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, idiopathic, 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 limited to the mucosa and submucosa, which mainly affect the sigmoid colon and rectum, and can also affect the descending colon and even the

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 developing colorectal cancer [4].

Though the etiology of UC has not been defined clearly [5], accumulating evidences have revealed that UC was correlated with the inappropriate mucosal immune response toward luminal bacterial flora [6]. Till now, clinic treatments for UC mainly comprise four aspects: aminosalicylates, glucocorticoid (GC), immune-suppressants 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]. MSCs are exhibiting unique superiorities and potentials in treatment of ulcer colitis [16]. But, at the same time, the deficiencies of MSCs are gradually emerging, such as the invasive obtaining process, less availability, poor proliferation capacity and related complications [17]. These restrictions sharply limit its application as a clinical therapy.

Endometrial regenerative cells (ERCs), a new type of mesenchymal-like stromal cells, were firstly isolated from human menstrual blood by Meng *et al* in 2007 [18]. ERCs were demonstrated to possess the similar phenotypic markers with MSCs, but surmount the limits of traditional MSCs and with more outstanding advantages, including diverse differentiation potentials, immunomodulatory characteristics, non-invasive obtaining process and high proliferative capacity without karyotypic abnormality [19]. We and others have previously reported the forcible therapeutic effects of ERCs 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 [20–25]. Moreover, there was no serious immunological rejections emerging against xeno-ERCs in these ERC-treated animal models [22]. Therefore, ERCs are being gained more and more attention in treatment of immune-disordered diseases, and its beneficial efficacy is being recognized by more and more researchers.

Dickkopf-1 (DKK1), which can be secreted by MSCs [26], is a kind of secreted glycoprotein. DKK1 has been recognized as the special inhibitor of Wnt/ β -catenin signaling pathway [27], relying on competing with Wnt ligands for receptors and inducing the endocytosis of binding DKK1-receptor complex [28, 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

this pathway, β -catenin is a central component and its existence is essential for the activation of Wnt/ β -catenin 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 properties in various immune-related inflammations and auto-immune diseases, comprising cancer, rheumatoid arthritis, enteritis and so on [34–36]. Wnt/ β -catenin pathway is playing a major role in regulating the development and differentiation of immunocytes [30] and also exhibiting encouraging and promising anti-inflammatory effects in chronic disease, such as inflammatory bowel disease [35–37].

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 20–24 g (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 all 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

Human ERCs were isolated and cultured from menstrual blood which was collected from healthy women (20–30 years old) with a sterilized menstrual cup on the first day of menstruation, in accord with the ethical approval from Tianjin Medical University (Tianjin, China). After about 2 weeks, cells expanded to 80–90% area of dishes and displayed a spindle-shaped morphology. Then we split these cells and passaged down as the rate of 1:3.

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 2.5 ml culture media. In an attempt to alter DKK1 secretion, we added glucocorticoid (GC, 100 nM, as the stimulator) or IL-1 β (10 ng/ml, as the inhibitor) [38] 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 (100 nM) or IL-1 β (10 ng/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 [17]. 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. 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 accord to the scoring system directed by Murthy *et al* [39].

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 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; another 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 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 [40]: (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 PBS. Then we lysed the erythrocytes with red blood cell lysis buffer, flushed the splenocytes twice and resuspended splenocytes 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). 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, Beijing, China). Supernatants in culture media of ERCs were collected and prepared for measuring the DKK1 secretion level. Homogenates of intestinal tissues in each group were gathered 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.). The pureness and concentration of RNA were determined with an UV spectrophotometer at the spectrum of 260 and 280 nm. cDNA was generated from the obtained RNA by using a Fastquant RT kit (KR106, Tiangen Biotech Co. Ltd). Real-time quantitative PCR (RT-PCR) was carried out by using SuperReal Color Premix kit (FP216, Tiangen Biotech Co. Ltd.), according to the recommended protocol. The primer sequences involved were designed as follows:

human-GADPH: forward, 5'-ACAACTTTGGTATCGTGGAAGG-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'-GAGTAGCTGCAGGGGTCCTC-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'-CGGCTGCTTGAAGTAAACAGG-3';

mouse iNOs: forward, 5'-GTTCTCAGCCCAACAATACAAGA-3',

reverse, 5'-GTGGACGGGTCGATGTCAC-3';

mouse SOD: forward, 5'-CAGACCTGCCTTACGACTATGG-3',

reverse, 5'-CTCGGTGGCGTTGAGATTGTT-3'.

Each sample was performed in triplicates on MJ Research DNA Engine Opticon 2 PCR cyclers (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 was measured by ELISA and its concentration was shown significantly different in each generation of ERCs. Among which, the 5th generation of ERCs were recorded with the lowest DKK1 expression when compared with other generation cells (Fig. 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 groups, 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 Fig. 1B and 1C, the DKK1 expression was strikingly decreased in IL-1 β -treated ERC group (unmodified ERCs vs. IL-1 β -treated ERCs: $p < 0.05$, Fig. 1B; $p < 0.05$, Fig. 1C), but when ERCs were pre-treated with glucocorticoid (GC), DKK1 expression boosted up (unmodified ERCs vs. GC-treated ERCs: $p < 0.01$, Fig. 1B; $p < 0.001$, Fig. 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 induction with DSS, all mice exhibited colitis with bloody stool, weight loss and lethargy before ERCs administration. Following the treatment of unmodified ERCs, bloody stool (Fig. 2A) and body weight loss were found with moderate relieve and improvement (Fig. 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 (Fig. 2A; Fig. 2B, vs. unmodified ERCs, $p < 0.01$), while diminished when treated with DKK1^{high}-ERCs (Fig. 2A; Fig. 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 Fig. 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 Treatment Reduced 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 Figs. 2D and 2E. We found that the average length of colons in untreated group is 5.3 cm ($n = 6$), 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 (6.3 cm; Fig. 2E, DKK1^{low}-ERC group vs. unmodified ERC group, $p < 0.01$) and the length in DKK1^{high}-ERC group is

5.5 cm, lower than the unmodified ERC group (Fig. 2E, DKK1^{high}-ERC group vs. unmodified ERC group, $p < 0.001$), it 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 (Fig. 2F-d), the pathological condition of colon was strikingly better, which showed 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 (Fig. 2G, $p < 0.001$), and unmodified ERC group was lower than the DKK1^{high}-ERC group (Fig. 2G, $p < 0.001$). Given together, these results indicate that DKK1^{low}-ERCs could exhibited therapeutic effects in relieving histopathological damages in DSS-induced colitis, and ERCs induced alleviating role is, at least in part, mediated by DKK1 low expression.

DKK1^{low}-ERCs Reduced Th1 and Th17, but Enhanced Th2 and Treg Populations 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 Fig. 3A and 3B, we analyzed the proportion of CD4⁺IFN- γ ⁺Th1, CD4⁺IL-4⁺Th2, CD4⁺IL17⁺Th17 and CD4⁺CD25⁺FoxP3⁺Treg cells respectively. The statistical results in Fig. 3C-3F showed that the percentage of CD4⁺IFN- γ ⁺Th1 and CD4⁺IL17⁺Th17 cells were significantly decreased in unmodified ERC group when compared with that in untreated group (Fig. 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 mature DCs in different groups, splenocytes were stained with anti-CD11c antibody and antigen presenting-related antibodies (MHCII, CD86) for further analysis. As indicated in Fig. 4, the two population of mature DCs (CD11c⁺MHCII⁺, CD11⁺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⁺, $p < 0.05$; CD11⁺CD86⁺, $p < 0.01$). But, in DKK1^{high}-ERC group, these two population of mature DCs were raised (DKK1^{high}-ERC group vs. unmodified ERC group: CD11c⁺MHCII⁺, $p < 0.01$; CD11⁺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

To determine whether each treatment has an influence in regulating macrophage phenotype in colitis mice, anti-CD68 antibody and anti-CD206 antibody were used to measure macrophage type 2 cell (M2) phenotype in spleens. Compared with that in untreated group, M2 population was obviously raised in unmodified ERC group (Fig. 4B and 4E: untreated group *vs.* unmodified ERC group, $p < 0.001$). Moreover, the percentage 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 mediates ERC-based therapy in regulating macrophage phenotypes and promote immunosuppressive M2 phenotype cell increase, which would help diminish the injury from acute immune response in colons.

DKK1^{low}-ERCs Altered the Expressions of Cytokine Profiles in Colons

In an attempt to address the effects of DKK1 on ERC-mediated immunoregulation, we measured different cytokine productions in colons which are associated with the development of UC. As showed in Fig. 5, it 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: Fig. 5A, $p < 0.001$; Fig. 5B, $p < 0.001$; Fig. 5C, $p < 0.01$; Fig. 5D, $p < 0.001$). While, in DKK1^{low}-ERC group, the levels of IL-4 and IL-10 were raised (DKK1^{low}-ERC group *vs.* unmodified ERC group: Fig. 5A, $p < 0.01$; Fig. 5B, $p < 0.01$; Fig. 5C, $p < 0.01$; Fig. 5D, $p < 0.001$), but plunged apparently in DKK1^{high}-ERC group (DKK1^{high}-ERC group *vs.* unmodified ERC group: Fig. 5A, $p < 0.05$; Fig. 5B, $p < 0.01$; Fig. 5C, $p < 0.05$; Fig. 5D, $p < 0.01$). Moreover, the IFN- γ level in colonic tissues was reduced in unmodified ERC group (Fig. 5E, $p < 0.001$; Fig. 5F, $p < 0.001$; *vs.* untreated group), and significantly decreased in DKK1^{low}-ERC group (Fig. 5E, $p < 0.05$; Fig. 5F, $p < 0.01$; *vs.* unmodified ERC group), but increased in DKK1^{high}-ERC group (Fig. 5E, $p < 0.001$; Fig. 5F, $p < 0.01$; *vs.* unmodified ERC group). In all, these data indicate that down-regulating the DKK1 secretion of ERCs, could regulate the inflammation profiles of IL-4, IL-10 and IFN- γ in colons, which thus contribute to a better therapeutic effect in DSS-induced colitis.

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

To further verify the inflammatory mediator changes in colons, we compared the mRNA expression levels of the related factors. As shown in Fig. 6, the data revealed that pro-inflammatory mediators in colons, including TNF- α , MPO, COX-2 and iNOs, 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, $p < 0.01$). Moreover, it was also 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, it indicated that DKK1^{low}-ERCs strikingly polished up the therapeutic effects on colitis and these effects might rely on promoting anti-inflammatory factors expression and inhibiting pro-inflammatory production.

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 Fig. 7, the β -catenin protein level in colons and spleens were increased in unmodified ERC group when compared with that in untreated group ($p < 0.001$, Fig. 7A; $p < 0.001$, Fig. 7B). However, in DKK1^{low}-ERC group, the β -catenin level was further significantly raised ($p < 0.001$, Fig. 7A; $p < 0.001$, Fig. 7B; vs. unmodified ERC group), but in DKK1^{high}-ERC group, it declined apparently ($p < 0.05$, Fig. 7A; $p < 0.01$, Fig. 7B; vs. unmodified ERC group). In addition, when we analyzed the β -catenin mRNA expression changes in colons, it showed that the trend (Fig. 7C) was consistent with the changes in protein level (Fig. 7A). Taken together, it suggested that DKK1^{low}-ERCs, eventually increased the β -catenin expression, which exhibited anti-inflammatory effects in colitis, and thus reducing DKK1 expression could enhance 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 [26, 41]. DKK1 possesses a conservative gene sequence⁴¹ and its expression in stromal cells can be down-regulated by IL-1 β or up-regulated by glucocorticoid stimulation [38]. Relying on competing with Wnt-ligands for receptors, DKK1 can uniquely inhibit the Wnt/ β -catenin signaling transduction, which exhibits anti-inflammatory effects in the development of ulcer colitis [42]. 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 weakening 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 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 MSCs²⁶, and the expression in different groups 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 revealed that in DKK1^{low}-ERC 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, 43]. Comparing with that in DKK1^{high}-ERC (pretreated with GC) group, we confirmed that DKK1 really plays a vital role in affecting therapeutic function of ERCs on experimental colitis and this action may be associated with the activation of Wnt/ β -catenin pathway.

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 [44]. 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 (ERCs treated with IL-1 β) 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 DKK1, also participates in the immune-regulatory function of ERCs, and it can affect the immunosuppressive effects of ERCs on CD4⁺T cells.

Among immunocytes activated, macrophages and DC cells, were also revealed acting a role in the development of DSS-induced colitis [45]. Macrophages, were illustrated with a diverse plasticity, and its differentiation can be driven by the surrounding settings [46]. M1 phenotype cells, which are known as pro-inflammatory macrophages, have the ability to generate interleukin (IL)-1 β , tumor necrosis factor alpha (TNF- α), and nitric oxide (NO) to exacerbate the inflammation [47]. 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 [48]. DCs, the major part of antigen-presenting cells (APCs), can exert its effects by costimulatory molecules (MHC class II and CD86) expressed on their cytomembranes [49], promoting the migration of immune cells into damaged colons, and then result in inducing T cell-mediated immune response.

Thus, in the present study, we evaluated the population changes of macrophages and DCs of splenocytes. The results showed that the number of mature DCs (CD11c⁺MHCII⁺ or CD11c⁺CD86⁺) in DKK1^{low}-ERC group was significantly lower than that of other groups. Meanwhile, the population of M2 phenotype macrophages (CD68⁺CD206⁺) rised up to the highest level among those of experimental groups. 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 and exert effective therapeutic roles in DSS-induced colitis.

Accumulating documents have illustrated that inflammatory mediators in colons, such as IL-4, IL-10, SOD, IFN- γ , TNF- α , COX-2, MPO and iNOs, which were produced by infiltrating immune cells, macrophages and epithelial cells, orchestrated the pathogenesis of ulcer colitis temporally and specially [50, 51]. The

changed inflammatory mediator profiles, induced by administration of DSS solution, contribute to promoting the immunocyte proliferations or differentiations, disrupting the balance between the immune system and antigens, and thus ultimately render the inflammatory cascade and the destruction in the colonic tissues [52, 53]. ERCs, injected into UC paradigms, were reported to have an ability to ameliorate production and accumulation of inflammatory mediators in colons, and thus polish up the immunomodulatory properties in relieving the local tissue injury [20]. But, whether down-regulating DKK1 expression in ERCs, could also modulate the imbalance of inflammatory mediator profiles, and exit a much more provoking effect has not been illustrated till now. Therefore, in our present study, we determined different inflammatory mediators in colonic tissues and try to address the relationship between DKK1^{low}-ERC treatment and inflammatory mediator productions.

As shown in results (Fig. 5), we found a significant higher expression of IL-4 and IL-10 in DKK1^{low} ERC group, both at the protein and RNA level. Correspondingly, a lower expression of IL-4 and IL-10 in the DKK1^{high}-ERC group was witnessed, compared with that in unmodified ERC group. These changes indicated that DKK1, secreted by ERCs, could negatively modulate IL-4 and IL-10 profiles in colons. As known, IL-4 is an anti-inflammatory cytokine which was illustrated with plenty of protective effects in colons, through the IL-4/signal transducer and activator of transcription 6 (Stat6) signaling pathway [54]. The increased IL-4 assists in inducing T helper 2 cell (Th2) responses, inhibiting Th17 cell development and polarizing macrophages toward M2 phenotype [54–56], and thus prompts its ability in maintaining the mucosal surface integrity, modulating intestinal immune responses and harboring the goblet cell functions [57]. Similar with IL-4, IL-10 was also reported to act as an anti-inflammatory factor in the pathogenesis of UC [58]. The expression of IL-10 was reported with the ability in suppressing the antigen presentations and the synthesis of pro-inflammatory cytokines. Meanwhile, its high expression could also inhibit T cell activation and function [53], and thus attenuate UC development.

IFN- γ , another pro-inflammatory factor, could exacerbate the inflammations cascade in colitis, which was enhanced by DSS administration and decreased by ERC treatment [59]. IFN- γ has been recorded that it could cause intestinal barrier impairments and detriments in colons via pathological T cell effects [60]. In our current study, we determined that the expression of IFN- γ was markedly decreased in DKK1^{low}-ERC group, while raised in DKK1^{high}-ERC group (compared with that in unmodified ERC group). It suggested that DKK1 could contribute to the ERC-mediated immune regulation by reducing the production of IFN- γ in colons.

In addition, we also measure the relevant inflammatory cytokines and inducible enzymes at the transcriptional level, which reflected the degree of local tissue inflammation. TNF- α , mainly generated by macrophages and neutrophils, is reported with overexpression in UC patients' colons and correlated with the intensity and severity of mucosal inflammation [61]. MPO is a significant peroxidase produced by neutrophils, and recognized as a criterion to reflect the activations of neutrophils. MPO activity could indicate the activity of inflammatory bowel disease (IBD), acclaimed by Chadwick *et al* [62]. COX-2 and iNOs, which are inducible enzymes and produced by macrophages and epithelial cells, were observed

accumulating in inflammation sites and involved in UC pathogenesis [63]. In the present study, we witnessed there was a lower mRNA expression of these factors (TNF- α , IFN- γ , MPO, COX-2 and iNOs) in DKK1^{low}-ERC group. While, when we exerted the DKK1^{high}-ERC treatment, these pro-inflammatory mediators tended to be elevated straightly.

In addition, we also tested the superoxide dismutases (SOD) mRNA expression in different groups. SOD, which is regarded as a critical antioxidant enzyme, could catalyze the dismutation of toxic superoxides and scavenge superoxide radicals [64]. In our present study, we found that in DKK1^{low}-ERC group, the SOD level presented the highest level when compared with other groups. Taking together, by analyzing inflammatory cytokines and inducible enzymes changes, we believed that downregulating the DKK1 secretion of ERCs indeed alleviated inflammatory mediator expressions 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 ultimately 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 Fig. 7, 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 Wnt β -catenin pathway could be activated and β -catenin expression would be promoted, 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 that downregulating the DKK1 secretion of ERCs could significantly enhance their therapeutic effects in the treatment of ulcer colitis.

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 1

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.

Figure 2

Figure 2

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 [40] 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 3

Figure 3

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 4

Figure 4

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 5

Figure 5

DDK1^{low}-ERCs altered the expressions of cytokine profiles in colons. The concentrations of IL-4, IL-10 and IFN-γ in colonic tissues were determined by ELISA kit and RT-PCR. The protein concentration and mRNA expression of IL-4 (A-B), IL-10 (C-D) and IFN-γ (E-F) were shown respectively. Data were presented as mean ± standard deviation (n=6, *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 6

Figure 6

DKK1^{low}-ERCs altered mRNA expressions of inflammatory mediators in colons. The relative mRNA expression levels of inflammatory mediators were performed by Real-Time PCR. (A) TNF- α , (B) MPO, (C) COX-2, (D) iNOs and (E) SOD mRNA expression level. Data are mean $\pm$ SD of three separate experiments

(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 7

Figure 7

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 $\pm$ SD (*p < 0.05, **p <

0.01, *** $p < 0.001$). Statistical analysis was performed by one-way ANOVA followed by the LSD test, $n = 6$.