suggest that sex hormones may have different function on immune cells in different tissues and organs.

Previous studies have shown that estrogens can alter the transcription of inflammatory genes [37–39]. More importantly, treatment with estrogens increases the frequency and numbers of TNF-α-producing $CD8^+$ T cells in mice [46]. Consistently, we found that estrogens could promote the secretion of TNF-α *in vivo*. As is known, TNF-α is a key inflammatory cytokine involved in the pathogenesis of SLE [33,34] and estrogens contribute to the pathogenesis of SLE [12–14]. Thus, estrogens may contribute to the pathogenesis of SLE through promoting the secretion of TNF-α. More studies are needed to reveal the mechanism through which estrogens effect TNF-α secretion.

In summary, we demonstrate for the first time that 17β-estradiol promotes the secretion of TNF-α *in vivo* and contributes the accumulation of MDSCs in blood. This study may help us to understand the role of estrogens in the progression and development of inflammatory and autoimmune diseases. Further investigation into the related signaling pathways and potential mechanisms underlying the effects of estrogens are required to fully understand the pathogenic function of estrogens.

Supplementary Data

Supplementary data is available at *ABBS* online.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (No. 31370899) and the Ministry of Science and China Postdoctoral Science Foundation (No. 2013M531331).

References

1. McClelland EE, Smith JM. Gender specific differences in the immune response to infection. *Arch Immunol Ther Exp (Warsz)* 2011, 59: 203–213.
2. Choudhry MA, Bland KI, Chaudry IH. Trauma and immune response--effect of gender differences. *Injury* 2007, 38: 1382–1391.
3. Straface E, Gambardella L, Brandani M, Malorni W. Sex differences at cellular level: "cells have a sex". *Handb Exp Pharmacol* 2012, 214: 49–65.
4. Giampietri C, Petrungaro S, Filippini A, Ziparo E. Sex-related differences in death control of somatic cells. *J Cell Mol Med* 2013, 17: 550–551.
5. Fan H, Dong G, Zhao G, Liu F, Yao G, Zhu Y, Hou Y. Gender differences of B cell signature in healthy subjects underlie disparities in incidence and course of SLE related to estrogen. *J Immunol Res* 2014, 2014: 814598.
6. Abdullah M, Chai PS, Chong MY, Tohit ER, Ramasamy R, Pei CP, Vidyadaran S. Gender effect on *in vitro* lymphocyte subset levels of healthy individuals. *Cell Immunol* 2012, 272: 214–219.
7. Sankaran-Walters S, Macal M, Grishina I, Nagy L, Goulart L, Coolidge K, Li J, *et al*. Sex differences matter in the gut: effect on mucosal immune activation and inflammation. *Biol Sex Differ* 2013, 4: 10.
8. Rubtsov AV, Rubtsova K, Kappler JW, Marrack P. Genetic and hormonal factors in female-biased autoimmunity. *Autoimmun Rev* 2010, 9: 494–498.
9. Gonzalez DA, Diaz BB, Rodriguez Perez Mdel C, Hernandez AG, Chico BN, de Leon AC. Sex hormones and autoimmunity. *Immunol Lett* 2010, 133: 6–13.
10. Sokka T, Toloza S, Cutolo M, Kautiainen H, Makinen H, Gogus F, Skakic V, *et al*. Women, men, and rheumatoid arthritis: analyses of disease activity, disease characteristics, and treatments in the QUEST-RA study. *Arthritis Res Ther* 2009, 11: R7.
11. Masi AT, Kaslow RA. Sex effects in systemic lupus erythematosus: a clue to pathogenesis. *Arthritis Rheum* 1978, 21: 480–484.
12. Nalbandian G, Kovats S. Understanding sex biases in immunity: effects of estrogen on the differentiation and function of antigen-presenting cells. *Immunol Res* 2005, 31: 91–106.
13. Josefsson E, Tarkowski A, Carlsten H. Anti-inflammatory properties of estrogen. I. *In vivo* suppression of leukocyte production in bone marrow and redistribution of peripheral blood neutrophils. *Cell Immunol* 1992, 142: 67–78.
14. Xie H, Hua C, Sun L, Zhao X, Fan H, Dou H, Hou Y. 17beta-estradiol induces CD40 expression in dendritic cells via MAPK signaling pathways in a minichromosome maintenance protein 6-dependent manner. *Arthritis Rheum* 2011, 63: 2425–2435.
15. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* 2012, 12: 253–268.
16. Ochoa AC, Zea AH, Hernandez C, Rodriguez PC. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. *Clin Cancer Res* 2007, 13: 721s–726s.
17. Delano MJ, Scumpia PO, Weinstein JS, Coco D, Nagaraj S, Kelly-Scumpia KM, O'Malley KA, *et al*. MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis. *J Exp Med* 2007, 204: 1463–1474.
18. Solito S, Falisi E, Diaz-Montero CM, Doni A, Pinton L, Rosato A, Francescato S, *et al*. A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. *Blood* 2011, 118: 2254–2265.
19. Liu CY, Wang YM, Wang CL, Feng PH, Ko HW, Liu YH, Wu YC, *et al*. Population alterations of L-arginase- and inducible nitric oxide synthase-expressed CD11b+/CD14(-)/CD15+/CD33+ myeloid-derived suppressor cells and CD8+ T lymphocytes in patients with advanced-stage non-small cell lung cancer. *J Cancer Res Clin Oncol* 2010, 136: 35–45.
20. Peranzoni E, Zilio S, Marigo I, Dolcetti L, Zanovello P, Mandruzzato S, Bronte V. Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr Opin Immunol* 2010, 22: 238–244.
21. Ribechini E, Greifenberg V, Sandwick S, Lutz MB. Subsets, expansion and activation of myeloid-derived suppressor cells. *Med Microbiol Immunol* 2010, 199: 273–281.
22. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009, 9: 162–174.
23. Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, *et al*. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* 2004, 6: 409–421.

24. Haile LA, von Wasielewski R, Gamrekelashvili J, Kruger C, Bachmann O, Westendorf AM, Buer J, *et al*. Myeloid-derived suppressor cells in inflammatory bowel disease: a new immunoregulatory pathway. *Gastroenterology* 2008, 135: 871–881, 881 e871–875.
25. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 2009, 182: 4499–4506.
26. Meyer C, Sevko A, Ramacher M, Bazhin AV, Falk CS, Osen W, Borrello I, *et al*. Chronic inflammation promotes myeloid-derived suppressor cell activation blocking antitumor immunity in transgenic mouse melanoma model. *Proc Natl Acad Sci USA* 2011, 108: 17111–17116.
27. Sinha P, Chornoguz O, Clements VK, Artemenko KA, Zubarev RA, Ostrand-Rosenberg S. Myeloid-derived suppressor cells express the death receptor Fas and apoptose in response to T cell-expressed FasL. *Blood* 2011, 117: 5381–5390.
28. Van Ginderachter JA, Beschin A, De Baetselier P, Raes G. Myeloid-derived suppressor cells in parasitic infections. *Eur J Immunol* 2010, 40: 2976–2985.
29. Zhang H, Wang S, Huang Y, Wang H, Zhao J, Gaskin F, Yang N, *et al*. Myeloid-derived suppressor cells are proinflammatory and regulate collagen-induced arthritis through manipulating Th17 cell differentiation. *Clin Immunol* 2015, 157: 175–186.
30. Yi H, Guo C, Yu X, Zuo D, Wang XY. Mouse CD11b^{+}Gr-1^{+} myeloid cells can promote Th17 cell differentiation and experimental autoimmune encephalomyelitis. *J Immunol* 2012, 189: 4295–4304.
31. Guo C, Hu F, Yi H, Feng Z, Li C, Shi L, Li Y, *et al*. Myeloid-derived suppressor cells have a proinflammatory role in the pathogenesis of autoimmune arthritis. *Ann Rheum Dis* 2014, doi: 10.1136/annrheumdis-2014-205508.
32. Trigunaite A, Khan A, Der E, Song A, Varikuti S, Jorgensen TN. Gr-1(high) CD11b+ cells suppress B cell differentiation and lupus-like disease in lupus-prone male mice. *Arthritis Rheum* 2013, 65: 2392–2402.
33. Weckerle CE, Mangale D, Franek BS, Kelly JA, Kumabe M, James JA, Moser KL, *et al*. Large-scale analysis of tumor necrosis factor alpha levels in systemic lupus erythematosus. *Arthritis Rheum* 2012, 64: 2947–2952.
34. Mak A, Tang CS, Ho RC. Serum tumour necrosis factor-alpha is associated with poor health-related quality of life and depressive symptoms in patients with systemic lupus erythematosus. *Lupus* 2013, 22: 254–261.
35. Sade-Feldman M, Kanterman J, Ish-Shalom E, Elnekave M, Horwitz E, Baniyash M. Tumor necrosis factor-alpha blocks differentiation and enhances suppressive activity of immature myeloid cells during chronic inflammation. *Immunity* 2013, 38: 541–554.
36. Zhao X, Rong L, Li X, Liu X, Deng J, Wu H, Xu X, *et al*. TNF signaling drives myeloid-derived suppressor cell accumulation. *J Clin Invest* 2012, 122: 4094–4104.
37. Stein B, Yang MX. Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta. *Mol Cell Biol* 1995, 15: 4971–4979.
38. Cvoro A, Tzagarakis-Foster C, Tatomer D, Paruthiyil S, Fox MS, Leitman DC. Distinct roles of unliganded and liganded estrogen receptors in transcriptional repression. *Mol Cell* 2006, 21: 555–564.
39. Straub RH. The complex role of estrogens in inflammation. *Endocr Rev* 2007, 28: 521–574.
40. Marriott I, Huet-Hudson YM. Sexual dimorphism in innate immune responses to infectious organisms. *Immunol Res* 2006, 34: 177–192.
41. Adrie C, Azoulay E, Francais A, Clech C, Darques L, Schwebel C, Nakache D, *et al*. Influence of gender on the outcome of severe sepsis: a reappraisal. *Chest* 2007, 132: 1786–1793.
42. Breedveld F. TNF antagonists opened the way to personalized medicine in rheumatoid arthritis. *Mol Med* 2014, 20(Suppl 1):S7–S9.
43. Cheng P, Corzo CA, Luetteke N, Yu B, Nagaraj S, Bui MM, Ortiz M, *et al*. Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *J Exp Med* 2008, 205: 2235–2249.
44. Friedberg J, Jacobsen E, Neuberg D, Kutok J, Munoz O, Boussiotis V, Reynolds H, *et al*. Targeting the follicular lymphoma microenvironment through blockade of TNFalpha with etanercept. *Leuk Lymphoma* 2008, 49: 902–909.
45. Wilson JA. Tumor necrosis factor alpha and colitis-associated colon cancer. *N Engl J Med* 2008, 358: 2733–2734.
46. Robinson DP, Hall OJ, Nilles TL, Bream JH, Klein SL. 17beta-estradiol protects females against influenza by recruiting neutrophils and increasing virus-specific CD8 T cell responses in the lungs. *J Virol* 2014, 88: 4711–4720.