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Original Article

PD-L1 is required for human endometrial regenerative cells-associated attenuation of experimental colitis in mice

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Abstract: Endometrial regenerative cells (ERCs) are easily isolated from menstrual blood, and can be cultured in large amounts. Although, ERCs can ameliorate DSS-induced colitis in mice, the molecular mechanism underlying ERCs-mediated immunosuppression is unclear. This study was aimed to assess the function of PD-L1 expressed on ERCs in colitis attenuation. ERCs with and without anti-PD-L1 mAb-pretreatment were administered to mice by injection at 2, 5 and 8 days after colitis induction by DSS treatment. Blood, spleen and colon samples were obtained 15 days post-DSS-induction. Then, clinicopathological alterations, cytokine levels, immune cell types and cell tracking were assessed. ERCs or ERCs preincubated with anti-PD-L1 antibody were co-cultured with splenocytes, whose phenotypes was analyzed by flow cytometry. We found that PD-L1 on ERCs was upregulated by IFN-γ stimulation. The transplanted PKH26-labeled ERCs were engrafted to the lung, liver, spleen and injured colon. Interestingly, ERC-based therapy markedly attenuated mouse colitis, but blockade of PD-L1 on ERCs with a specific monoclonal antibody conferred severe colitis to the animals. These effects of PD-L1 inhibition on colitis were associated with reduced amounts of pro-inflammatory cytokines and infiltrated immune cells, including $CD3^+CD4^+$ T lymphocytes, $CD3^+CD8^+$ T lymphocytes, $CD11c^+MHC\text{-}II^+$ Dendritic cells and $F4/80^+$ macrophages, both in vivo and in vitro, as well as with elevated levels of anti-inflammatory cytokines and regulatory immune cells, including $CD4^+CD25^+Foxp3^+$ Tregs and $F4/80^+CD206^+$ macrophages. These findings demonstrated that ERCs-based treatment promotes immune tolerance in mouse colitis, in association with PD-L1, thus indicating that PD-L1 modulates immunosuppression by ERCs.

Keywords: Colitis, endometrial regenerative cells, immunosuppression, mice, PD-L1

Introduction

Ulcerative colitis (UC) is a major type of inflammatory bowel disease (IBD) featuring chronic, relapsing, nonspecific inflammatory reactions in the colorectal area [1]. UC prevalence and incidence are currently elevated in developed nations, and have been quickly increasing in other countries, especially in China, in past decades [2]. Although a clear-cut etiology has not been proposed for UC, it is generally admitted that this ailment is associated with genetic susceptibility, environmental factors, changes in the gut microbiota and dysregulation of immune response [3]. UC is routinely treated with anti-inflammatory medicines and immunosuppressors, and even surgical removal of the colon [1]. However, satisfactory results are rarely obtained. In recent years, there has been increasing evidence that shows mesenchymal stromal cells (MSCs) therapy to be effective in the treatment of UC [4].

MSCs are multipotent stromal cells with self-renewal potential. They promote tissue repair and wound healing, and show immunomodulatory and anti-inflammatory features in UC [5]. Endometrial regenerative cells (ERCs), isolated from menstrual blood as a novel group of MSCs, also display tissue repair and immunomodula-

tory functions. Compared with MSCs from many conventional sources, ERCs present further advantages, including rich source, noninvasive method for harvesting, high abundance, easy purification, rapid proliferation, relatively unlimited expandability, and the potential to differentiate into more lineages [6]. Moreover, ERCs can produce matrix metalloprotease and a large number of growth factors to promote tissue repair [7]. In our previous study, ERCs were selected for the treatment of UC, and the results showed that systemic infusion of ERCs attenuates experimental colitis in mice with significantly reduced disease activity index (DAI). The intra-colon levels of proinflammatory cytokines were decreased while there was an increase in anti-inflammatory cytokines. It was also found that immune reactive cells in the spleen decreased, while regulatory T cells increased [8]. However, the molecular mechanism underlying ERC-mediated immunosuppression in the colitis model remains largely unclear.

MSCs modulate some immune cells, including DCs, macrophages and T and B cells via secretion of soluble factors [5, 9, 10]. In addition, MSCs exert immunosuppressive effects on immune cells by direct cell-to-cell interactions (e.g. PD-L1 interacts with PD-1) [11], and production of surface adhesion molecules [12] and human leukocyte antigen-G [13]. MSC-expressed PD-L1 plays a significant role in MSC-mediated immune suppression [14]. Our previous research indicated that MSCs require PD-L1 for the induction of immune tolerance in a cardiac allotransplantation model [15]. Moreover, Song et al found that PD-L1-Fc markedly alleviates DSS associated acute colitis as well as T-cell dependent chronic colitis, by inducing colonic $CD4^+$ T cell and DC regulation [16]. We detected that ERCs as mesenchymal-like stem cells also express PD-L1. Thus, we speculate that PD-L1 also plays an important role in immunosuppressive effects during the ERCs treatment for colitis.

Materials and methods

Animals

BALB/c mice (Male, 8 weeks, 18-22 g; Aoyide Co., Tianjin, China) were maintained routinely under a cycle of 12 h-12 h light-dark in the vivarium of the Tianjin General Surgery Institute, with standard rodent chow and drinking water ad libitum. The study was approved by the Institutional Ethics Committee, following the Chinese Council on Animal Care guidelines.

Preparation of ERCs

The menstrual blood was collected from a 30-year-old healthy woman upon informed consent. ERCs were isolated following the isolation method as described by Y Lv et al previously [8]. In brief, menstrual blood was obtained by the Diva cup method in a solution supplemented with antibiotics. Approximately 10 ml of menstrual blood samples were used for ERCs isolation each time. Then, the Ficoll method was employed for mononuclear cell separation. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose containing 10% fetal bovine serum (FBS) overnight, with marginal adherence to tissue culture flasks. After 2 weeks of culture (with 2 medium changes weekly), adherent cells with a fibroblast-like morphology were obtained. A culture was started with about 1×10^7 adherent cells. Phenotypic analysis of ERCs was performed by flow cytometry (Becton Dickinson FACS Canto II, Franklin Lakes, NJ, USA) after staining with antibodies against CD34, CD45, CD90 and CD105 (Becton Dickinson and Company, http://www.BD.com/), respectively, as directed by the manufacturer. In addition, ERCs were positive for PD-L1 (BioLegend, http://www.biolegend.com/) as evaluated by FACS. In functional experiments, PD-L1 on ERCs was inhibited by anti-PD-L1 antibodies (BioLegend, http://www.biolegend.com/) as reported in previous studies [15, 17, 18]. ERCs at passages 3-4 were used for in vitro and in vivo.

Induction and assessment of DSS-associated colitis

The mouse DSS colitis model was established according to previous study [19], by administration of 3% Dextran sulfate sodium (DSS) (MW 5000 kDa; Sigma-Aldrich, St Louis, United States) in drinking water for 7 consecutive days. Weight, stool consistency and occult blood assessments were carried out daily.

In vivo treatment and experimental groups

Thirty-two animals were randomly divided into four groups (n=8), including sham (Group 1; drinking water for 14 days), colitis model (Group 2; 3% DSS for 7 days, and drinking water for 7 days), ERCs treatment (Group 3; DSS models intravenously administered ERCs in PBS at

1×10^6/0.2 mL/mouse at 2, 5 and 8 days, respectively), and anti-PD-L1-pretreated ERCs group (Group 4; DSS models intravenously administered anti-PD-L1 mAb-pretreated ERCs in PBS at 1×10^6/0.2 mL/mouse at 2, 5 and 8 days, respectively).

Gross inspection and DAI

Body weight, stool consistency and stool features were evaluated during the experiment. DAI was employed to assess intestinal inflammation [20]. The animals were euthanized at 15 days after DSS initiation, and blood, spleen and colon specimens were obtained from different groups. The colon length was measured; after rinsing with PBS, each colon was divided and employed for histology, mRNA purification, and protein extraction.

Histological examination

Colon specimens were fixed with 10% formaldehyde, paraffin embedded and sectioned at 5 μm for hematoxylin and eosin staining. The samples were histologically scored in a blinded manner based on inflammation severity (scores of 0 to 3), extent of injury (0 to 3), crypt damage (0 to 4), and percent involvement (1-4); the overall histopathology score was obtained as a sum of the above subscores [21].

Immunohistochemistry staining

To assess the amounts of neutrophils, T cells and M2 macrophages (total or activated), the colonic sections were stained with antibodies raised against mouse Ly6g, CD3 and CD206 (Abcam, Cambridge, MA), separately. Briefly, 5 μm sections of paraffin embedded samples were deparaffinized and rehydrated, followed by antigen retrieval (microwave) and treatment with 3% H_2O_2. After blocking with 5% bovine serum albumin (BSA), the samples were exposed to antibodies raised against mouse Ly6g, CD3 and CD206 overnight at 4°C, respectively. In negative controls, the primary antibodies were omitted. A Streptavidin-Biotin Complex (SABC) kit was employed for detection; imaging was photographed (Olympus Imaging America, Center Valley, PA).

Real-time quantitative PCR

TNF-α, IFN-γ, IL-10 and TGF-β mRNA amounts in colon specimens were assessed by real-time quantitative PCR (MJ Research Inc., USA), with the following PCR primers: TNF-α, forward 5'-CATCTTCTCAAAATTCGAGTGACAA-3' and reverse 5'-TGGGAGTAGACAAGGTACAACCC-3'; IFN-γ, forward 5'-GCCGCGTCTTGGTTTTGCAG-3' and reverse 5'-TACCGTCCTTTTGCCAGTT-CCTCCA-3'; IL-10, forward 5'-AGAAGCATGGC-CCAGAAATCA-3' and reverse 5'-GGCCTTGTA-GACACCTTGGT-3'; TGF-β, forward 5'-CAGCA-ACAATTCCTGGCGATAC-3' and reverse 5'-GC-TAAGGCGAAAGCCCTCAAT-3'.

ELISA

The protein levels of TNF-α, IFN-γ, IL-10 and TGF-β in colon tissue and serum specimens were evaluated with specific ELISA kits (eBioscience, http://www.ebioscience.com/) as instructed by the manufacturer.

Flow-cytometry

To quantitate $CD3^+CD4^+$ T, $CD3^+CD8^+$ T and $CD4^+CD25^+Foxp3^+$ T lymphocytes, as well as $CD11c^+MHC\text{-}II^+$ DCs, macrophages ($F4/80^+$) and the sub-population of macrophages positive for $CD206^+$ in spleen specimens, the cell concentration of suspensions was adjusted to 1×10^7 cells/mL before incubation with fluorescent antibodies targeting CD3, CD4, CD8 (BioLegend, http://www.biolegend.com/), CD25, Foxp3, CD11c, MHC-II, F4/80 and CD206 (eBioscience, http://www.ebioscience.com/), respectively. Flowjo was used for data analysis.

Co-cultures of ERCs with splenocytes

In order to assess PD-L1's role in ERCs immunoregulation in vitro, splenocytes were co-cultured with ERCs blocked with anti-PD-L1 mAb or not, via transwell plates. ERCs (10^4 cells/well) were pre-incubated with anti-PD-L1 antibody or isotype-control antibody (20 μg/mL) for 1 h, and co-cultured with splenocytes obtained from BALB/c mice. In non-contact assays, 3 μm pore-Transwell was employed for ERCs separation from splenocytes. To assess whether Tregs differentiation is modulated by ERCs, splenocytes (2×10^5/well) were co-cultured in different groups, and administered a mixture of anti-CD3 antibody (100 ng/mL) and anti-CD28 antibody (200 ng/mL) for 72 h [15]. For $CD4^+$ T and $CD8^+$ T cell quantitation, splenocytes were co-cultured with ERCs by using the same grouping as

above, and administered lipopolysaccharide (LPS) (20 μg/mL) [15]. DCs were stimulated with ConA (10 μg/mL) for assessment [22], while macrophages were stimulated with IL-4 (20 ng/mL) [23]. Analysis was performed by flow-cytometry (Becton Dickinson FACS Canto II, United States).

ERCs labeling and in vivo tracking

To track ERCs in vivo after treatment, cells labeling with PKH26 Fluorescent Cell Linker Kit (Sigma-aldrich, St Louis, USA) were performed as directed by the manufacturer. Then, PKH26-labeled ERCs (6×10^6 cells/0.2 mL) were injected via tail vein into normal and colitis mice on day 5, respectively. After sacrifice 48 h following treatment, mouse liver, lung, kidney, spleen and colon specimens were obtained and snap-frozen in liquid nitrogen. Cryosections (4 μm) were assessed by fluorescence microscopy for ERCs detection.

Statistical analysis

SPSS 20.0 software (SPSS Inc., USA) was used for all analyses. Data are mean ± SD, and were assessed by one-way analysis of variance (ANOVA) followed by post hoc least significant difference (LSD) test. $P<0.05$ indicated statistical significance.

Results

Phenotypic identification of ERCs and PD-L1 expression on ERCs

ERCs are plastic adherent cells. Flow cytometric analysis revealed the expression of CD105 and CD90 while lacking of CD34 and CD45 (**Figure 1A**). These results were consistent with previous reports on ERCs [7]. For assessing PD-L1 levels and its response to IFN-γ in ERCs, these cells were treated with IFN-γ (PeproTech, Rocky Hill, USA) or medium control. Basal PD-L1 levels were detectable on ERCs and were increased with the IFN-γ concentration (0.5-5 ng/mL) in a dose-dependent manner (**Figure 1B**).

PD-L1 is required for ERCs-mediated attenuation of colitis

It was demonstrated that ERCs as a novel cell therapy significantly alleviate DSS-induced colitis in our previous report [8]. To investigate the PD-L1's role in ERC-based therapy, ERC-expressed PD-L1 was neutralized with anti-PD-L1 mAb. As shown in **Figure 2**, untreated mice with colitis progressively showed the following symptoms: body weight loss, lethargy, and loose and even bloody stool (**Figure 2A**), with the highest DAI (**Figure 2C**) and histopathological scores (**Figure 2D**). This was accompanied by severe inflammatory reactions featured by epithelial and crypt structural damage, disarranged glandular, less goblet cell renewal and pronounced inflammatory cell infiltration into the mucosal layers (**Figure 2B**). Compared with the colitis model group, ERCs therapy strikingly relieved the above clinical signs (**Figure 2A**), including the DAI ($P<0.05$ vs. model group; **Figure 2C**) and histopathological scores ($P<0.05$ vs. model group; **Figure 2D**). Overt bowel dilation was absent. Colon lengths in the ERC-treated group were starkly greater in comparison with those of the model group (**Figure 2A**). In addition, mucosal hyperemia and edema were attenuated, with blunted ulceration. Histologically, the colon showed improved epithelial and crypt structures, with remarkably reduced infiltration of inflammatory cells and larger goblet cell amounts (**Figure 2B**). However, functional blockage of ERCs by pretreatment with anti-PD-L1 mAb markedly reduced the therapeutic effects of ERCs in clinical signs (**Figure 2A**). Our results also showed that DAI and histopathological score alterations in mice administered anti-PD-L1 mAb-pretreated ERCs (*ERCs) were more pronounced than in the ERC-treatment group (ERCs) (both DAI and histopathological scores, $P<0.05$ for ERCs vs. *ERCs; **Figure 2C** and **2D**). These findings indicated that functional PD-L1 blockage via anti-PD-L1 mAb markedly reduced the efficacy of ERCs-based treatment of colitis.

ERCs reduce the amounts of neutrophil, $CD3^+$ T cell and enhance M2 macrophage levels in the colon via PD-L1

To assess whether ERCs treatment via PD-L1 could influence inflammatory cell infiltration in the mouse colitis model, intra-colon neutrophils (Ly6g-positive), intra-colon $CD3^+$ T cells and $CD206^+$ (a biomarker for M2 macrophages) cells infiltrated were evaluated among different groups. ERCs administration markedly decreased the amounts of neutrophils and $CD3^+$ T cells, while enhancing $CD206^+$ cells in

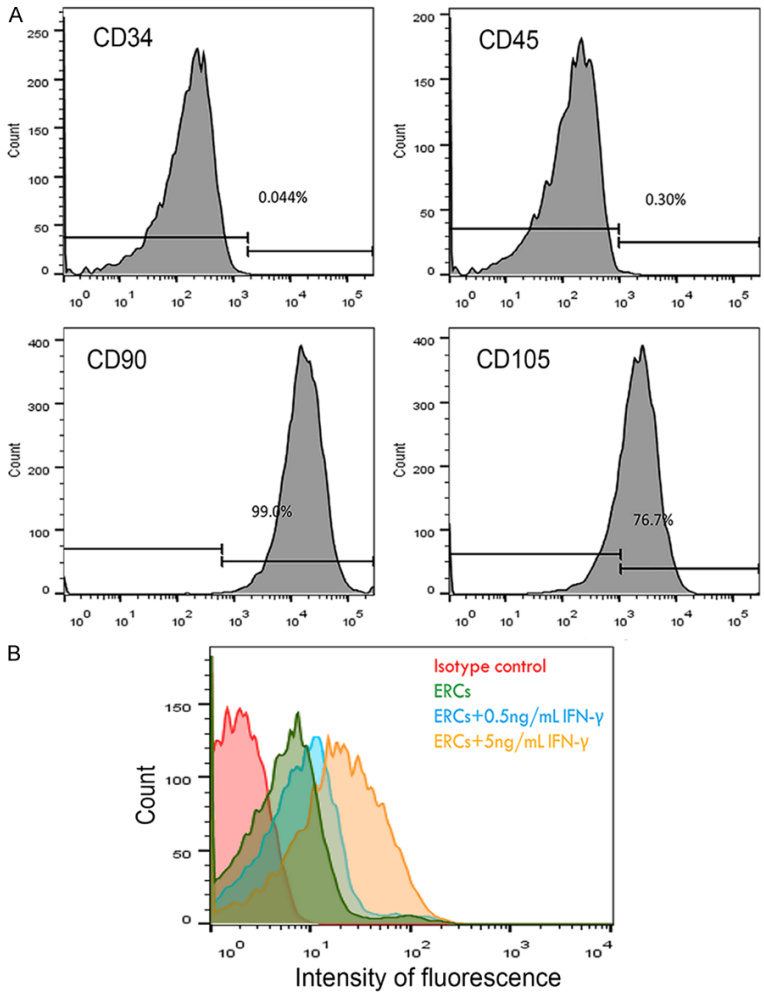

Figure 1. A. Representative flow cytometric analysis of surface expression of phenotypic markers in ERCs. ERCs express CD105, CD90, while lacking of CD34, CD45. B. PD-L1 expression of ERCs was analyzed by using the FACS. Basal PD-L1 levels were found on ERCs and dose-dependently increased with the IFN-γ concentration (0.5-5 ng/mL). The result was representative in three separate experiments.

colon samples in comparison with control colitis mice (**Figure 2B**). However, after pretreatment of ERCs with anti-PD-L1 mAb, neutrophil and $CD3^+$ T cell amounts were significantly increased, and those of $CD206^+$ cells significantly decreased (**Figure 2B**). These results suggested that ERCs significantly suppressed colonic inflammation via PD-L1.

PD-L1 is required for ERCs-induced changes of pro-inflammatory and anti-inflammatory cytokines in mouse colitis

In a previous report, we demonstrated that ERCs concomitantly downregulate pro-inflammatory cytokines and upregulate anti-inflammatory cytokines [8]. In this study, pro-inflammatory cytokine (TNF-α, IFN-γ) amounts in serum (protein) as well as the colon (mRNA and protein) were overtly elevated in control mice with colitis (**Figure 3**); therefore, administration of ERCs resulted in markedly decreased pro-inflammatory cytokine levels (both TNF-α and IFN-γ, $P<0.05$, Untreated vs. ERCs; **Figure 3A-C**). Compared with the amounts in ERCs-treated animals, the levels of intra-colonic pro-inflammatory cytokines were enhanced in ERCs pre-conditioned with anti-PD-L1 mAb (both TNF-α and IFN-γ, $P<0.05$, ERCs vs. *ERCs; **Figure 3A-C**). Meanwhile, administration of ERCs resulted in enhanced levels of anti-inflammatory cytokines (IL-10, TGF-β) in serum and colon samples (both IL-10 and TGF-β, $P<0.05$, Untreated vs. ERCs; **Figure 3A-C**). However, functional blockade of ERCs-expressing PD-L1 with anti-PD-L1 mAb resulted in reduced levels of anti-inflammatory cytokines compared with the ERCs-treatment group (both IL-10 and TGF-β, $P<0.05$, ERCs vs. *ERCs; **Figure 3A-C**). These findings indicated PD-L1 in ERCs-based therapy has a critical function in regulating cytokine profiles.

ERCs via PD-L1 reduce the amounts of $CD4^+$ T and $CD8^+$ T cell in cell culture and animal model

To determine PD-L1's effects on T cell populations in ERCs-treated mice with colitis, we used flow-cytometric analysis to assess $CD4^+$ T and $CD8^+$ T cell changes in the spleen in vivo or in co-cultures of ERCs with splenocytes in vitro. As depicted in **Figure 4**, the population of splenic $CD4^+$ T and $CD8^+$ T cells showed markedly reduced in ERCs-treated animals compared with untreated mice with colitis (both

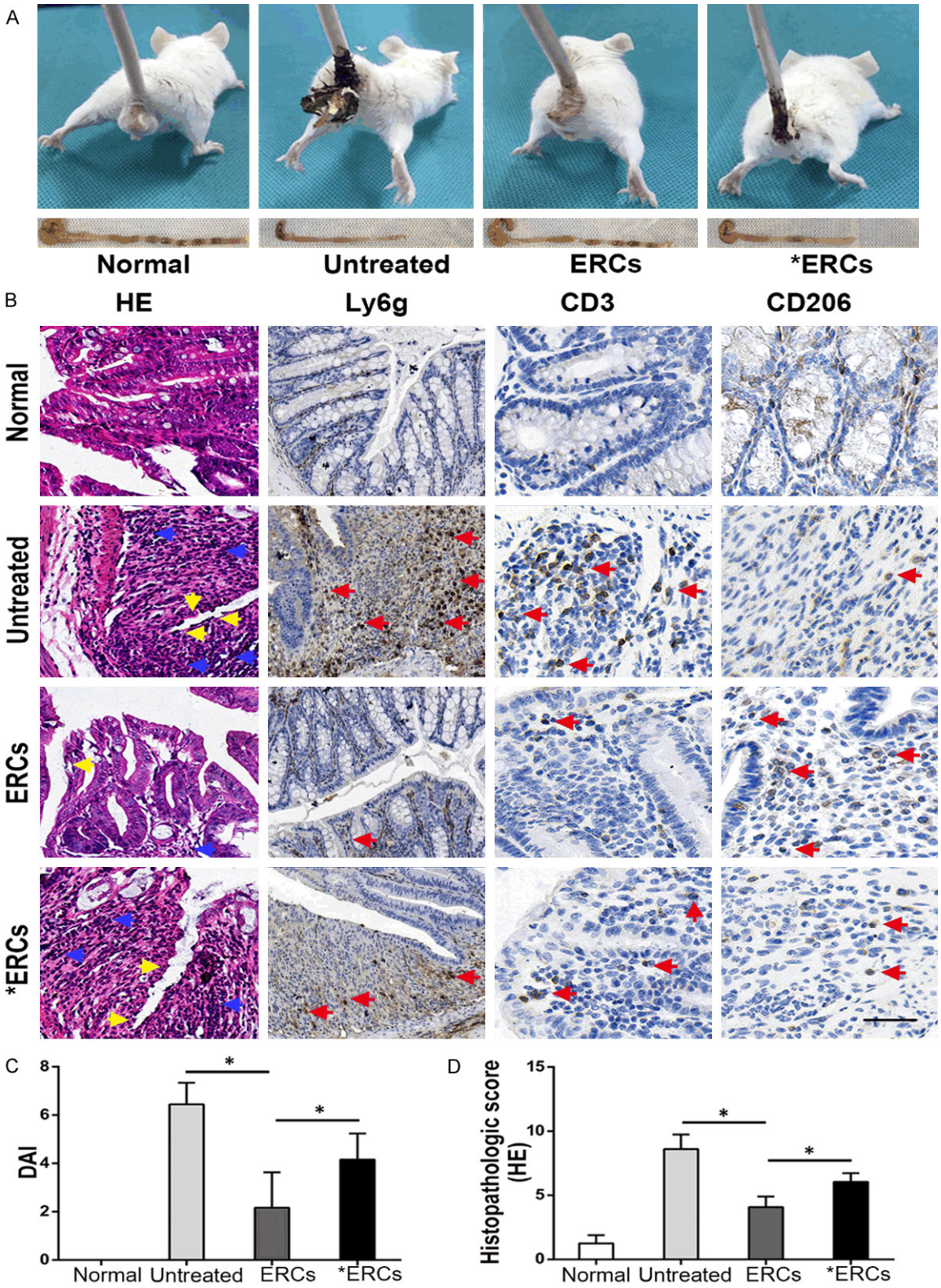

Figure 2. A. In vivo, the representative photos of mice with general performance were shown from different groups. B. Microscopic observation of the colon from different groups. In the untreated group, it was characterized by damaged epithelium (yellow arrow) and massive inflammatory cells infiltration (blue arrow) into the mucosa and submucosa. In ERCs-treated group, the colon showed improved epithelial and crypt structures (yellow arrow) and remarkably reduced infiltration of inflammatory cells (blue arrow). ERCs suppress intra-colon Ly6g$^+$ cells and CD3$^+$ T cells

(pro-inflammatory) infiltration (red arrow) and promote CD206^{+} cells (anti-inflammatory) infiltration (red arrow) in the colitis mice. However, the blockage of ERCs with anti-PD-L1 mAb significantly impaired the therapeutic efficacy. C. The comparison of DAI was analyzed in different groups on day 15 (n=8 per group). Bar=100 μm. D. The comparison of the histopathological scores was performed in different groups on day 15 (n=8 per group). ERCs indicated ERCs treatment. *ERCs indicated anti-PD-L1 antibody pretreated ERCs treatment. *P<0.05.

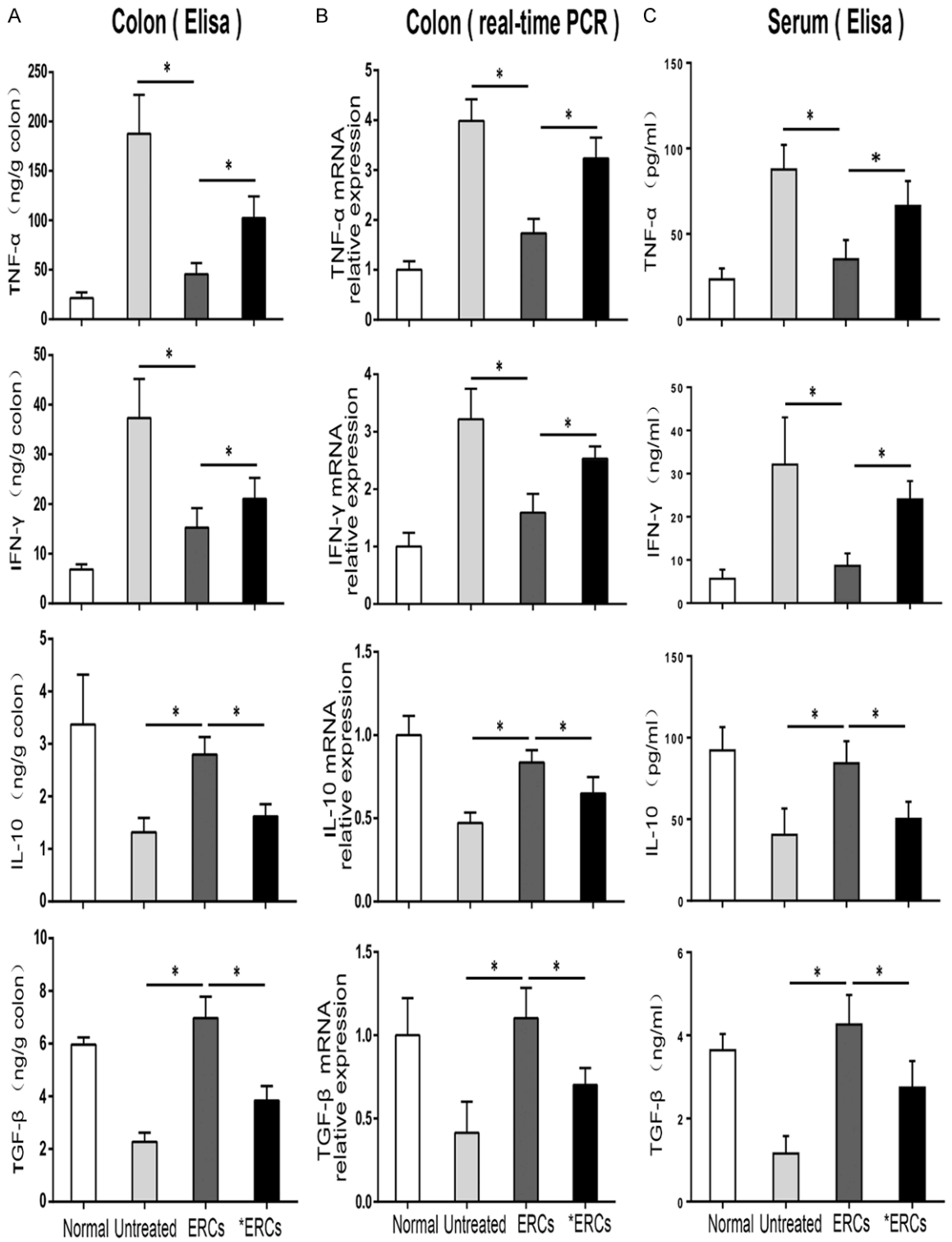

Figure 3. Comparison of the colonic cytokine protein levels (A), colonic mRNA transcriptional levels (B) and serum cytokine protein levels (C) were performed in different groups on day 15 (n=8 per group). ERCs indicated ERCs treatment. *ERCs indicated anti-PD-L1 pretreated ERCs treatment. *P<0.05.

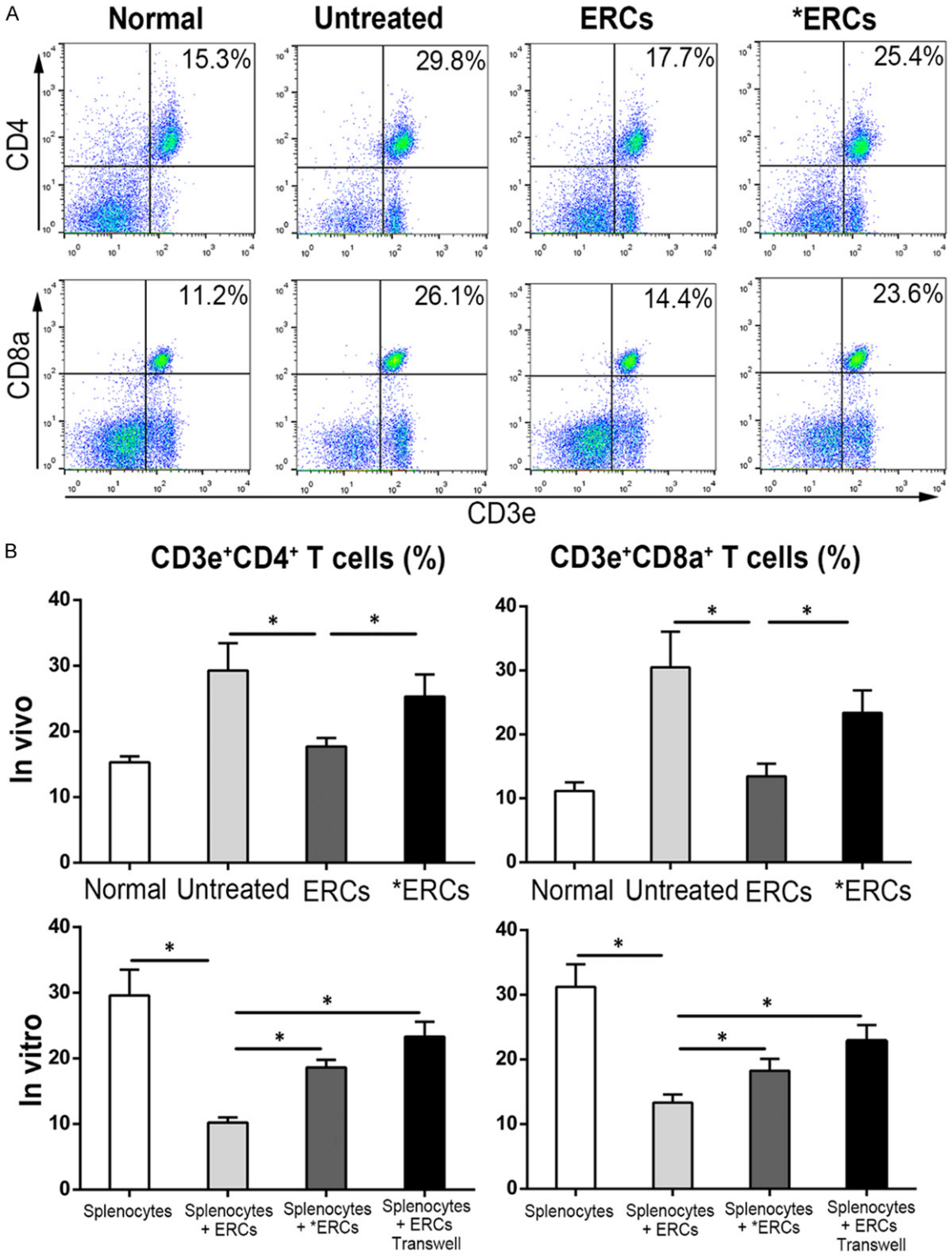

Figure 4. ERCs can suppress CD4+ T and CD8+ T cell populations through PD-L1. The proportion of CD4+ T and CD8+ T cells was detected by using FACS in different groups. A. Dot plots of CD4+ T and CD8+ T cells in vivo. B. Percentage of CD4+ T and CD8+ T cells in vivo (n=8 per group) and in vitro (n=6 per group). ERCs indicated ERCs-treated group. *ERCs indicated anti-PD-L1 antibody pretreated ERCs treatment. *P<0.05.

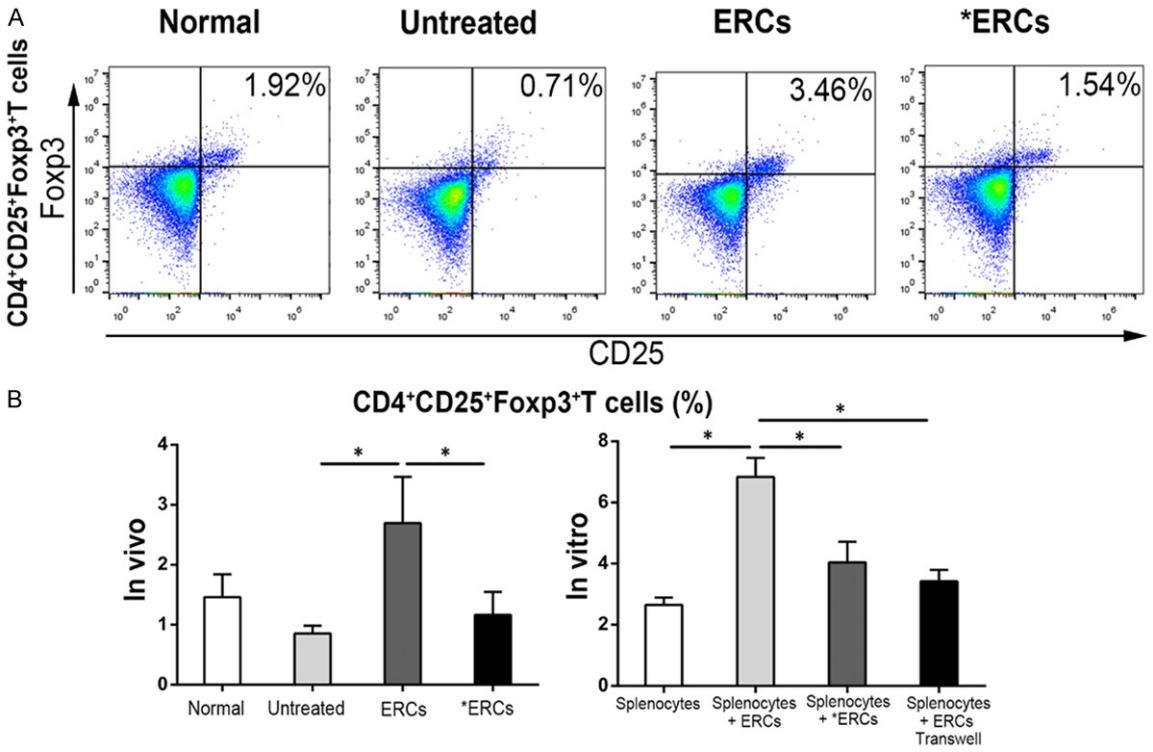

Figure 5. ERCs enhance the percentage of $CD4^+CD25^+Foxp3^+$ Tregs via PD-L1 and induce the protection against colitis. Flow cytometric analysis of $CD4^+CD25^+Foxp3^+$ Tregs was performed in different groups. A. Dot plot of $CD4^+CD25^+Foxp3^+$ Tregs in vivo. B. Percentage of $CD4^+CD25^+Foxp3^+$ Tregs in vivo (n=8 per group) and in vitro (n=6 per group). ERCs indicated ERCs-treatment. *ERCs indicated anti-PD-L1 pretreated ERCs treatment. *P<0.05.

$CD4^+$ T and $CD8^+$ T, P<0.05, Untreated vs. ERCs; **Figure 4B**). However, the numbers of splenic $CD4^+$ T and $CD8^+$ T cells were significantly increased by pretreatment of ERCs with anti-PD-L1 mAb (both $CD4^+$ T and $CD8^+$ T, P<0.05, ERCs vs. *ERCs; **Figure 4B**). In vitro, addition of ERCs resulted in lower $CD4^+$ T and $CD8^+$ T cell numbers in co-cultures stimulated with LPS (both $CD4^+$ T and $CD8^+$ T, P<0.05, Splenocytes Vs. Splenocytes + ERCs; **Figure 4B**). These effects, however, were specifically blunted by neutralization with anti-PD-L1 mAb (both $CD4^+$ T and $CD8^+$ T, P<0.05, Splenocytes + ERCs vs. Splenocytes + *ERCs; **Figure 4B**) or separation of ERCs from splenocytes in a transwell plate (both $CD4^+$ T and $CD8^+$ T, P<0.05, Splenocytes + ERCs vs. Splenocytes + ERCs transwell; **Figure 4B**).

ERCs via PD-L1 increase Treg amounts

Tregs significantly contribute to immune suppression [24]. As depicted in **Figure 5**, ERCs-treated colitis mice had starkly elevated numbers of splenic $CD4^+CD25^+Foxp3^+$ cells (Tregs) (P<0.05, Untreated vs. ERCs; **Figure 5B**). In contrast, Tregs were significantly decreased after pre-treatment with anti-PD-L1 of ERCs (*ERCs) compared with the ERCs-treatment group (P<0.05, ERCs vs. *ERCs; **Figure 5B**). Similarly, Treg numbers were higher in co-culture in presence of ERCs (P<0.05, Splenocytes Vs. Splenocytes + ERCs; **Figure 5B**). Meanwhile, when the co-cultured ERCs were pre-treated with anti-PD-L1 antibody, $CD4^+CD25^+Foxp3^+$ Tregs were reduced (P<0.05, Splenocytes + ERCs vs. Splenocytes + *ERCs; **Figure 5B**). In addition, Treg levels were also decreased by employing a transwell plate (P<0.05, Splenocytes + ERCs vs. Splenocytes + ERCs transwell; **Figure 5B**).

ERCs via PD-L1 decrease the percentage of $CD11c^+MHC\text{-}II^+$ DCs in vivo and in vitro

DCs are critical for regulating immune responses in the antigen-rich gastrointestinal environment [25]. As shown in **Figure 6**, the $CD11c^+MHC\text{-}II^+$ DCs population in the spleen was markedly decreased after administration of ERCs in comparison with control animals with colitis (P<0.05, Untreated vs. ERCs; **Figure**

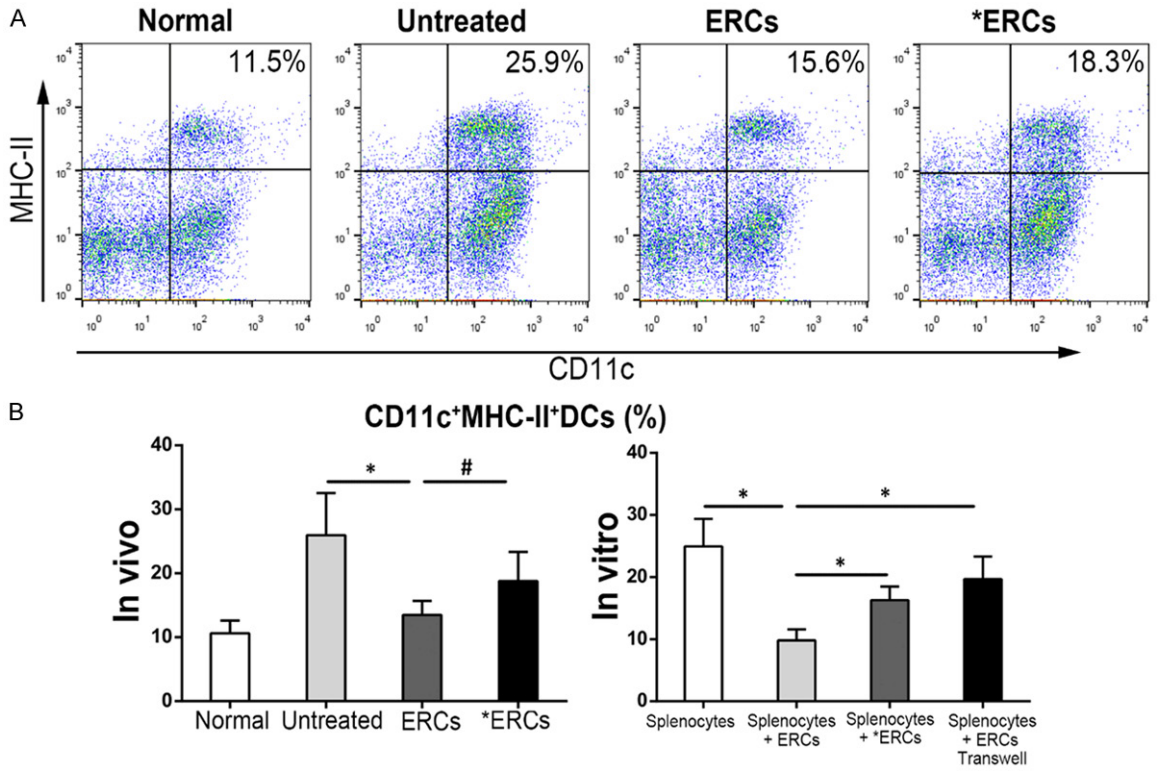

Figure 6. ERCs regulate the $CD11c^+MHC\text{-}II^+$ DCs percentage via PD-L1. The analysis of $CD11c^+MHC\text{-}II^+$ DCs was performed by using FACS in different groups. A. Dot plot of $CD11c^+MHC\text{-}II^+$ DCs in vivo. B. Percentage of $CD11c^+MHC\text{-}II^+$ DCs in vivo (n=8 per group) and in vitro (n=6 per group). ERCs indicated ERCs-treatment. *ERCs indicated anti-PD-L1 mAb-pretreated ERCs-treatment. $^*P<0.05$. $^\#P>0.05$.

6B). Meanwhile, the number of these cells in the spleen induced by anti-PD-L1 mAb pretreated ERCs was increased, but not significantly, in comparison with the ERCs-treatment group ($P>0.05$, ERCs vs. *ERCs; **Figure 6B**). Addition of ERCs in vitro resulted in decreased numbers of $CD11c^+MHC\text{-}II^+$ DCs in co-cultures ($P<0.05$, Splenocytes + ERCs vs. Splenocytes; **Figure 6B**); these cells were more abundant when anti-PD-L1 antibody pretreated ERCs were co-cultured with splenocytes ($P<0.05$, Splenocytes + ERCs vs. Splenocytes + *ERCs; **Figure 6B**). Meanwhile, the number of $CD11c^+MHC\text{-}II^+$ DCs was increased in ERCs and splenocytes co-culture performed in a transwell plate ($P<0.05$, Splenocytes + ERCs vs. Splenocytes + ERCs transwell; **Figure 6B**). Collectively, these data suggested that ERCs treatment decreases DCs maturation via PD-L1.

ERCs via PD-L1 reduce total macrophage amounts, but relatively increase the number of M2 macrophages in vivo and in vitro

Macrophages have critical functions in body protection from the luminal content at an extremely large interface [26]. To assess whether ERCs-associated colitis alleviation is related to macrophage profile, and the potential effect of PD-L1 on macrophage amounts, total macrophages positive for F4/80 and the M2 macrophage subtype with double F4/80 and CD206 staining were evaluated in vitro and in vivo by the flow-cytometric analysis (**Figure 7**). Interestingly, ERCs efficiently reduced total macrophage numbers compared with control values ($P<0.05$, Untreated vs. ERCs; **Figure 7B**). However, M2 macrophages were relatively more abundant in the ERCs-treatment group than control mice with colitis ($P<0.05$, Untreated vs. ERCs; **Figure 7B**). Compared with the ERCs-treatment group, the percentage of splenic total macrophages in mice administered ERCs-pretreated with anti-PD-L1 mAb was enhanced but showed no significant difference ($P>0.05$, ERCs vs. *ERCs; **Figure 7B**); meanwhile, the M2 sub-population was relatively reduced, with a significant difference ($P<0.05$, ERCs vs. *ERCs; **Figure 7B**). Similarly, ERCs reduced the number of total macrophages after co-culture of splenocytes with ERCs ($P<0.05$, Splenocytes vs. Splenocy-

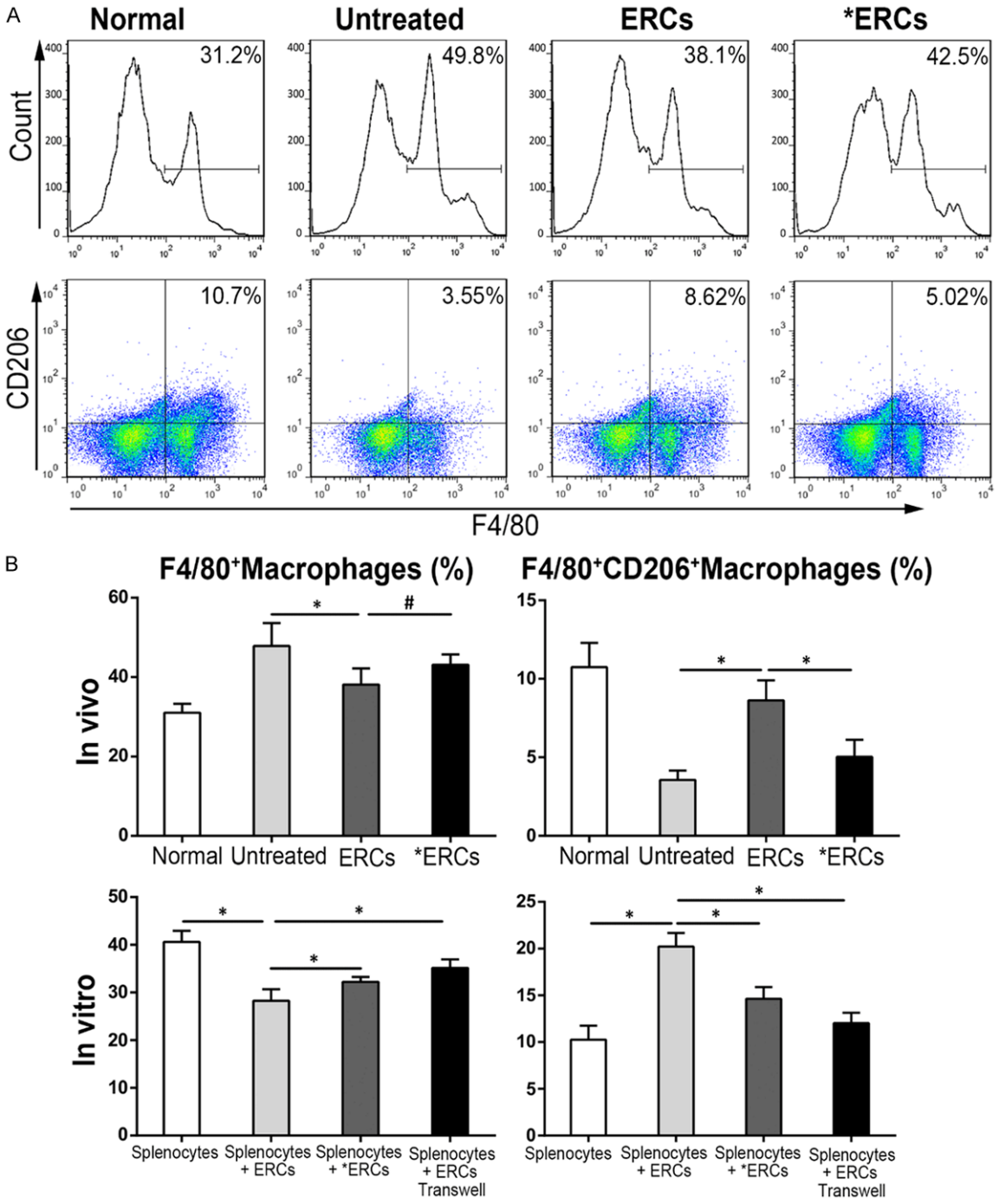

Figure 7. ERCs regulate the number and type of macrophage via PD-L1. Total macrophages positive for F4/80 and the M2 macrophage subtype with double F4/80 and CD206 staining were evaluated in vitro and in vivo flow-cytometrically. A. Flow cytometry of total macrophages positive for F4/80 and M2 macrophage subtype with double F4/80 and CD206 in vivo. B. Percentage of total macrophages and M2 macrophages in vivo (n=8 per group) and in vitro (n=6 per group). ERCs indicated ERC-treatment. *ERCs indicated anti-PD-L1 mAb-pretreated ERCs-treatment. *P<0.05. #P>0.05.

tes + ERCs; **Figure 7B**), but relatively increased the M2 macrophage population (P<0.05, Splenocytes vs. Splenocytes + ERCs; **Figure 7B**). However, the percentage of total macrophages in the co-culture was correspondingly enhanced by using anti-PD-L1 antibody-pre-

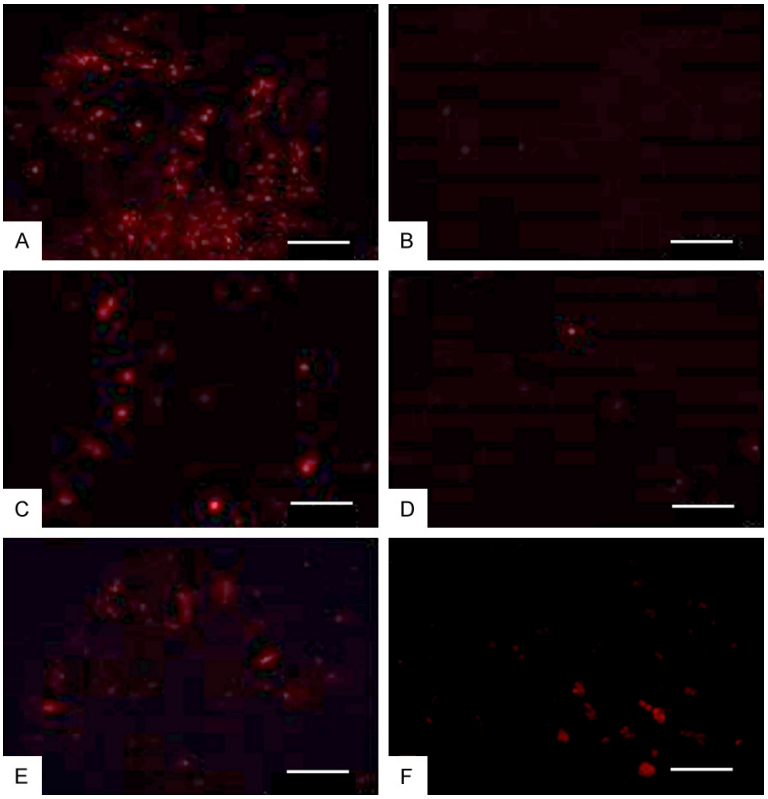

Figure 8. The figure shows the distribution of ERCs in experimental animals under fluorescence microscopy. ERCs were injected into both of the normal control mice and colitis mice via the tail vein, and the internal organs were sacrificed after 48 hours. The fluorescence intensity of the colitis mice was showed in the following order: lung (A), liver (C), spleen (E), colon (F). No fluorescence was observed in the kidney (B). Fluorescence intensity of colon in the colitis group (F) was stonger than the normal group (D). Bar=100 μm.

treated ERCs (P<0.05, Splenocytes + ERCs vs. Splenocytes + *ERCs; **Figure 7B**) or separating ERCs from splenocytes in transwell plates (P<0.05, Splenocytes + ERCs vs. Splenocytes + ERCs transwell; **Figure 7B**). The M2 macrophage sub-population in co-culture was reduced after neutralization with anti-PD-L1 mAb (P<0.05, Splenocytes + ERCs vs. Splenocytes + *ERCs, **Figure 7B**) or separating ERCs from splenocytes in transwell plates (P<0.05, Splenocytes + ERCs vs. Splenocytes + ERCs transwell; **Figure 7B**).

In vivo ERCs tracking after engraftment

To assess whether PHK26-ERCs could be engrafted in the colon tissue of colitis mice, ERCs were administered by tail-vein injection into normal control and colitis mice, which were sacrificed after 48 hours. Interestingly, PKH26-positive ERCs were found by fluorescence microscopy in colon (injured tissue) and spleen (lymphoid organ) samples from ERCs-treated mice (**Figure 8**). Moreover, fluorescence intensity of labeled ERCs after 48 hours had the following order: lung, liver, spleen and colon. There was no fluorescence observed in the kidneys. Fluorescence intensities in colon samples from mice with DSS-induced colitis were greater than those of normal control animals.

Discussion

MSC-based therapy constitutes an efficient method in treating IBD [4]. ERCs, as an attractive stem cell type obtained from menstrual blood, show several advantages over MSCs of other origins. ERCs are harvested from female donors in a non-invasive manner, representing an ethically compliant substitute of autologous stem cells. Meanwhile, ERCs can be rapidly and massively expanded while maintaining a normal karyotype until the 68th doubling [7]. In our previous studies, ERCs were shown to regulate the immune system and induce immune tolerance [27-30]. In the present study, intravenously injected ERCs ameliorate clinical signs and histological alterations in the colon of DSS-colitis mice. Intact epithelium and crypts in the colon of normal mice were observed by microscopy, which showed intestinal layers orderly arranged, large amounts of goblet cells, and limited inflammatory cells. Untreated mice displayed significant inflammatory alterations, including epithelial and crypt damage, glandular disarrangement, reduced goblet cell amounts, and pronounced inflammatory cells (including neutrophils, T lymphocytes and macrophages) infiltration into the mucosal layers. In mice administered ERCs, the colon showed markedly improved symptoms. However, the molecular mechanism of ERCs-mediated immunosuppression remains unclear.

MSCs modulate the functions of certain immune cells by means of soluble factor secretion or direct cell-to-cell interactions [12]. MSCs derived from the bone marrow (BM) or umbilical cord (UC) constitutively express PD-L1, which plays a significant role in MSC-associated immune suppression [14, 31]. Our previous research has shown that PD-L1 expression is a requirement in BM-MSCs for immune tolerance to heart transplants together with rapamycin administration [15]. Furthermore, the negative effects of MSCs-expressing PD-L1 neutralization on graft survival are related to decreased amounts of regulatory immune cells and increased levels of alloantibodies (IgG and IgM), both in the graft and blood stream. In cell cultures, blockade of MSC-associated antibody synthesis and B cell proliferation is associated with PD-L1 as well as interactions of $CD19^+$ B cells with MSCs [15]. Therefore, PD-L1 plays an important role in MSC-mediated immunosuppression.

Our research showed that PD-L1 was also expressed on the cell surface of ERCs. PD-L1 levels in ERCs increased with the stimulating factor concentration. Meanwhile, PD-1 as a PD-L1 receptor is found at high levels in activated T lymphocytes, B lymphocytes, DCs and macrophages [32-36]. PD-1 and PD-L1 interactions modulate immune cells and induce immune tolerance. Indeed, ligand binding to PD-1 results in suppressed T cell receptor (TCR) signaling via reduction of CD3z chain phosphorylation and Zap-70 binding [37]. PD-1/PD-L1 signaling can downregulate both Ras and Bcl-xL, which regulate cell growth and survival, respectively [38]. In addition, PD-1 suppresses phosphatidylinositol 3-kinase (PI3K)/Akt signaling by reducing PI3K activation [39]. This further downregulates mechanistic target of rapamycin (mTOR) and increases the half-life of FoxO1 [40]. In this setting, inflammatory cells show reduced proliferation and effector function, resulting in a dysfunctional phenotype.

PD-1/PD-L1 signaling is important in inducing immune suppression and establishing a therapeutic strategy for autoimmune diseases [41-43]. For example, mice not expressing PD-1 develop systemic autoimmune diseases spontaneously [44]. In addition, PD-1-associated inhibitory signals are important in alleviating colon inflammation, and PD-L1-Fc constitutes a very potential therapeutic tool for treating IBD [16]. Thus, we evaluated the role of PD-L1 in ERCs-mediated treatment of colitis. ERCs could reduce $Ly6g^+$ cells and $CD3^+$ T cells infiltration and enhance $CD206^+$ cells infiltration in the colon. ERCs reduced levels of pro-inflammatory cytokines and enhanced levels of anti-inflammatory cytokines in the colon and serum. However, blockade of ERCs with anti-PD-L1 mAb disrupted their immunosuppressive properties. In addition, the distribution of ERCs in colitis mice remains unknown; therefore, xenogeneic PHK26 labeled ERCs were grafted via tail vein, and found to migrate to the lung, liver, spleen, colon tissue in the mouse colitis model. The colon tissue in the colitis group showed significantly higher fluorescence intensity compared with that of the normal group. Most ERCs were distributed in the lung since they first pass through the lung after intravenous injection. Since the distribution of early transplanted cells is associated with the degree of capillary richness in different organs, elevated fluorescence was found in the liver. Meanwhile, large amounts of fluorescently labeled cells were detected in the spleen; therefore, splenic immune cell populations were assessed to examine the association of ERCs with systemic immune reactions. ERCs circulation in the body and the chemotactic effects of inflammatory factors would cause ERCs to gradually migrate to the diseased colon tissue. Therefore, fluorescence intensity in colon samples from the colitis group was much stronger than that of the normal group.

$CD4^+$ T and $CD8^+$ T lymphocytes as well as Tregs induced by ERCs-based therapy were markedly alleviated by pre-treatment of ERCs with mAb targeting PD-L1 in vitro and in vivo. ERCs downregulated pro-inflammatory cytokines while upregulating anti-inflammatory cytokines. Moreover, compared with the ERCs-treatment group, colonic pro-inflammatory cytokine amounts were enhanced while anti-inflammatory immune mediators were reduced when ERCs were pre-conditioned with mAb against PD-L1. Most studies suggest that PD-L1 exerts negative regulation of T cells and inhibits inflammation via PD-1 [45-47], and our study is consistent with these studies. Tregs comprise 5 to 10% of all $CD4^+$ T cells in healthy human and rodents, as important players in

peripheral tolerance which suppress effector T (Teff) cells and reduce tissue damage associated with immune reactions [48]. Binding of PD-1 to PD-L1 enhances the suppressive ability of Tregs as well as their interactions with Teffs [43]. In presence of TGF-β, PD-L1-Ig induces an important production of iTregs from naive $CD4^+$ T cells. Meanwhile, PD-L1 blockade decreases Treg amounts and reduces $CD4^+$ $Foxp3^-$ cell differentiation into IL-17 positive T cells [49]. Furthermore, PD-L1-Ig administration upregulates Foxp3 and increases suppressive function in iTregs [50].

DCs are critical for immune regulation in the gut. DCs activation is a prerequisite for DSS-associated colitis [51]. In agreement, DCs accumulate at sites of inflammation in patients with IBD [25]. As shown above, addition of ERCs markedly reduced the number of $CD11c^+MHC\text{-}II^+$ DCs in co-culture, which, however, was reversed by neutralization with anti-PD-L1 mAb in vitro. Splenic $CD11c^+MHC\text{-}II^+$ DCs were enhanced by pretreatment of ERCs with anti-PD-L1 mAb although no statistical significance was found in vivo; this result may be related to sample size, indicating the need to further enlarge the animal number. Previous evidence reveals PD-1 on DCs negatively modulates their functions since mice not expressing PD-1 show a greater ability in innate protection of the animals from Listeria infection and elevated IL-12 and TNF-γ synthesis [36]. Moreover, Song et al. demonstrated that in DSS colitis, PD-L1-Fc activates PD-1/PD-L1 signaling pathway primarily in DCs, causing DC dysfunction and therefore reducing the severity of colon inflammation [16].

Macrophages play critical roles in tissue homeostasis and remodeling [52], with various functions and phenotypes in distinct metabolic and immune microenvironments [53]. Two macrophage subsets are broadly acknowledged, including the classically (M1) and alternatively (M2) activated subgroups [54]. Macrophages have anti-inflammatory phenotype and function in the normal intestinal lamina propria [55]. However, in individuals with active IBD, macrophages are more abundant in the intestine, expressing pro-inflammatory phenotypic and functional characteristics at the sites of mucosal inflammation [56]. As shown above, administration of ERCs resulted in increased amounts of M2 cells, which likely have a critical function in the alleviation of DSS-induced colitis. This corroborates previous findings that inflammation suppression in UC might be associated with induced M2 polarization [57]. Besides, a previous study also showed that MSCs regulate the M1/M2 balance and may contribute to immune-tolerance [58]. However, the molecular mechanism of ERC-mediated M2 polarization remains unclear. It has been demonstrated that PD-L1 plays a significant role in MSC-associated immune suppression. Binding of PD-L1 and PD-1 acts in a negative feedback mechanism to inhibit immune reactions. Since PD-1 is found on the macrophage surface, alleviation of DSS-associated colitis by PD-L1-Fc administration could be modulated by interaction with macrophages, controlling downstream macrophage effects [16]. Therefore, we also evaluated the regulating effects of ERC-expressing PD-L1 on macrophages. The results showed M2 cells were relatively reduced after pretreatment of ERCs with anti-PD-L1 mAb. Indeed, PD-L1 interaction with PD-1 leads M2 polarization and thus repair of the intestinal mucosa.

Conclusion

ERCs can provide an immuno-privileged environment for the regulation of immune responses. The present study showed that PD-L1 plays a critical role in mediating ERCs therapy of colitis. However, the in-depth molecular mechanism of ERCs in immune regulation is complex and deserves further investigation.

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Disclosure of conflict of interest

None.

Abbreviations

CD, Crohn's disease; DAI, Disease activity index; DCs, Dendritic cells; DSS, Dextran sulfate sodium; ERCs, Endometrial regenerative cells; HLA, Human leukocyte antigen; IBD, Inflammatory bowel disease; IL, Interleukin; LP, Lamina propria; MHC, Major histocompatibility complex; MSCs, Mesenchymal stromal cells; M, Macrophages; PBS, Phosphate buffered saline; SPF, Specific pathogen free; TGF-β, Transforming growth factor-β; Th, Helper T cell; TNF, Tumor necrosis factor; Tregs, Regulatory T cells; UC, Ulcerative colitis.

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