BMSCs improve TNBS-induced colitis in rats through inducing Tregs differentiation by expressing PD-L1

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Abstract

Objective: Bone marrow-derived mesenchymal stem cells (BMSCs) are a kind of stem cells with high differentiation potential and immunomodulatory ability, which has a broad prospect in the treatment of inflammatory bowel disease. The aim of this study is to investigate whether BMSCs could improve TNBS-induced colitis in Sprague-Dawley (SD) rats through inducing Tregs differentiation by expressing PD-L1.

Methods: BMSCs were isolated and identified by flow cytometry before being transfected with PD-L1 siRNA recombinant plasmids. Then SD rats were randomly divided into 4 groups. Colitis induced by TNBS (sigma Aldrich) except normal group. On the fourth day of modeling, the rats in BMSCs control group and PD-L1 siRNA BMSCs group were injected with corresponding BMSCs through tail vein for 1 week, the dose was 5×10^6 cells. The normal group and model group were given the same volume of PBS.

Results: PD-L1 siRNA BMSCs and BMSCs could reach colon tissue in TNBS-induced colitis. BMSCs control group significantly improved the clinical symptoms and histopathological severity of TNBS induced colitis, but PD-L1 siRNA BMSCs group did not. We found that the percentage of Tregs in spleen and mesenteric lymph nodes decreased, the expression of PD-L1, IL10, PTEN was down-regulated, and the expression of p-Akt and p-mTOR was up-regulated in colon tissue after PD-L1 siRNA intervention.

Conclusion: This study suggested that BMSCs can induce the differentiation of Treg through inhibiting Akt/mTOR pathway by expressing PD-L1, which can significantly improve the symptoms and pathological damage of ulcerative colitis rats and affect the immune function.

1. Introduction

Ulcerative colitis (UC), a disease characterized by chronic inflammation and ulceration in colorectum with typical symptoms of recurrent abdominal pain and bloody mucous diarrhea, is diagnosed by colonoscopy and pathological examination. UC, previously considered as a low-risk lesion, has been gradually increasing in incidence in Asia in recent years, and is regarded as an important risk factor for colorectal cancer[1, 2].Though being studied for several years, the etiology and pathogenesis of UC are still unknown, while most researchers think they are related to environmental factors, genetic susceptibility and immune disorder. Compelling data revealed that the most critical factor contributing to UC is immune disorder, exactly Intestinal mucosal immune disorder[3]. As we all known, the central part of intestinal immune dysfunction is the imbalance of pro-inflammatory cells and anti-inflammatory cells, which is mainly reflected between regulatory T cell (Treg) and T helper (Th17) cell. Treg, an important anti-inflammatory cell, is proved to play pivotal role in improving UC by activation of forkhead box P3 (FOXP3) and secretion of interleukin-10 (IL-10)[4, 5].

Bone marrow-derived mesenchymal stem cells (BMSCs), a kind of non-hematopoietic stem cells existing in bone marrow, are widely used to study of stem cell-based therapy. For their multi-directional differentiation and high proliferation potentials, BMSCs could be induced directly into neural cells,

osteoblasts, fat cells and so on under specific conditions[6, 7]. Although they only account for 0.001% - 0.01% in bone marrow monocytes, they can be expanded more than 1 million times or 6 generations in vitro[8]. More and more researches reported that BMSCs show great immunosuppressive potentials and can be used to treat inflammation-mediated diseases including inflammatory bowel disease (IBD)[9]. Our previous study demonstrated that BMSCs can relieve TNBS-induced colitis and raise the percentage of Tregs in blood of rats, so we think that BMSCs alleviated colitis by promoting Tregs differentiation. However, the regulatory mechanism between BMSCs and Tregs remains to be elucidated[10, 11].

PD-L1(also called CD279 or B7-H1), a ligand of programmed cell death-1 (PD-1), is a member of the CD28/B7 superfamily and widely expressed in various organizations[12, 13]. The PD-1/PD-L1 co-stimulatory signal is mainly involved in the central and peripheral immune tolerance of CD4+ T cells, and can regulate the balance of effector T cells and Tregs in the progression of multistage autoimmune diseases[13, 14]. Some studies show that PD-L1 is the most important factor which is able to induced naive T cells to differentiate into Tregs by inhibiting Akt/mTOR signal pathway[15]. At the same time, BMSCs are reported to inhibit activation and proliferation of CD4+ T cells by PD-1/PD-L1 pathway[16]. So based on those findings above, we hypothesized that BMSCs alleviated colitis of rats model by inducing differentiation of Tregs via PD-L1 expressed on them.

2. Materials And Methods

Isolation, culture and identification of rat BMSCs

BMSCs were isolated from 3-week-old healthy male SD rats as described previously. BMSCs were collected from femurs and tibias by flushing marrow cavities and cultured in culture flask using low-glucose complete cell culture medium consisting of α-minimum essential medium (α-MEM; Gibco, Invitrogen Corp., Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Invitrogen Corp.). The cells were kept in atmosphere of 5% CO_2 at 37°C. Removing no-adherent cells at every time medium changed and collecting adherent cells using 0.25% trypsin solution (Gibco, Invitrogen Corp.) when passaged, and the passage 2 (P2) BMSCs were used for identification and the following experiments. For flow cytometry identification, the anti-rat CD-29, CD-90, CD-45, CD-11b antibody (BioLegend, San Diego, CA, USA) were used as surface markers. And the adipogenic and osteogenic differentiation potentials of BMSCs were researched after identification as described previously.

Plasmid construction and cell transfection

PD-L1 siRNA serial number is NM_001191954. 1 in Gene Bank, and then we designed and synthesized PD-L1 siRNA expression cassettes (PD-L1 -SECs) according to certain principles. After establishing ligation between the purified siRNA expression cassettes and the psiLentGene^{TM} vector, the appropriate amount of ligation products were transformed into efficient sensory cells E.coil DH5α, positive recombinant clones were selected, and the recombinant plasmids were transfected into BMSCs. The BMSCs were inoculated on a 6-well plate one day before transfection, 5×10^4 per well, and the complete

medium was replaced with 1.9mL serum-free medium half an hour before transfection. At transfection time, the confluence of 2 (P2) BMSCs reached 70%-80% per well, incubated at 37℃ for 4-6h, and then the serum-free medium was replaced with complete medium for further culture. After 48 hours, PCR and western blot were used to detect the transfection efficiency, and Rat-PD-L1-175 was finally determined as the best PD-L1 siRNA. Its gene sequence is 5 '-ggaagacaaggaaguuauuca-3' and 5 '-Aauaacuuccuugucuuccuu-3'.

Animals

160-180g weight male SD rats were purchased from the experimental animal center of Huazhong University of Science and Technology (HUST; Wuhan, China), and kept under specific pathogen-free (SPF) conditions with freely eating and drinking. All the procedures and care of animals were strictly according to the guidelines of the Animal Research Institute Committee of Wuhan Service Technology Co., Ltd., China, and approved by the Institutional Animal Care and Use Committee (IACUC) of Wuhan Service Technology Co., Ltd., China..

Induction of UC models and treatment

After an adaptive feeding of one week, forty male rats were randomly assigned to four groups (n=10): normal, model, BMSCs control and PD-L1 siRNA BMSCs. TNBS (Sigma-Aldrich) was used to induce colitis according to Morris *et al.* On the fourth day of modeling，the rats in the BMSCs control group and the PD-L1 siRNA BMSCs group were injected corresponding BMSCs via tail vein at a dose of 5×10^6 cells in 1 ml phosphate-buffered saline (PBS), and the normal group and model group were given the same volume of PBS in the same way. One week after cells injection, all rats were anesthetized and then executed, colons were detached and stored for various studies.

Evaluation of inflammation

From molding, all rats were daily monitored weight loss, stool trait, and degrees of bloody stool to calculate the disease activity index (DAI). After colons were dissected, part of colonic tissue were resected for hematoxylin-eosin (HE), and histological evaluation was performed according to previous description.

Western blotting

To investigate the content of several proteins, we performed western blotting as described previously. We used anti-AKT antibody (1:500, Abcam)，anti-ser473 antibody (1:5000, Abcam, Cambridge, UK),anti-Thr308 antibody (1:2000, Proteintech), anti-mTOR antibody (1:5000, Abcam)， anti-pho-mTOR antibody (1:5000, Abcam), anti-PTEN antibody (1:1000, CST) ，anti-PD-L1 antibody (1:2000, Proteintech) for primary antibodies incubation. β-actin (1:6000, Abcam) was used as control to standardize the proteins expression.

Real-time quantitative PCR (RT-PCR)

RT-PCR was used for quantifying the expression of PD-L1, IL-10 mRNA, and the procedure was the same as described previously. All primer sequences are shown in Table 1. Target gene expression was normalized to β-actin and calculated with the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primer sequences used for polymerase chain reaction

Gene name	Primer sequences (5'to 3')		
PD-L1	Forward		GGAAGACAAGGAAGUUAUUCA
	Reverse		AAUAACUUCCUUGUCUUCCUU
IL-10	Forward		CACTGCTATGTTGCCTGCTCTT
	Reversβ-actin	Forward	GTCTGGCTGACTGGGAAGTGG
	Reverse		TGCTATGTTGCCCTAGACTTCG
			GTTGGCATAGAGGTCTTTACGG

Flow cytometry

Monocytes were isolated from peripheral blood as described previously. After incubated with anti-CD4 and anti-CD45 (BD Biosciences, San Diego, USA) at 4˚C for 30 min in the dark, the cells were stained with anti-FOXP3 (BD Biosciences) and then analyzed by flow cytometry.

Enzyme-linked immunosorbent assay (ELISA)

To detect the level of IL-10, we collected colon homogenate supernatants for sandwich enzyme-linked immunosorbent assay using rat IL-10 ELISA kit (NeoBioscience, Shenzhen, China) according to the manufacture's protocol.

Statistical analysis

Statistical analysis was performed by SPSS 22.0 software. All data are presented as means ± standard deviation (SD). One-way ANOVA or Dunnett's test (equal variances were not assumed) was used for assessing statistical significant, and a value of P□0.05 was regarded as significant difference.

3. Results

Identification of BMSCs

To identify the cells' purity and differentiation potential, we used flow cytometry to analyse the bio-marker on their surface and exposed them to specific medium for verifying pluripotent. The flow cytometric

analysis revealed that the cells were positive for CD29 and CD90, the surface markers of bone marrow progenitor cell, but negative for CD11b and CD45, the surface markers of hematopoietic cell(Fig.1A). P2 BMSCs were used for induction, a large number of lipid deposition were shown with Oil Red O staining after adipogenic induction (Fig.1B), and calcium nodules which are showed as red spots with alizarin red staining were observed after osteogenic induction(Fig.1C).

PD-L1 siRNA down-regulated PD-L1 expression in BMSCs

To determine the effect of transfection, fluorescent labeling, qRT-PCR and western blotting of PD-L1 were performed in the PD-L1 siRNA BMSCs and the BMSCs control.The expression of GFP in transfected BMSCs was observed under fluorescence microscope (Fig.2A).After 72 hours of plasmid transfection, the levels of PD-L1 mRNA and protein in BMSCs transfected with PD-L1 siRNA were significantly lower than those in the control group(Fig.2B).In other words,the plasmid was successfully transfected and did down-regulate the expression of PD-L1. To determine whether the homing ability of BMSCs was different after virus transfection,the migration efficiency of BMSCs labeled by GFP in colon tissue was observed by immunofluorescence microscope. The average fluorescence intensity of PD-L1 siRNA BMSCs group was 16.346, and that of null-BMSCs group was 16.26. The results showed that there was no significant difference in the number of BMSCs colonized in the intestine between the two groups and no effect of virus transfection on homing of BMSCs (fig.2C).

Down-regulation of PD-L1 inhibited remission of TNBS-induced colitis by BMSCs

To evaluate the effect of PD-L1 in TNBS-induced colitis of rats, we monitored weight loss and calculated disease activity index (DAI) daily from molding. Rats in model group suffered the most serious weight loss and their DAI were the highest among all groups. For the cells therapy groups, the weight loss and DAI in the BMSCs control group decreased significantly than the PD-L1 siRNA BMSCs group compared with the model group(Fig.3A). As we all known, colonic inflammation leads to colon shortening. The colon length of model group, null-BMSCs group and PD-L1 siRNA BMSCs group were decreased by 21.47%, 5.93% and 13.05% respectively compared with the control group(Fig.3B,C), so the difference between two cells therapy groups was statistical significant. Histological analysis showed that there were mucosal erosion and large amount of inflammatory cellular infiltration in rat colon of model group, and cells therapy mitigated these damages and lower the histological score. However, the BMSCs control group indicated less inflammation than PD-L1 siRNA BMSCs group(Fig.3D,E). Taken together, the results reveal that BMSCs inhibit the intestinal inflammation in TNBS-induced rats via PD-L1.

Down-regulation of PD-L1 in BMSCs decreased Tregs in TNBS-induced colitis

To investigate that whether BMSCs promote Tregs differentiation via PD-L1, we analyzed the percentage of Tregs in monocytes cells of spleen and mesenteric lymph nodes. As anti-inflammatory cells, Tregs has been verified to decreased in rat model of UC induced by TNBS, and BMSCs transplantation can promote Tregs in UC rats according to our previous studies[11]. Our results showed that we reproduced the results of our previous experiments and PD-L1 inhibition in BMSCs decreased Tregs compared with BMSCs

control (Fig.4A, B). What is more, we detected the levels of IL-10, the signature transcription cytokine and effect cytokine of Tregs, in colonic tissue. PD-L1 siRNA BMSCs down-regulated the level of IL10 by PCR and ELISA compared with BMSCs control (Fig.5A). In general, PD-L1 exerts an important role in promoting Tregs differentiation by BMSCs.

Down-regulation of PD-L1 inhibited Akt/mTOR pathway

Akt/mTOR pathway is one of the down-stream of PI3K signal channels, and participates in regulating the differentiation of T naive cells, which are induced to Tregs through inhibiting Akt/mTOR pathway. For further verification of whether PD-L1 on BMSCs promotes Tregs and relieves inflammation via Akt/mTOR pathway, western blotting of Akt phosphorylation and mTOR phosphorylation were performed. The results display that the levels of phosphor-Akt (Ser473), phosphor-Akt (Thr308) and phosphor-mTOR in the PD-L1 siRNA BMSCs group were obviously increased compared with the BMSCs control group(Fig.5B,C,D). We also detected the level of PTEN (phosphate and tension homology deleted on chromosome ten), which is important for antagonizing PI3K signaling. As is shown in Fig.5E, the expression of PTEN was contrary to the level of Akt/mTOR pathway. Therefore, our results revealed BMSCs induced Tregs by activating Akt/mTOR pathway via PD-L1 in TNBS-treated rats.

4. Discussions

Ulcerative colitis (UC), together with the crohn's disease (CD), belongs to inflammatory bowel disease (IBD). The typical pathologic manifestations of UC are mucosal ulcer, submucosal edema, lymphocytes infiltration and fibrosis, which are considered to be related to T cell-mediated immune regulation disorder, though not being fully understood[17, 18]. For studying the pathogenesis and therapeutic methods, many animal models are implemented. In this research, we used TNBS to induce colitis of rats, which is the mimic of human UC in both symptoms and pathological processes.

For the past few years, mesenchymal stem cells (MSCs) therapy has attracted lots of attention owing to the immune-regulatory capacity and multi-lineage differentiation ability of MSCs. Currently, the application of MSCs therapy mainly focus on local or systematic transplantation, combining with genetic modification and tissue engineering[19-21]. And more attractive thing is that BMSCs, one of the most important representations of MSCs, have been proved of significant treatment prospect for IBD[11, 22]. The locations of IBD are mainly happened in gut, a unique immune organ, and it would be damaged when the balance between pro-inflammatory cells and anti-inflammatory cells was destroyed[23, 24]. Some pro-inflammatory cells such as Th1 and Th17, differentiated by naive T cells, exert influence on inducing and maintaining intestinal inflammation by secreting effector cytokines, while Tregs, another kind of cells derived from Naive T cells, are important anti-inflammatory cells which suppress the function of pro-inflammatory cells[25-27]. Extensive evidence reveals that Tregs decrease in UC, and promoting the differentiation of Tregs alleviates the colitis of animal models[11, 28, 29]. So the therapeutic effects of both BMSCs and Tregs raise much interests of researchers worldwide. Casiraghi et al. [30]has testified that transplantation of MSCs induces Tregs *in vivo*, and our preliminary experiment

shows the percentage of Tregs increased after administration of BMSCs in TNBS-induce colitis[11]. However, some experiments show that transplantation of MSCs enhances tumor growth in some animal model which is considered to be related to their multi-differentiation potential[31, 32]. So the more understanding of complicated interaction between MSCs and Tregs, the greater therapeutic effect we would get, and this study aimed at clarifying possible approaches of regulation between they two based on our previous findings.

Recently, the functions of PD-1 and its ligand PD-L1 in regulating immunological tolerance and autoimmunity caused significant concern of researchers[13]. Several studies have demonstrated that PD-1/PD-L1 pathway exerts pivotal roles in physiological and pathological processes including activation and differentiation of T cells, oncogenesis, and some chronic inflammation[33-35]. Both of them are found to expressed on T cells, B cells, macrophages and some dendritic cells (DCs), meanwhile, PD-L1 is found to constitutively expressed on MSCs on murine[36, 37]. That is to say, BMSCs might promote the activation of naive T cells and induce them to differentiate into Tregs via PD-1/PD-L1 pathway. In the differentiation process of naive T cells, it is the interaction between PD-1 and PD-L1 that influences the generation of Tregs[38]. Furthermore, extensive studies indicate that Akt signaling, which is necessary for activaton and proliferation of naive T cells, is dispensible for development and function of Tregs[39-41]. During the activation and proliferation of T cells, The binding of PD-1 on the surface of naive T cells and PD-L1 leads to the phosphorylation of ITIM and ITSM on cytoplasmic domain and the recruitment of SHP-1 and SHP-2, SHP-1 and SHP-2 inhibit the activation of PI3K and interdict the phosphorylation of Akt, the down-stream of PI3K[36], and then suppress the Akt/mTOR signaling cascade, consequently influencing on the “molecular switch” in naive T cells to inducing development of Tregs[42]. Simultaneously, the inhibition of PI3K blocks proliferation and survival of T cells, which maintains the function of Tregs[43]. In this study, our data revealed that BMSCs administration inhibited Akt/mTOR pathway, up-regulated the expression of PTEN, the inhibitory signal of PI3K, and raised percentage of Tregs, as well as IL-10. However, the results in PD-L1 siRNA BMSCs group were opposite.

In conclusion, BMSCs can improve TNBS-induced colitis by expressing PD-L1, and its mechanism is mainly related to the inhibition of Akt / mTOR pathway which can induce Treg differentiation. PD-L1 is an important target of BMSCs in the treatment of UC.

Declarations

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Authors' Contributions

Fei Gao: Software, Formal analysis, Writing - original draft. **Dandan Cui Heng Fan**: Writing - review & editing, Software, Methodology. **Zhexing Shou:** Conceptualization, Funding acquisition. **Dongmei Zuo, Jia Yang,**: Investigation, Methodology, Data curation. **Ting Yu**: Validation. **Yujin Liu**: Methodology. **Feng Zhu, Si Chu, Chunzhu Wei**: Formal analysis, Validation.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics Approval and Consent to Participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of Wuhan Service Technology Co., Ltd., China.

Consent for Publication.

Not applicable.

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Not applicable.

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Figures

Figure 1

Identification of bone marrow-derived mesenchymal stem cells (BMSCs).(A) Flow cytometric characterization of BMSCs at passage 2. **(B)** Adipogenic differentiation of BMSCs stained with Oil Red O. Original magnifcation, x 400. **(C)** Osteogenic differentiation of BMSCs stained with alizarin red. Original magnifcation, x400.

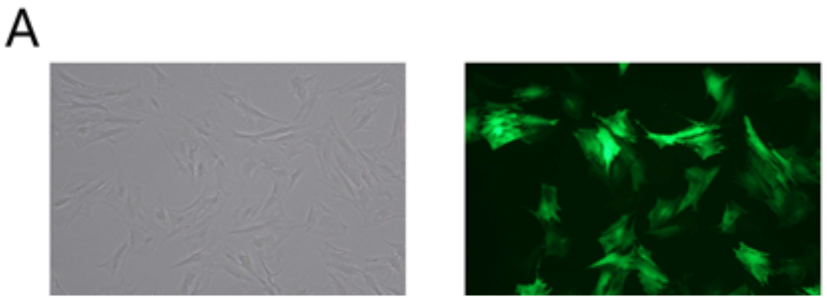

Figure 2

Expression of PD-L1 is down-regulated in PD-L1 siRNA-transfected bone marrow-derived mesenchymal stem cells (BMSCs).(A) The expression of fluorescence-labeled PD-L1 in transfected bone marrow stromal cells was observed under optical and fluorescence microscope. Original magnifcation,x400.**(B)**Western blotting analysis of PD-L1 protein in lentivirus-transfected BMSCs and levels of PD-L1 protein and mRNA in lentivirus-transfected BMSCs. **(C)** GFP-lentivirus transfected BMSCs under fluorescence microscopes. Original magnifcation,x40. Data are expressed as means ± SD ($n_{=}3$). ***P < 0.001 vs. null-BMSCs.

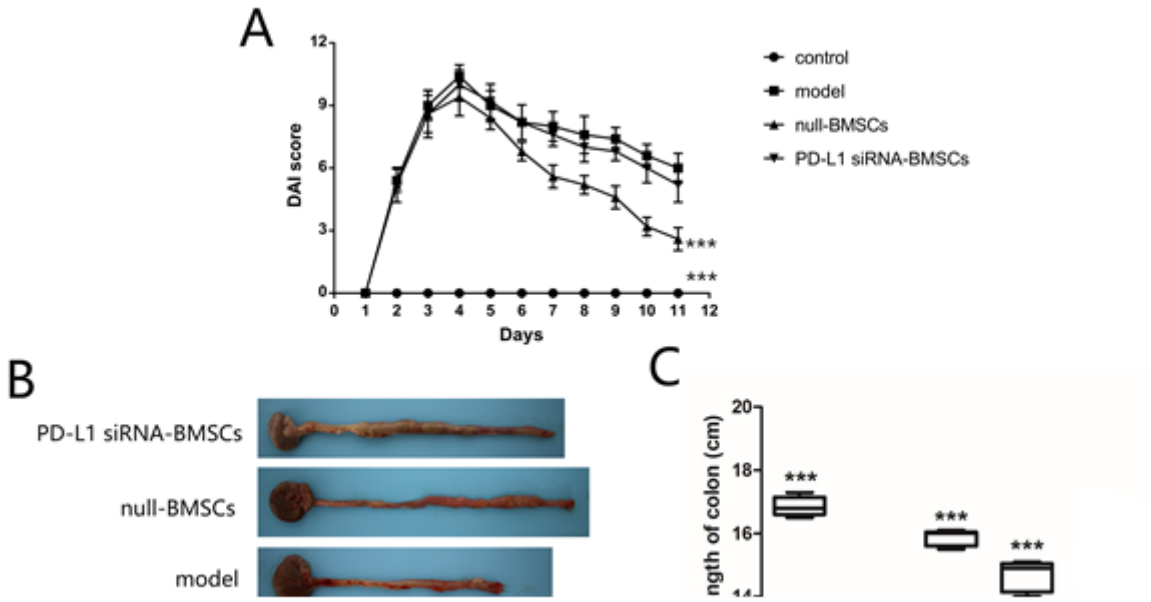

Figure 3

Expression of PD-L1 enhances the protective effects of bone marrow-derived mesenchymal stem cells (BMSCs) in rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. (A) The disease activity index (DAI) of rats was monitored daily. **(B)** Colons of rats from different treatments are shown. **(C)** Colonic length of rats under different treatments.**(D)** Colon specimens stained with hematoxylin and eosin were analyzed. Original magnification, x 40. **(E)**Colonic histological scores.Data are expressed as means ± SD (n=5).**P < 0.01, ***P < 0.001 vs. model group.

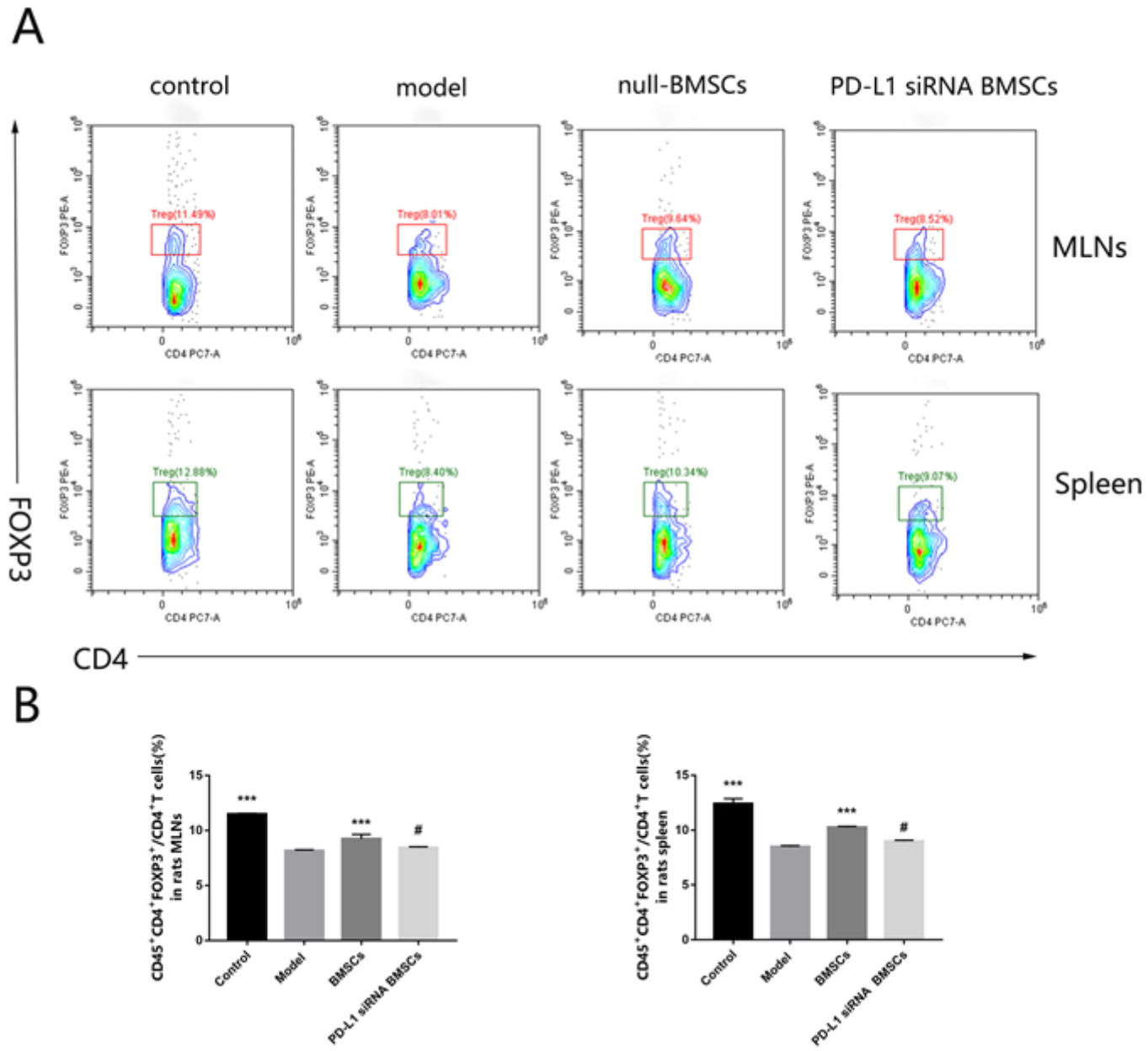

Figure 4

Expression of PD-L1 up-regulates Tregs cells in rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. (A)Flow cytometry analyzed the frequencies of Tregs in mesenteric lymph nodes (MLNs) and spleen. (B) Average frequencies of Tregs in MLNs and spleen. Data are expressed as means ± SD (n=3). #P>0.05，***P < 0.001 vs. model group.

Figure 5

（A）Levels of IL-10 in colon tissues of each group were measured by enzyme-linked immunosorbent assay (Elisa) and quantitative real-time polymerase chain reaction (qRT-PCR) analyses of IL-10 mRNA

expression in colons.**(B)** Western blotting analysis of phosphor-Akt (Ser473) ,phosphor-Akt (Thr308),AKT, phosphor-mTOR,mTOR and PTEN protein.**(C)** Levels of Ser473 protein and Thr308 protein in colon tissues of each group. **(D)** Levels of phosphor-mTOR protein in colon tissues of each group. **(E)** Levels of PTEN protein in colon tissues of each group. Data are expressed as means ± SD (n=3). *P < 0.05,**P < 0.01, ***P < 0.001 vs. model group.