

Downregulation of Dickkopf-1 Augments the Therapeutic Effects of Endometrial Regenerative Cells on Experimental Colitis

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Research

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Abstract

Background We have demonstrated that endometrial regenerative cells (ERCs) are mesenchymal-like stromal cells and can attenuate experimental colitis, however, its underlying mechanism needs further investigation. Dickkopf-1 (DKK1), a glycoprotein secreted by mesenchymal stromal cells (MSCs), is a classical inhibitor of Wnt/ β -catenin pathway which is closely associated with the development of colitis. Therefore, the objective of this study was to investigate whether ERCs could also secrete DKK1, and whether the downregulation of DKK1 (DKK1 low -ERCs) would enhance the therapeutic effects of ERCs in attenuation of experimental colitis. **Methods** BALB/c mice were given 3% dextran sodium sulfate (DSS) for 7 consecutive days and free tap water for 3 days sequentially to induce experimental colitis. Unmodified ERCs, IL-1 β -treated ERCs (DKK1 low -ERCs) and glucocorticoid-treated ERCs (DKK1 high -ERCs) were injected (1 million/mouse/day, i.v.) on day 2, 5 and 8 respectively. Colonic and splenic samples were harvested on day 10 after DSS-induction. **Results** It was found that DKK1 low -ERC treatment markedly attenuated colonic damage, body weight loss and colon-length shortening in colitis mice. Compared with other treatments, cell populations of CD4 + IL-4 + Th2, CD4 + CD25 + FOXP3 + Treg, and CD68 + CD206 + macrophages in spleens were also significantly upregulated in DKK1 low -ERC group ($p < 0.05$). In addition, lower expression of pro-inflammatory (TNF- α and IFN- γ), but higher levels of anti-inflammatory cytokines (IL-4 and IL-10) and β -catenin were detected in colons in DKK1 low -ERC group ($p < 0.01$ vs. other groups). **Conclusions** DKK1 low -ERCs display augmented immunoregulatory ability and therapeutic effects in DSS-induced colitis.

Background

Ulcerative colitis (UC) is a chronic, relapsing and non-specific inflammatory disease of the intestine. It is usually characterized by abdominal pain, diarrhea, fecal urgency and bloody stools[1]. Histologically, the lesions of ulcerative colitis are mostly confined to the mucosa and submucosa, which mainly involve the sigmoid colon and rectum, as well as the descending and entire colon[2]. In the last decades, the incidence and prevalence of UC have experienced a dramatic upward trend in Europe and America[3]. But, up to now, there are still no effective measures to control its development. Moreover, the consistent inflammation in colons may also increase the risk of suffering colorectal cancer[4].

Though the etiology of UC has not been defined clearly[5], accumulating evidences indicate that UC is correlated with the inappropriate immune response of mucosa toward luminal bacterial flora[6]. Till now, clinic treatments for UC mainly comprise four aspects: aminosalicylates, glucocorticoid (GC), immunosuppressants and biologics. Disappointingly, these agents are mostly used to relieve symptoms or for maintenance, and sometimes are not well tolerated[7]. Moreover, long-term administrations are always accompanied with various toxic effects[8, 9]. Therefore, seeking a novel therapy to supplement the existing treatment is in urgent need.

Mesenchymal stromal cells (MSCs) were primarily recorded as a group of non-hematopoietic, self-renewing, plastic-adherent and fibroblast-like stromal cells[10]. Plenty of evidences have demonstrated

that MSCs possess the immunomodulatory and anti-inflammatory specialties[11], assuming migrating to the injury sites to promote the tissue repair and modulating the function of immunocytes, such as T cells, B cells, dendritic cells (DCs) and macrophages[12-15]. Rely on these above properties, MSCs are exhibiting unique potentials in attenuating the development of ulcer colitis. But, at the same time, the deficiencies of MSCs are gradually emerging, such as the invasive obtaining process and related complications, less availability, and poor proliferation capacity [16]. These restrictions sharply limit its application as a clinical therapy.

Endometrial regenerative cells (ERCs), a new type of mesenchymal-like stromal cells, were isolated from human menstrual blood by Meng *et al* in 2007[17]. ERCs possess the similar phenotypic markers with MSCs (high expression of CD29, CD44 and CD90 molecules, but low of CD45), but surmount the limits of traditional MSCs. Compared with MSCs, ERCs were with more outstanding advantages, including diverse differentiation potentials, immunomodulatory properties, non-invasive obtaining process and high proliferative capacity without karyotypic abnormality[18]. We and others have previously reported the forcible therapeutic effects of ERC for immune-related diseases such as ulcerative colitis, acute liver injury, critical limb ischemia, renal ischemia reperfusion injury, pulmonary fibrosis, myocardial infarction, and so on[19-24]. Moreover, no serious immunological rejections were emerging against the human derived ERCs when we used these cells to treat animal models[21]. Therefore, ERCs are being gained more and more attention in immune-disordered diseases, and its beneficial efficacy is being recognized by more and more researchers.

Dickkopf-1 (DKK1), is a kind of secreted glycoprotein which can be secreted by MSCs as well[25]. DKK1 possesses a conservative gene sequence and previous studies have proved its expression in stromal cells can be down-regulated by IL-1 β or up-regulated by glucocorticoid stimulation[26]. Relying on competing with Wnt ligands for LRP5/6 receptors and inducing the endocytosis of binding DKK1-receptor complex[27, 28], DKK1 has been recognized as the special inhibitor of Wnt/ β -catenin signaling pathway[29]. As known, Wnt pathway is an ancient, complicated and highly-conserved signaling system, proceeding in various cells[30]. There exist about three pathways consisting in the Wnt signaling transduction: Wnt/ β -catenin pathway, plane cell polar pathway and Wnt-Ca²⁺ pathway. Among them, Wnt/ β -catenin pathway is the main method of signaling transduction, which is also known as the canonical Wnt signaling[30]. In Wnt/ β -catenin pathway, β -catenin is a central component and its existence is essential for the activation of this signaling[31]. Proved by previous reports, Wnt/ β -catenin pathway is involved in regulating cell differentiation, embryonic development, tissue regeneration and other important physiological processes[32, 33]. However in recent years, accumulating researches have revealed its another important role which was associated with the immunoregulatory porperties in various immune-related inflammations and auto-immune diseases, comprising cancer, rheumatoid arthritis, enteritis and so on[34-36]. Wnt/ β -catenin pathway is participating in regulating the development and differentiation of immunocytes[30] and exhibiting encouraging and promising anti-inflammatory effects in chronic disease, such as inflammatory bowel disease[37-39].

In present study, our results demonstrated that ERCs, as mesenchymal-like stromal cells, could secrete an considerable amount of DKK1, and ERCs with downregulated DKK1 secretion (DKK1^{low}-ERCs) exhibited more powerful therapeutic effects in attenuation of experimental colitis.

Methods

Animals

Male adult BALB/c mice, aged 8–10 weeks and weighing 20-24g (Aoyide Co., Tianjin, China), were caged in a comfortable experimental condition in the Animal Care Facility, Tianjin General Surgery Institute (Tianjin, China). Mice were provided with 1 week to adapt the new surroundings and free access to ample tap water and mouse food constantly. Total experiments were fulfilled based on the protocols approved by the Animal Care and Use Committee of Tianjin Medical University (Tianjin, China), according to the Chinese Council on Animal Care guidelines.

ERC preparation

Primary human ERCs were isolated from menstrual blood by a density gradient centrifugation method in accord with previous study[40]. In brief, the mononuclear cells were firstly separated from menstrual blood and then suspended in the Dulbecco's modified Eagle's medium (DMEM) high glucose which was supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. Then cells were seeded in 10 cm dishes and cultured at the 37°C 5% CO₂ condition incubator. ERCs would adhere to the bottom of dishes after overnight incubation and the culture medium was changed every two days to wash away the non-adhered cells. Two weeks later, when cells expanded to 80-90% area of dishes and displayed a spindle-shaped morphology, we split and passaged down the ERCs as the rate of 1:3. Typical cell surface markers of ERCs were detected by a flow cytometry as previous study described [17].

In vitro, we harvested the 3rd to 7th generation of ERCs, divided each generation of cells into 3 groups (unmodified ERCs, GC-treated ERCs, and IL-1 β -treated ERCs), and inoculated them at a concentration of 3.5×10^5 /ml with 2.5ml culture media. In an attempt to alter DKK1 secretion, we added glucocorticoid (GC, 100 nM, as the stimulator) or IL-1 β (10ng/ml, as the inhibitor)[26] into respective groups. After cultured for 48 hours, supernatants in each group were collected to implement ELISA analysis. Fifth generation of ERCs, the most suitable candidate for treatments, were prepared for RT-PCR test to further analysis the DKK1 secretion changes.

For *in vivo* use, we selected the 5th generation of ERCs, and pre-treated these candidate cells with GC (100nM) or IL-1 β (10ng/ml) for 48 hours respectively. Then ERCs were harvested, washed and suspended at a concentration of 1×10^6 /ml for the following therapy.

Experimental groups

The experimental colitis was induced by supplying the mice with 3% (wt/vol) DSS (MP Biochemicals) dissolved tap-water as previous studies described[16]. In this current study, 24 BALB/c mice were randomly allocated into 4 groups: untreated group, unmodified ERC group, DKK1^{high}-ERC group (GC-treated ERCs), DKK1^{low}-ERC group (IL-1 β -treated ERCs) (n=6). All experimental groups were firstly supplied with 3% (wt/vol) DSS (MP Biochemicals) soluted water for seven days, and then replaced with the non-DSS tap water. ERCs or pre-treated ERCs were suspended in phosphate buffered saline (PBS) and injected into experimental mice (1×10^6 cells/ml/mouse, *i.v.*) on day 2, 5 and 8, respectively. Untreated group was also given the equal volume of PBS as control. Mice body-weights, general conditions and fecal characters were monitored and kept into records daily, convenient for the Disease Activity Index (DAI) assessment and other statistical calculations. DAI is an indicator for disease activity which can comprehensively reflect the severity of inflammation in mice. Its score was calculated by assessing weight loss, fecal character and stool blood, accord to the scoring system (Min = 0, Max = 4) directed by Murthy *et al*[41].

On day 10, all mice were sacrificed after being fasted for 8 hours. Colons were dissected carefully from ileocecal junction verge to anus, and their lengths were measured. Then, samples were washed with PBS to clean away the contents and longitudinally severed into two parts. One part was fixed in 10% formalin buffer preparing for pathology analysis, and the other was reserved at -80 °C for other experiments. Spleen samples were also harvested and split into two parts. One was immediately ground in PBS for FACS; the other was stored at -80°C for ELISA test.

Pathological examination

Colons were obtained, cleaned with PBS, and fixed in 10% buffered formaldehyde on day 10 after DSS-induction. Undergoing processes of dehydration and paraffin embedding, samples were sectioned on an ultra-microtome (LEICA, Germany) at a thickness of 5 μ m for haematoxylin and eosin (H&E) staining. Histopathological scores were evaluated and calculated in a double-blinded manner, based on the following criteria[42]: (a) inflammation severity: 0 (physiologic inflammation), 1 (mild inflammation or prominent lymphoid aggregates), 2 (moderate inflammation), 3 (moderate inflammation associated with crypt loss), 4 (severe inflammation with crypt loss and ulceration). (b) crypt damage: 0 (no destruction), 1 (1–33% of crypts destroyed), 2 (34–66% of crypts destroyed), 3 (67– 100% of crypts destroyed). The two respective scores, inflammation severity and crypt damage, were summed together to drive the histopathological scores for evaluating colonic inflammation (maximum score 7).

Flow cytometry analysis

Mouse spleens were respectively ground, filtered with sterilized meshes and suspended in 2 ml precooled PBS. Then we added RBC Lysis Solution (1x) (Biolegend Inc., San Diego, CA, USA) into splenic suspensions to lyse erythrocytes, washed twice and resuspended the splenocytes with PBS to a concentration of 1×10^7 /ml. Fluorescent monoclonal antibodies against mouse CD4, IFN- γ , IL-4, IL-17, CD25, FOXP3, CD68, CD206, CD11c, MHCII, and CD86 were applied to detect the populations of Th1 (CD4⁺IFN- γ ⁺), Th2 (CD4⁺IL-4⁺), Th17 (CD4⁺IL-17⁺), Treg (CD4⁺CD25⁺FOXP3⁺), macrophage (CD68⁺CD206⁺) and DC (CD11c⁺MHCII⁺/CD86⁺) cells by FACS Canto II flow cytometer (BD Biosciences, America), as previously described[43]. The absolute number of immune cells detected in Figure 3 and Figure 4 were at the average level of 5×10^4 and the percentages of TH1, TH2, TH17 and TREG were showed on CD4⁺ gated cells. In addition, to accurately identify the subpopulation of Th1, Th2 and Th17 CD4⁺ T cells, splenocytes were firstly incubated with cell stimulation cocktail (including phorbol-12-myristate-13-acetate (PMA), ionomycin, brefeldin A, and monensin) (ebioscience Inc., San Diego, CA, USA) for 5 hours before being stained with fluorescent antibodies. The statistics of various immunocyte proportions were analyzed by Flowjo X software.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out according to the manufacturer's instructions (Boster, Wuhan, China). Supernatants in culture media of ERCs were collected and prepared for measuring the DKK1 secretion level. Equal quality (30mg) of same area intestinal tissues in each group were gathered and grounded with high efficiency tissue lysate buffer (RIPA) and phenylmethyl sulfonylfluoride (PMSF) (Solarbio, Beijing, China) for testing the level of IFN- γ , IL-4, IL-10 and β -catenin. Splenic tissues frozen in -80°C were also ground for detecting the β -catenin expression level. The reaction absorbance was determined at 450 nm with the Microplate Reader (Tecan, Mannedorf, Switzerland) and each sample was performed in duplicates to lessen the error.

Real-time polymerase chain reaction (RT-PCR)

To determine the transcriptional changes of inflammatory mediators and β -catenin in colons, colonic total RNA was extracted with an RNAprep Pure Tissue Kit (DP431, Tiangen Biotech Co. Ltd., Beijing, China). The pureness and concentration of RNA were determined with an UV spectrophotometer (SANYO, Japan) at the spectrum of 260 and 280nm. cDNA was generated from the obtained RNA by using a Fastquant RT kit (KR106, Tiangen Biotech Co. Ltd., Beijing, China). Real-time quantitative PCR (RT-PCR) was carried out by using SuperReal Color Premix kit (FP216, Tiangen Biotech Co. Ltd., Beijing, China), according to the recommended protocol. The primer sequences involved were designed as follows:

human-GADPH: forward, 5'-ACAACCTTTGGTATCGTGGAAGG-3',

reverse, 5'-AAGTGGTCGTTGAGGGCAATG-3';

human-DKK1: forward, 5'-ATAGCACCTTGGATGGGTATTCC-3',
reverse, 5'-CTGATGACCGGAGACAAACAG-3';

mouse GAPDH: forward, 5'-AGGTCGGTGTGAACGGATTTG-3',
reverse, 5'-TGTAGACCATGTAGTTGAGGTCA-3';

mouse β -catenin: forward, 5'-GAGTAGCTGCAGGGGTCTC-3',
reverse, 5'-GGACAGCAGCTGCGTATGTT-3';

mouse IFN- γ : forward, 5'-GCCGCGTCTTGGTTTTGCAG-3',
reverse, 5'-TACCGTCCTTTTGCCAGTTCCTCCA-3';

mouse IL-4: forward, 5'-ACAGGAGAAGGGACGCCAT-3',
reverse, 5'-GAAGCCCTACAGACGAGCTCA-3';

mouse IL-10: forward, 5'-AGAAGCATGGCCCAGAAATCA-3',
reverse, 5'-GGCCTTGTAGACACCTTGGT-3';

mouse TNF- α : forward, 5'-CCCTCACACTCAGATCATCTTCT-3',
reverse, 5'-GCTACGACGTGGGCTACAG-3';

mouse COX-2: forward, 5'-TGAGCAACTATTCCAAACCAGC-3',
reverse, 5'-GCACGTAGTCTTCGATCACTATC-3';

mouse MPO: forward, 5'-AGTTGTGCTGAGCTGTATGGA-3',
reverse, 5'-CGGCTGCTTGAAGTAAAACAGG-3';

mouse iNOs: forward, 5'-GTTCTCAGCCCAACAATACAAGA-3',
reverse, 5'-GTGGACGGGTCGATGTCAC-3';

mouse SOD α forward, 5'-CAGACCTGCCTTACGACTATGG-3',
reverse, 5'-CTCGGTGGCGTTGAGATTGTT-3'.

Each sample was performed in triplicates on MJ Research DNA Engine Opticon 2 PCR cycler (BIO-RAD, USA). The expressions of target genes among different groups were calculated with the comparative

$2^{-\Delta\Delta CT}$ method.

Statistical analysis

Experimental data was presented as mean \pm standard deviation (SD) and analyzed by SPSS 19.0. Data variance was evaluated by using one-way analysis of variance (ANOVA) (groups ≥ 3) or unpaired two-tailed student's *t* test (groups=2) after normality test. The differences between groups were considered significant with *p* values ≤ 0.05 in statistics.

Results

Pretreatment with Glucocorticoid or IL-1 β Changed the DKK1 Secretion by ERCs

To determine whether ERCs would secrete DKK1 and whether this secretion would be affected by glucocorticoid (GC) or IL-1 β , the level of DKK1 in the supernatant of different pre-treated ERCs was measured by ELISA and its concentration was shown significantly different in Figure 1. Among which, the 5th generation of ERCs were recorded with the lowest DKK1 expression when compared with other generation cells (Figure 1A; **p* < 0.05, ***p* < 0.01, ****p* < 0.001). Furthermore, in each generation, the DKK1 secretory amount in IL-1 β -treated ERC group was shown lower than that in unmodified ERC group and GC-treated ERC group respectively. Specifically, to further clarify whether there exists statistical difference between different pre-treated ERCs, culture supernatant and homogenate of 5th generation of ERCs were collected and analyzed for the DKK1 expression at the protein and mRNA level separately. As shown in Figure 1B and 1C, the DKK1 expression was strikingly decreased in IL-1 β -treated ERC group (unmodified ERCs vs. IL-1 β -treated ERCs: *p* < 0.05, Figure 1B; *p* < 0.05, Figure 1C), but when ERCs were pre-treated with glucocorticoid (GC), DKK1 expression boosted up (unmodified ERCs vs. GC-treated ERCs: *p* < 0.01, Figure 1B; *p* < 0.001, Figure 1C).

DKK1^{low}-ERCs Markedly Ameliorated the Symptoms of DSS-induced Colitis

In present study, we used the 5th generation of ERCs for the following in vivo experiments due to its lowest DKK1 expression. After the fifth day of DSS induction, mice begin to exhibit significant bloody stool, weight loss and lethargy. But, in the unmodified ERC group, following the treatment of ERCs, bloody stool (Figure 2A) and body weight loss were found with moderate relieve and improvement (Figure 2B, unmodified ERC group vs. untreated group, *p* < 0.001). Moreover, the therapeutic effects were further improved when the colitis mice were treated with DKK1^{low}-ERCs (IL-1 β -treated ERCs) (Figure 2A; Figure 2B, vs. unmodified ERCs, *p* < 0.01), while diminished when treated with DKK1^{high}-ERCs (GC-treated ERCs) (Figure 2A; Figure 2B, vs. unmodified ERCs, *p* < 0.01). In addition, we have also analyzed the Disease Activity Index (DAI) score in each group. As shown in Figure 2C, the DAI score was apparently decreased in unmodified ERC group, when compared with that in untreated group (unmodified ERC group vs. untreated group, *p* < 0.001). While in DKK1^{high}-ERC group, the DAI score raised up (*p* < 0.05, DKK1^{high}-ERC

group vs. unmodified ERC group), indicating the severe colon injury, but significantly decreased in DKK1^{low}-ERC group (DKK1^{low}-ERC group vs. unmodified ERC group, $p < 0.05$). Given the above results, it suggests that DKK1 is closely associated with the therapeutic effect of ERCs on the development of DSS-induced colitis.

DKK1^{low}-ERCs Relieved the Histopathological Damage of DSS-induced Colitis

To observe the changes of colonic morphology, colon samples were collected and their lengths were measured as shown in Figures 2D and 2E. We found that the average length of colons in untreated group is 5.3cm (n=6), indicating a significant reduction in colon length due to the severe intestinal inflammation. While, the average length in DKK1^{low}-ERC group is 7.0 cm, higher than unmodified ERC group (6.3cm; Figure 2E, DKK1^{low}-ERC group vs. unmodified ERC group, $p < 0.01$) and the length in DKK1^{high}-ERC group is 5.5cm, lower than the unmodified ERC group (Figure 2E, DKK1^{high}-ERC group vs. unmodified ERC group, $p < 0.001$), which suggested that tissue injury and structural damage were obviously alleviated in DKK1^{low}-ERC group. Pathological examination also confirmed the above findings. DSS intake caused severe injury, while in DKK1^{low}-ERC group (Figure 2F a-d), the pathological condition of colon was strikingly better, showing slight damages to crypt structure, glands and epithelium cells, mild inflammatory cell infiltration and less goblet cells loss. Also, the histopathological score of DKK1^{low}-ERC group was lower than that of the unmodified ERC group (Figure 2G, $p < 0.001$), and unmodified ERC group was lower than the DKK1^{high}-ERC group (Figure 2G, $p < 0.001$). Given together, these results indicate that DKK1^{low}-ERCs could exhibit optimized therapeutic effect in relieving histopathological damages in DSS-induced colitis, and this optimized effect of ERCs is, at least in part, mediated by DKK1 low expression.

DKK1^{low}-ERCs Reduced Th1 and Th17, but Enhanced Th2 and Treg Proportions in Colitis Mice

To determine whether DKK1 is involved in the immunomodulatory effects of ERCs on Th1, Th2, Th17 and Treg cell populations, splenocytes from each group were prepared and stained for FACS analysis. As shown in Figure 3A and 3B, we analyzed the proportions of CD4⁺IFN- γ ⁺Th1, CD4⁺IL-4⁺Th2, CD4⁺IL17⁺Th17 and CD4⁺CD25⁺FoxP3⁺Treg cells respectively. The statistical results in Figure 3C-3F showed that the proportions of CD4⁺IFN- γ ⁺Th1 and CD4⁺IL17⁺Th17 cells were significantly decreased in unmodified ERC group when compared with that in untreated group (Figure 3C and 3E: Th1, $p < 0.001$; Th17, $p < 0.001$) and further decreased in DKK1^{low}-ERC group (unmodified ERC group vs. DKK1^{low}-ERC group: Th1, $P < 0.05$; Th17, $P < 0.05$). Whereas, the proportion of CD4⁺IL-4⁺Th2 cells and CD4⁺CD25⁺FoxP3⁺ Treg were increased in DKK1^{low}-ERC group (DKK1^{low}-ERC group vs. unmodified ERC group: Th2, $p < 0.05$; Treg, $p < 0.001$), while reduced in DKK1^{high}-ERC group (DKK1^{high}-ERC group vs. unmodified ERC group: Th2, $p < 0.001$; Treg, $p < 0.05$). These results indicate that downregulating the secretion of DKK1 could augment the immunomodulatory function of ERCs, at least in regulating Th1/Th2 paradigm, Th17 and Treg populations.

DKK1^{low}-ERCs Reduced the Population of Mature DCs in Colitis Mice

To explore the changes of DCs in different groups, mature DCs in splenocytes gated by anti-CD11c were detected by high expressing antigen presenting-related marks (MHCII, CD86) for further analysis. As indicated in Figure 4, the two populations of mature DCs (CD11c⁺MHCII⁺, CD11c⁺CD86⁺) were both reduced in unmodified ERC group, while were further strikingly reduced in the DKK1^{low}-ERC group (DKK1^{low}-ERC group vs. unmodified ERC group: CD11c⁺MHCII⁺, $p < 0.05$; CD11c⁺CD86⁺, $p < 0.01$). But, in DKK1^{high}-ERC group, these two populations of mature DCs were raised (DKK1^{high}-ERC group vs. unmodified ERC group: CD11c⁺MHCII⁺, $p < 0.01$; CD11c⁺CD86⁺, $p < 0.001$). These data suggest that DKK1, may be also associated with the regulation of ERCs and enhance the capability of unmodified ERCs on inhibiting DC maturation and reducing total DCs generation.

DKK1^{low}-ERCs Increased the Population of M2 Phenotype Macrophages in Colitis Mice

M2 phenotype macrophages is one of the main subtypes of macrophages which plays the anti-inflammatory role in the pathogenesis of experimental colitis. Our previous study has revealed that ERCs could promote the differentiation of macrophages to M2 subtype. To determine whether DKK1 has an influence in regulating M2 in colitis mice, anti-CD68 and anti-CD206 antibodies were used to measure the proportion changes in spleens. Compared with that in untreated group, M2 population was obviously raised in unmodified ERC group (Figure 4B and 4E: untreated group vs. unmodified ERC group, $p < 0.001$). Moreover, the population of M2 cells was further increased to a higher level in DKK1^{low}-ERC group (DKK1^{low}-ERC group vs. unmodified ERC group, $p < 0.05$), while reduced in DKK1^{high}-ERC group (DKK1^{high}-ERC group vs. unmodified ERC group, $p < 0.05$). These results suggest that DKK1^{low}-ERCs mediate the colitis therapy in regulating macrophage phenotypes and promoting immunosuppressive M2 phenotype cell increase, which would help to diminish the injury from acute immune response in colons.

DKK1^{low}-ERCs Altered the Expressions of Inflammatory Mediators in Colons

In an attempt to address the effects of DKK1 on ERC-mediated immunoregulation, we measured different inflammatory mediator productions in colons which are associated with the development of UC. As showed in Figure 5A-D, the result demonstrated that IL-4 and IL-10 in colons, both the protein and mRNA expression, were apparently increased in unmodified ERC group (unmodified ERC group vs. untreated group: Figure 5A, $p < 0.001$; Figure 5B, $p < 0.001$; Figure 5C, $p < 0.01$; Figure 5D, $p < 0.001$). While, the levels of IL-4 and IL-10 were raised in DKK1^{low}-ERC group (DKK1^{low}-ERC group vs. unmodified ERC group: Figure 5A, $p < 0.01$; Figure 5B, $p < 0.01$; Figure 5C, $p < 0.01$; Figure 5D, $p < 0.001$), but plunged apparently in DKK1^{high}-ERC group (DKK1^{high}-ERC group vs. unmodified ERC group: Figure 5A, $p < 0.05$; Figure 5B, $p < 0.01$; Figure 5C, $p < 0.05$; Figure 5D, $p < 0.01$). Moreover, the IFN- γ level in colonic tissues was reduced in unmodified ERC group (Figure 5E, $p < 0.001$; Figure 5F, $p < 0.001$; vs. untreated group), and significantly decreased in DKK1^{low}-ERC group (Figure 5E, $p < 0.05$; Figure 5F, $p < 0.01$; vs. unmodified ERC group), but increased in DKK1^{high}-ERC group (Figure 5E, $p < 0.001$; Figure 5F, $p < 0.01$; vs. unmodified ERC group).

To further verify the inflammatory mediator changes in colons, we compared the mRNA expression levels of other related factors. As shown in Figure 5G-J, we found that pro-inflammatory mediators in colons, including TNF- α (Tumor necrosis factor-alpha), MPO (Myeloperoxidase), COX-2 (Cyclooxygenase-2) and iNOs (Inducible nitric oxide synthase enzyme), presented lower expression level in DKK1^{low}-ERC group (DKK1^{low}-ERC group vs. unmodified ERC group: TNF- α , $p < 0.05$; MPO, $p < 0.05$; COX-2, $p < 0.01$; iNOs, $p < 0.01$), but higher in DKK1^{high}-ERC group (DKK1^{high}-ERC group vs. unmodified ERC group: TNF- α , $p < 0.001$; MPO, $p < 0.01$; COX-2, $p < 0.01$; iNOs, $p < 0.01$). Meanwhile, we also tested the SOD (superoxide dismutases) mRNA expression in different groups (Figure 5K). SOD, which is regarded as a critical antioxidant enzyme, could catalyze the dismutation of toxic superoxides and scavenge superoxide radicals in colitis[44]. In our present study, we found that SOD mRNA expressions were significantly raised in DKK1^{low}-ERC group (DKK1^{low}-ERC group vs. unmodified ERC group, $p < 0.01$), but plummeted in DKK1^{high}-ERC group (DKK1^{high}-ERC group vs. unmodified ERC group, SOD, $p < 0.01$).

Taking together, these data indicate that down-regulating the DKK1 secretion of ERCs, could strikingly polished up the therapeutic effects on colitis and these effects might rely in part on promoting the expression of anti-inflammatory mediators and inhibiting the production of pro-inflammatory mediators.

DKK1^{low}-ERCs Increased β -catenin Expression in Colon and Spleen

To determine whether DKK1^{low}-ERCs have an influence on the activation of Wnt / β -catenin signaling, we measured the β -catenin level in colons and spleens, which is essential for this signaling transduction. As shown in Figure 6, the β -catenin protein level in colons and spleens were increased in unmodified ERC group when compared with that in untreated group ($p < 0.001$, Figure 6A; $p < 0.001$, Figure 6B). However, in DKK1^{low}-ERC group, the β -catenin level was further significantly raised ($p < 0.001$, Figure 6A; $p < 0.001$, Figure 6B; vs. unmodified ERC group), but in DKK1^{high}-ERC group, it declined apparently ($p < 0.05$, Figure 6A; $p < 0.01$, Figure 6B; vs. unmodified ERC group). In addition, when we analyzed the β -catenin mRNA expression changes in colons, it showed that the trend (Figure 6C) was consistent with the changes in protein level (Figure 6A). Taken together, it suggested that DKK1^{low}-ERCs, significantly increased the β -catenin expression, which exhibited anti-inflammatory effects in colitis, and thus eventually enhanced the therapeutic effects of ERCs in DSS-induced colitis.

Discussion

Wnt signaling antagonist DKK1, a secreted glycoprotein, expresses in many species, including human derived stromal cells and mouse colonic tissues[25, 45]. DKK1 possesses a conservative gene sequence and its expression in stromal cells can be down-regulated by IL-1 β or up-regulated by glucocorticoid stimulation[26]. Relying on competing with Wnt-ligands for LRP5/6 receptor, DKK1 can uniquely inhibit the Wnt/ β -catenin signaling transduction, which exhibits anti-inflammatory effects in the development of ulcer colitis[38]. Therefore, in present study, we hypothesized that ERCs, like other stromal cells, could also secrete DKK1, which blocks Wnt mediated anti-inflammatory signal transduction, and thus weaken

the immunoregulation of ERCs. Furthermore, we could optimize the therapeutic effect of ERCs on colitis by downregulating the expression of DKK1.

To carry out the current study, we firstly determined DKK1 levels in different groups, including unmodified ERC group, GC-treated ERC group and IL-1 β -treated ERC group. Our results showed that ERCs indeed secrete a considerable amount of DKK1, just like MSCs[46], and the expression of DKK1 could be markedly changed after ERCs stimulated by IL-1 β or glucocorticoid (GC), not only at the protein level but also at the level of RNA.

Next, we carried out the following experiments *in vivo* and found that in DKK1^{low}-ERC (ERC pretreated with IL-1 β) group, clinical characters and pathological manifestations of colitis were strikingly ameliorated, in which the immune-regulatory effects of ERCs may be enhanced by low DKK1 production, as a result of Wnt/ β -catenin pathway being activated[34, 47]. Comparing with that in DKK1^{high}-ERC (ERC pretreated with GC) group, we confirmed that DKK1 plays a vital role in affecting therapeutic function of ERCs on experimental colitis.

As we know, the balance between CD4⁺T cells (Th1/Th2, Th17 and Tregs) is essential for sustaining the intestinal homeostasis and closely involved in the development of UC[48]. In present study, we analyzed the CD4⁺T cell population changes in splenocytes by FACS, and found that, Th1 and Th17 populations in DKK1^{low}-ERC group, were the lowest among experimental groups, while the populations of Th2 and Tregs in DKK1^{low}-ERC group presented the highest. These data indicated that in the immune-regulatory function of ERCs, DKK1 participates in the immunoregulation effects on CD4⁺T cells.

Among immunocytes activated, DCs and macrophages, are also acting a role in the development of DSS-induced colitis[49]. DCs, the major part of antigen-presenting cells (APCs), exert its effects by MHC class II and CD86 expressed on their cytomembranes[50], promote the migration of immune cells into damaged colons, and then result in inducing T cell-mediated immune response. Macrophages, are illustrated with a diverse plasticity, and its differentiation can be driven by the surrounding settings[51]. Macrophages generally differentiated into two phenotypic subsets: M1 pro-inflammatory sub-type and M2 selectively activated anti-inflammatory sub-type[52]. M1 phenotype cells, which are known as pro-inflammatory macrophages, have the ability to generate tumor necrosis factor alpha (TNF- α), and nitric oxide (NO) to exacerbate the inflammation[53]. While, M2 phenotype cells exhibit immune-regulatory and anti-inflammatory effects by producing IL-10, which could promote tissue repairs by its negative immune regulation ability[54].

Thus, in the present study, we evaluated the proportion changes of macrophages and DCs in spleens. As the results shown, the proportion of mature DCs (CD11c⁺MHCII⁺ / CD11c⁺CD86⁺) in DKK1^{low}-ERC group was lower than that of other groups. Meanwhile, the proportion of M2 phenotype macrophages (CD68⁺CD206⁺) rised up to the highest. Taking together, these observations suggested that DKK1, secreted by ERCs, negatively affect the immunomodulatory effects of ERCs on macrophages and DCs. In

addition, when we downregulate the DKK1 expression of ERCs, this immunoregulatory mechanism tend to be active further and exert effective therapeutics in colitis.

Accumulating documents illustrated that inflammatory mediators in colons (IL-4, IL-10, SOD, IFN- γ , TNF- α , COX-2, MPO and iNOs) orchestrated the pathogenesis of ulcer colitis temporally and specially[55, 56]. IL-4, IL-10 and SOD are anti-inflammatory mediators with plenty of protective effects in colitis. IL-4 could assist in inducing T helper 2 cell (Th2) responses, inhibiting Th17 cell development and polarizing macrophages toward M2 phenotype[57-59]. IL-10 participates in suppressing the antigen presentations and the synthesis of pro-inflammatory cytokines in colitis[60]. SOD, is a critical antioxidant enzyme, which could catalyze the dismutation of toxic superoxides and scavenge superoxide radicals in colons[44]. On the contrary, IFN- γ , TNF- α , MPO, COX-2 and iNOs are pro-inflammatory factors which often strongly exacerbate the inflammations cascade in colitis[61-63]. ERCs, used for the immune-related diseases, were reported to have an ability to ameliorate the production and accumulation of inflammatory mediators, and thus to relieve the local tissue injury[19, 64]. But, whether down-regulating DKK1 expression in ERCs, could also modulate the imbalance of inflammatory mediator profiles, and exit a more provoking effect has not been illustrated till now. In our present study, as shown in results (Figure 5), we found a significant higher expression of anti-inflammatory mediators (IL-4, IL-10 and SOD) but lower of pro-inflammatory factors in DKK1^{low} ERC group, when compared with that in unmodified ERC group. Correspondingly, a lower expression of IL-4, IL-10 and SOD but high expression of pro-inflammatory mediators were witnessed in the DKK1^{high}-ERC group. Taking together, by analyzing inflammatory mediator changes, we believed that downregulating the DKK1 secretion of ERCs indeed improved inflammatory mediator profiles in the pathogenesis of UC.

Wnt/ β -catenin signaling was recorded with anti-inflammatory effects in the development of ulcer colitis[35]. As previous studies described, with the activating of canonical Wnt signaling, β -catenin would accumulate in cytoplasm and regulates the expressions of Wnt-related genes[65]. But, in absence of Wnt-ligand stimulus, cytosolic β -catenin will be degraded after rounds of ubiquitination and phosphorylation. Thus, we measured the β -catenin expressions, both at the protein level and the RNA level, to investigate the activity of canonical Wnt pathway. As shown in figure 6, β -catenin production was significantly increased in DKK1^{low}-ERC group, not only in colons but also in spleens. Intriguingly, high expression level of β -catenin was in accordance with the powerful immune regulation ability of DKK1^{low}-ERCs exhibiting in colitis. Thus, we concluded that reducing DKK1 expression in ERCs could weaken the antagonistic effect of DKK1 on Wnt signaling, and then the β -catenin accumulation could be promoted and Wnt β -catenin pathway would eventually be activated, so as to improve the immunoregulatory effect of ERCs and optimize their therapeutic effects in the process of ulcer colitis.

Conclusions

In summary, our present study demonstrated that DKK1^{low}-ERCs, obviously exert a more effective immune regulatory ability and better therapeutic effects in DSS-induced colitis. We confirmed DKK1^{low}-

ERCs significantly ameliorated the symptoms and pathological damages of DSS-induced colitis, and modulated the balance of immunocytes in spleens toward a tolerant status. DKK1^{low}-ERCs also apparently altered the inflammatory mediator profiles in colons, and thus, effectively suppressed the mucosal immune reaction and the detriments in colons. Taking together, these findings impressed us with an encouraging and viable method to significantly enhance the therapeutic effect of ERCs on ulcer colitis by downregulating the DKK1 secretion.

Abbreviations

DKK1: Dickkopf-1; ERCs: Endometrial regenerative cells; MSC: Mesenchymal stromal cells; PBS: Phosphate buffered saline; GC: Glucocorticoid; IL: Interleukin; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; DSS: Dextran sulfate sodium; DAI: Disease activity index; H&E: Hematoxylin and eosin; Th: Helper T cell; Tregs: Regulatory T cells; DCs: Dendritic cells; M2: Macrophage type 2 cells; APC: Antigen-presenting cells; FACS: Fluorescence activating cell sorter; IFN: Interferon; MHCII: Major histocompatibility complex-II; ELISA: Enzyme-linked immunosorbent assay; RT-PCR: Polymerase chain reaction; GADPH, Glyceraldehyde 3-phosphate dehydrogenase; TNF: Tumor necrosis factor; COX-2, Cyclooxygenase-2; MPO, Myeloperoxidase; iNOs: Inducible nitric oxide synthase enzyme; SOD: Superoxide dismutase; ANOVA: Analysis of variance; LSD: Least significant difference; SD, Standard deviation.

Declarations

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Authors' contributions

Dingding Yu, Yiming Zhao and Yonghao Hu are co-first authors on this paper. Dingding Yu conceived, designed and carried out the research, performed data analysis and interpretation, and drafted the manuscript. Yiming Zhao designed and carried out the research, performed data analysis, and drafted the manuscript. Yonghao Hu designed carried out the research, performed data analysis and paper revision.

Dejun Kong, Wang Jin, Yafei Qin, Baoren Zhang and Xiang Li performed the research and analyzed the data. Jingpeng Hao helped to review the data and the manuscript. Hongda Wang and Guangming Li contributed to experimental procedures. Hao Wang conceived and designed the study, provided financial and administrative support, helped in revising the manuscript and gave final approval of the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included within the article.

Ethics approval and consent to participate

All the experiments were fulfilled based on the protocols approved by the Animal Care and Use Committee of Tianjin Medical University (Tianjin, China), according to the Chinese Council on Animal Care guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

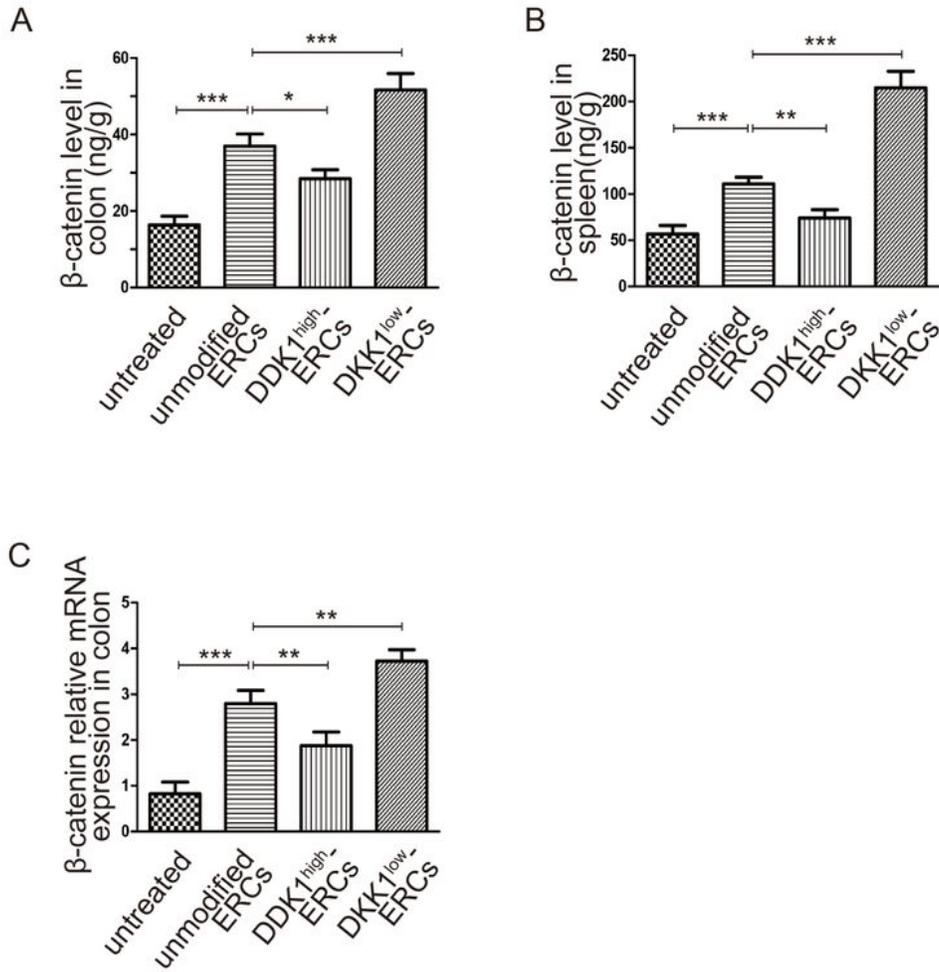


Figure 6

Figure 1

DDK1^{low}-ERCs increased β-catenin expression in colonic and splenic tissues. The protein level of β-catenin in colons (A) and spleens (B) were respectively detected. Furthermore, the mRNA expression (C) level of β-catenin in colon was also demonstrated. Data were presented as mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001). Statistical analysis was performed by one-way ANOVA followed by the LSD test, n = 6.

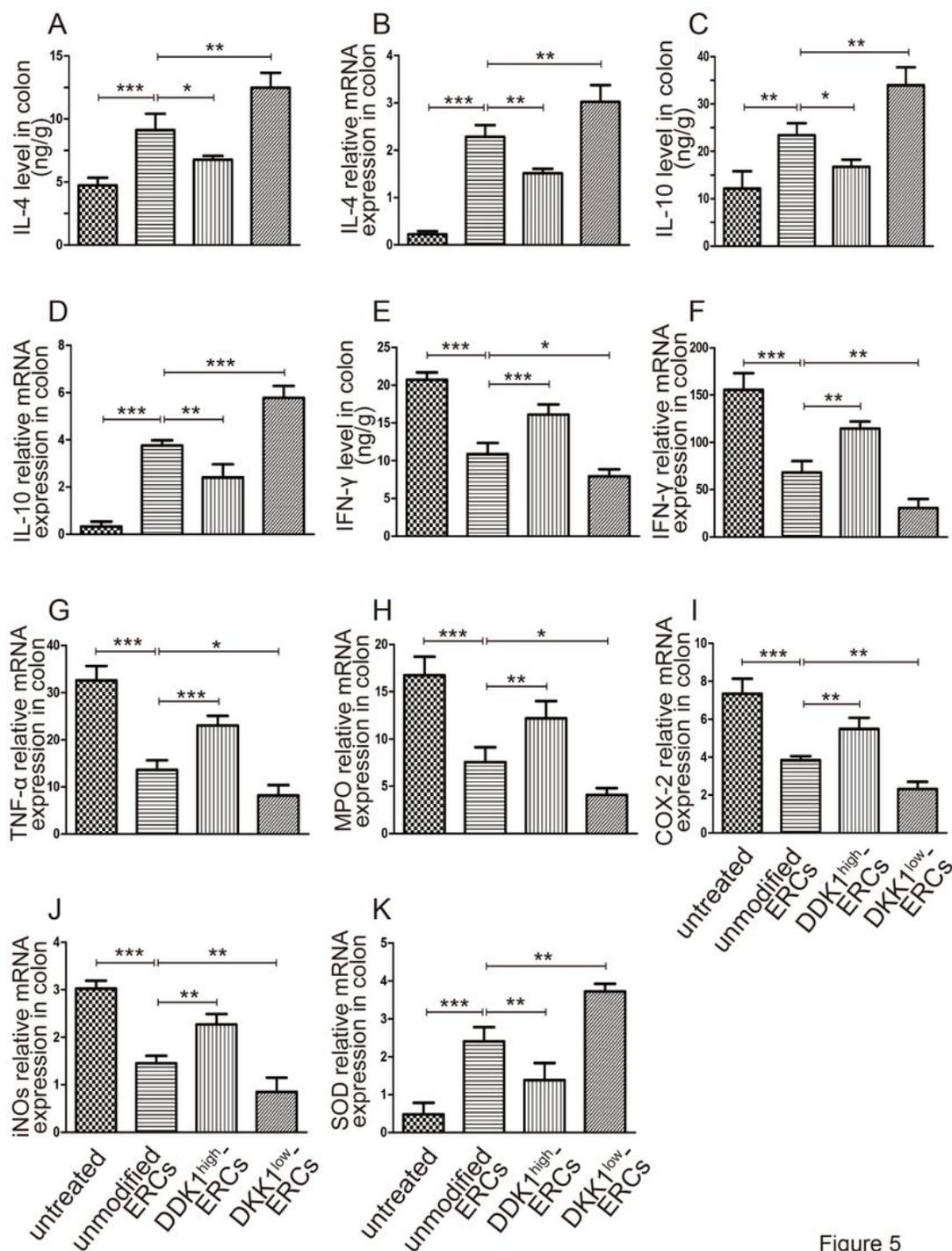


Figure 5

Figure 2

DDK1^{low}-ERCs altered the expressions of inflammatory mediators in colons. The concentrations of IL-4, IL-10 and IFN-γ in colonic tissues were determined by ELISA kit and the relative mRNA expression levels of inflammatory mediators were performed by Real-Time PCR. The expression of IL-4 (A-B), IL-10 (C-D), IFN-γ (E-F), TNF-α (G), MPO (H), COX-2 (I), iNOs (J) and SOD (K) were shown respectively. Data were

presented as mean \pm standard deviation, and P values were calculated by using one-way ANOVA followed by the least significant difference (LSD) test. (n=6, *p < 0.05, **p < 0.01, ***p < 0.001).

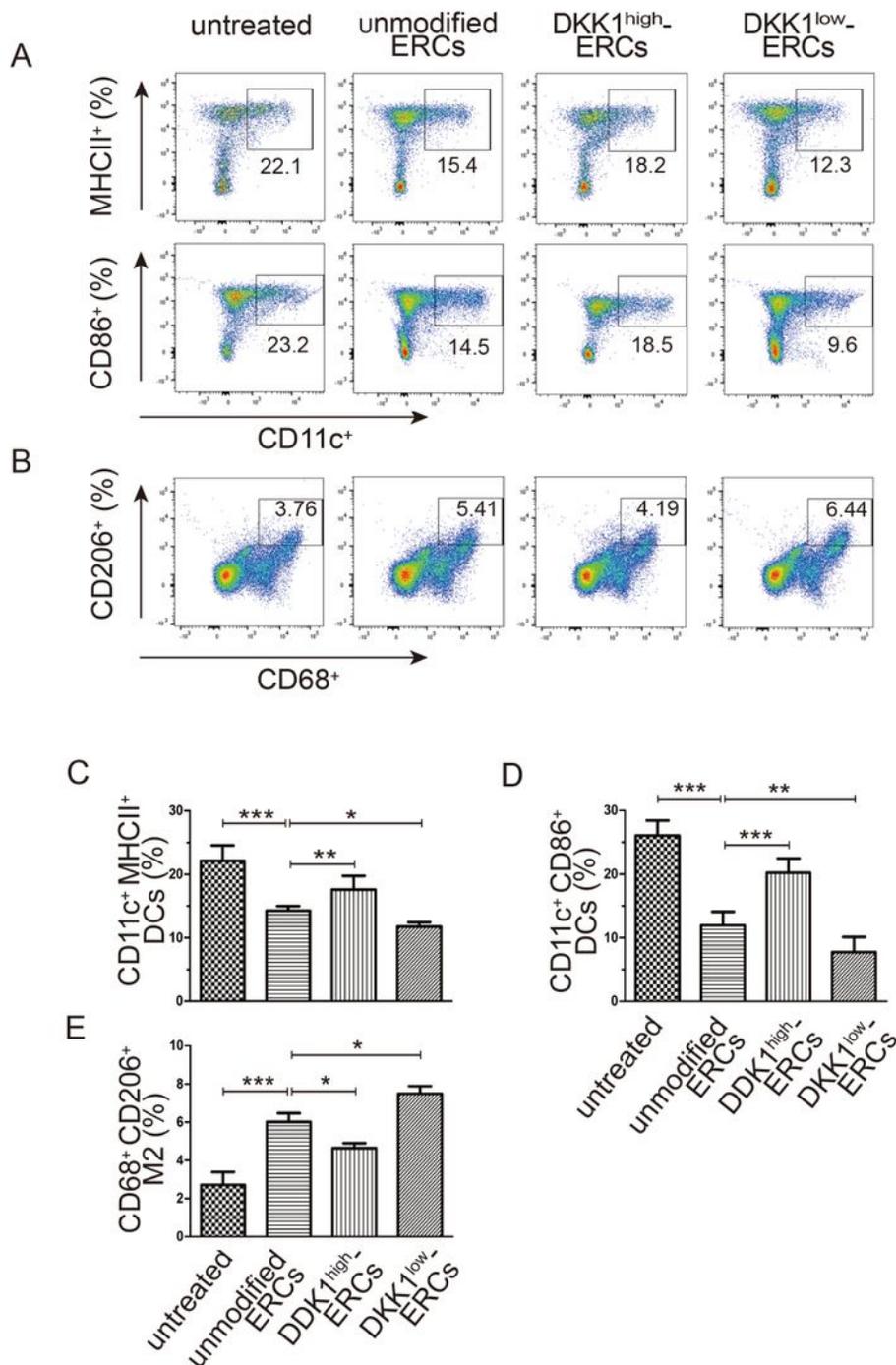


Figure 4

Figure 3

DKK1^{low}-ERCs reduced the population of mature DC, but increased M2 macrophages in colitis mice. To determine whether each treatment has an influence on regulating DC and macrophage phenotypes, anti-CD11c antibody and antigen presenting-related antibodies (anti-MHCII, anti-CD86) were used to measure

mature DCs, while anti-CD68 antibody and anti-CD206 antibody were used for M2 phenotype macrophages in spleens. (A) Representative dot plots of CD11c+MHCII+DCs and CD11c+CD86+DCs in spleens. (B) Dot plots of CD68+CD206+Macrophages. (C-E) Percentage of CD11c+MHCII+DCs, CD11c+CD86+DCs and CD68+CD206+Macrophages respectively. Data were mean \pm SD (n=6, *p < 0.05, **p < 0.01, ***p < 0.001). P values were analyzed by one-way ANOVA followed by the LSD test.

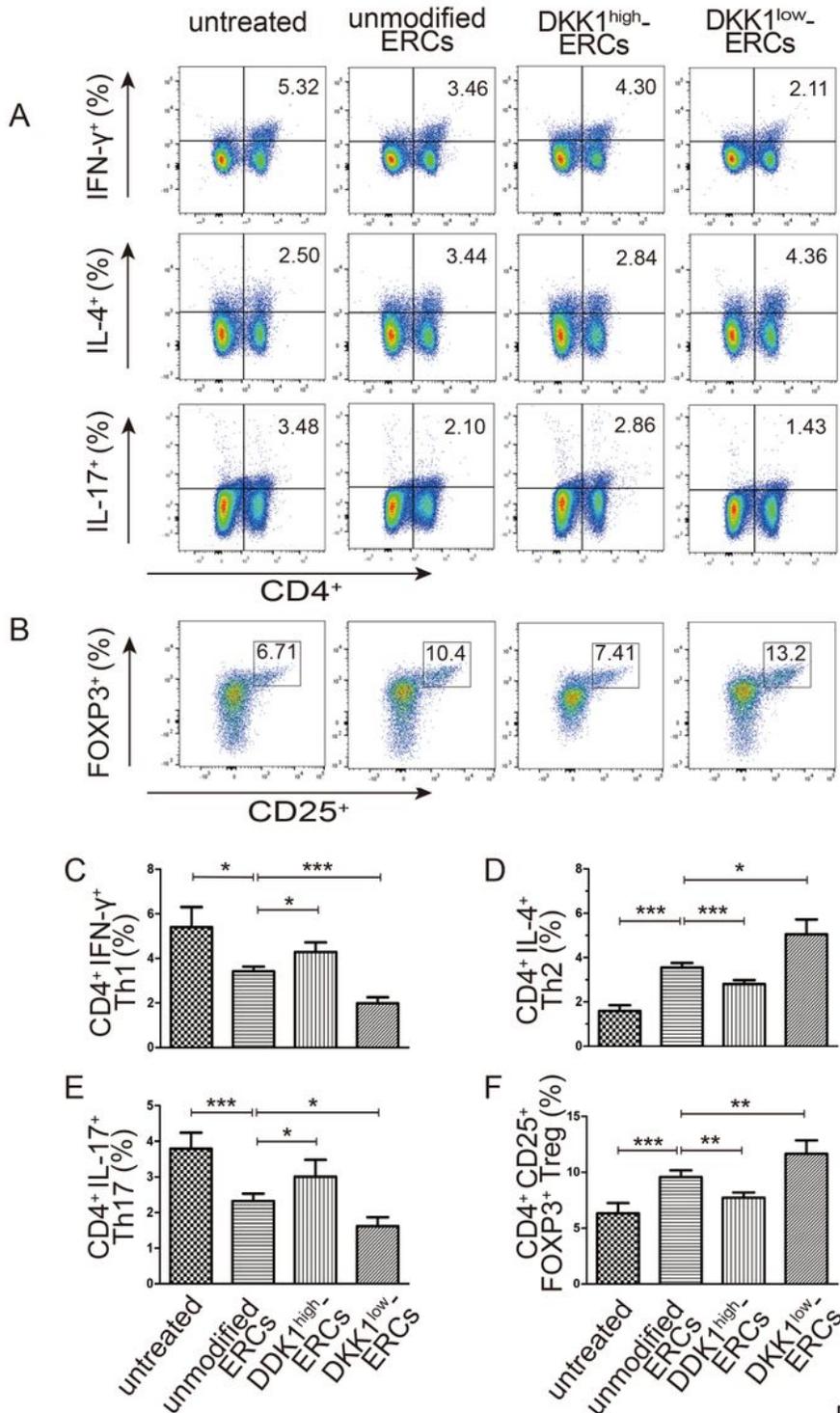


Figure 3

Figure 4

DKK1^{low}-ERCs reduced Th1 and Th17, but enhance Th2 and Treg populations in colitis mice. Splenocytes were collected on 10th day after DSS-induction. To accurately identify the subpopulation of Th1, Th2 and Th17 cells, splenocytes were firstly incubated with cell stimulation cocktail for 5 hours before stained with fluorescent antibodies. (A) Representative dot plots of CD4+IFN- γ +Th1, CD4+IL-4+Th2, CD4+IL-17+Th17 were shown while positive cells were counted from the quadrant Q2. (B) Dot plots of CD4+CD25+Foxp3+Tregs. (C) Percentage of CD4+IFN- γ +Th1. (D) Percentage of CD4+IL-4+Th2. (E) Percentage of CD4+IL-17+Th17. (F) Percentage of CD4+CD25+Foxp3+Tregs. Data were mean \pm SD (n = 6, *p < 0.05, **p < 0.01, ***p < 0.001). P values were calculated by one-way ANOVA followed by the LSD test.

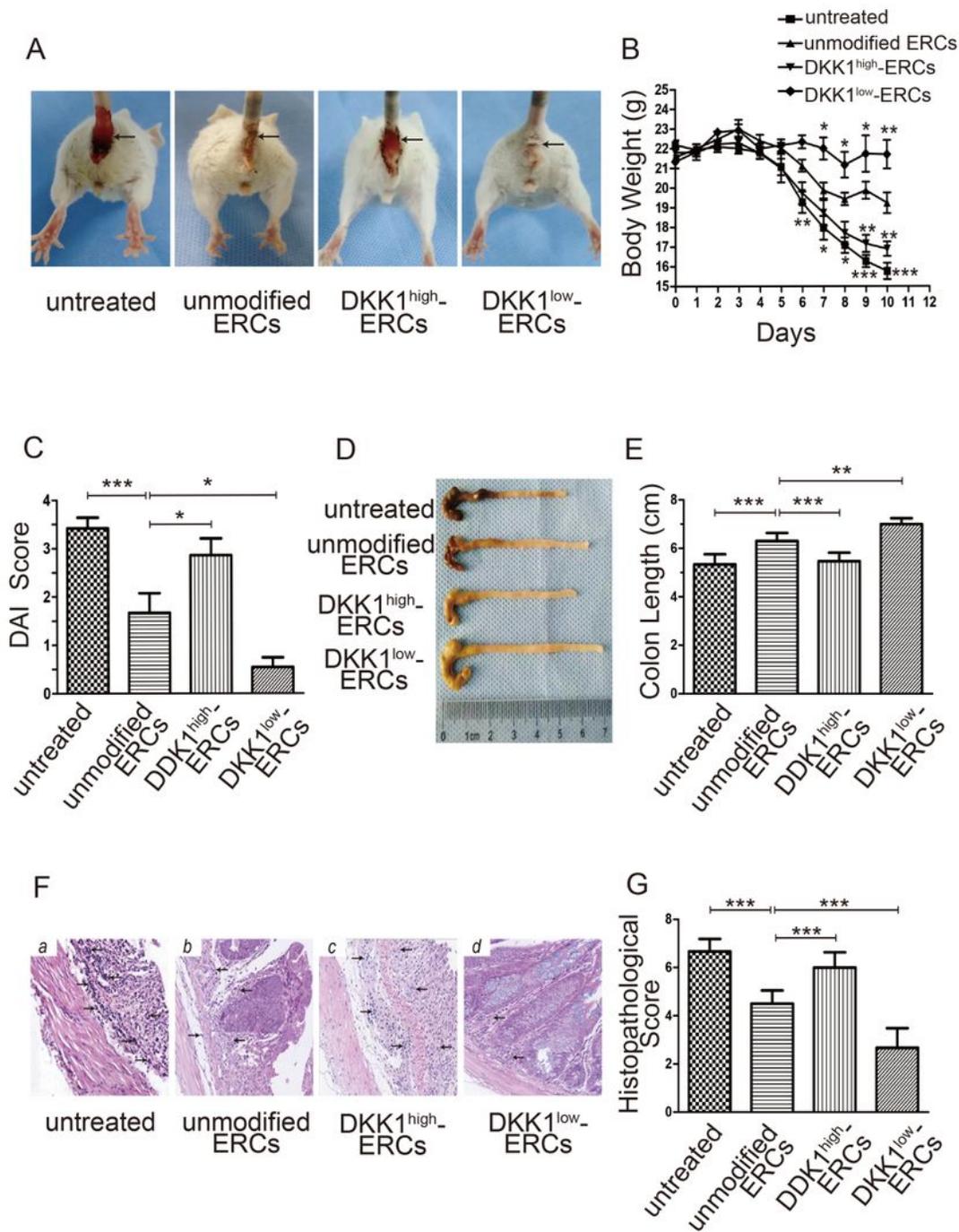


Figure 2

Figure 5

DKK1^{low}-ERCs alleviate the symptoms of DSS-induced colitis. (A) Representative pictures showing bloody stool were taken on 10th day after DSS-induction. The mice in DKK1^{low}-ERC group were in best condition than that in other groups. Body weight changes (B) and disease activity index (DAI) score (C) of each group mice were recorded daily. In DKK1^{low}-ERC group, the weight loss and DAI score were shown lesser than other groups. (D-E) The length of colon in each group were measured and analyzed on 10th

day (n=6). (F) Photomicrographs (200×, H&E staining) of representative histological sections of mouse colons in each group. Arrows indicated the inflammatory cells infiltration. (G) Histopathological scores were calculated according to the scoring system directed by Singh[42] to assess the colonic injury quantitatively. Data were presented as mean ± standard deviation (SD) (*p < 0.05, **p < 0.01, ***p < 0.001). Statistical analysis was calculated by using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test.

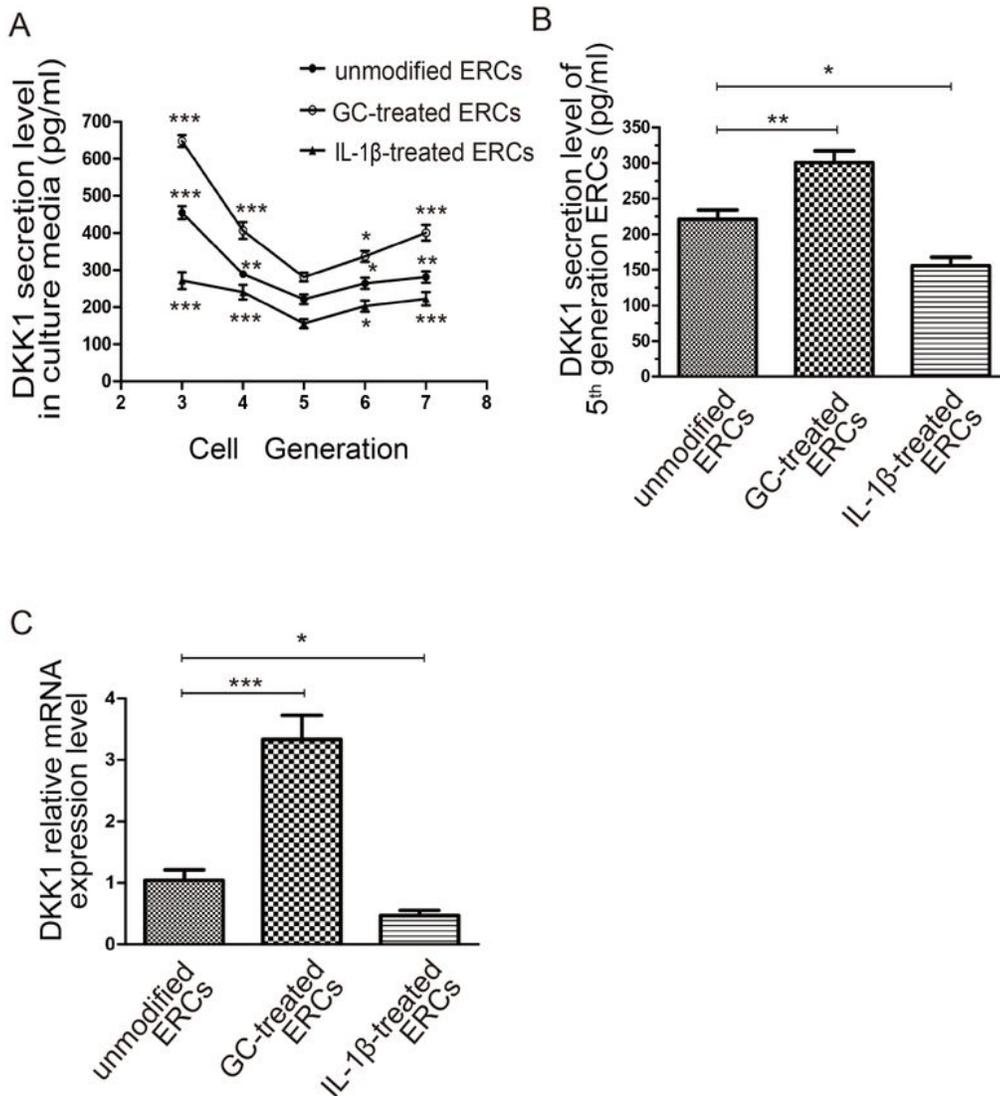


Figure 1

DKK1 was secreted by ERCs and can be decreased by IL-1 β or increased by GC. (A) The DKK1 level in supernatants of 3rd-7th generation were detected by ELISA kit. Each generation of cells was respectively treated with GC (100nM), IL-1 β (10ng/ml) or nothing for 48 hours (n=3). The 5th generation of ERCs were recorded with the lowest DKK1 expression when compared with other generations (vs. the 5th generation of ERCs, *p < 0.05, **p < 0.01, ***p < 0.001). (B) DKK1 level in 5th generation supernatants (n=3). (C) DKK1 mRNA expression in 5th generation of ERCs (n=3). Data were presented as mean \pm standard deviation (SD). Statistical analysis was calculated by using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test.