**ORIGINAL ARTICLE**

**Exosomes derived from interleukin-10-treated dendritic cells can inhibit trinitrobenzene sulfonic acid-induced rat colitis**

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**Abstract**

**Objective.** Inflammatory bowel disease (IBD), which mainly refers to Crohn’s disease and ulcerative colitis, is characterized by chronic inflammation of the gastrointestinal tract. Recent reports have demonstrated that exosomes derived from interleukin-10 (IL-10)-treated bone marrow-derived dendritic cells (DCs) can reduce the incidence and severity of established collagen-induced arthritis (CIA) in mice. Based on the essential role of IL-10 in the development of normal mucosal immunity, we investigated whether exosomes derived from DCs treated with IL-10 (known as IL-10-exosomes) can suppress the trinitrobenzene sulfonic acid (TNBS)-induced colitis. **Material and Methods.** We used the rat TNBS-induced colitis model to address the therapeutic potential of IL-10-exosomes in vivo. More specifically, a rectal enema of TNBS was administered to Wistar rats, and IL-10-exosomes were injected intraperitoneally on Day 3. **Results.** In the context of a high level of major histocompatibility complex class II (MHC II) and a low level of co-stimulatory molecule and membrane-bound IL-10 expression, IL-10-exosomes treatment substantially reduced all analyzed clinical, macroscopic, and histopathologic parameters of TNBS-induced colitis. The therapeutic effects of IL-10-exosomes were associated with a down-regulation mRNA expression of IL-2, IFN-γ and TNF-α in colon tissues. Importantly, treatment with IL-10-exosomes resulted in a pronounced up-regulation of IL-10mRNA expression in colon tissues and regulatory T cells (Tregs) in Colonic lamina propria. **Conclusions.** The results suggest that IL-10-exosomes treatment can suppress acute TNBS-induced colitis and may offer a promising new therapeutic strategy for IBD.

**Key Words:** Dendritic cell, exosomes, inflammatory bowel disease, interleukin-10

**Introduction**

Interleukin-10 (IL-10), a cytokine with anti-inflammatory properties, has a crucial role in preventing inflammatory and autoimmune pathologies [1]. Using gene transfer or injection of recombinant protein, IL-10 has been shown to be therapeutic in different murine models of autoimmune disease, including type I diabetes, collagen-induced arthritis (CIA), and murine models of inflammatory bowel disease (IBD). However, if treatment with the IL-10 is terminated, then these diseases progress and severity of symptoms return. Thus, there is still need for an effective therapy which is able to reverse the course of these autoimmune disease progression with a single or infrequent treatment [2,3].

Exosomes are small membrane vesicles (40–100 nm in diameter) that are secreted in the extracellular medium by many hemopoietic cells as well certain non-hemopoietic cells [4]. Exosomes derived from dendritic cells (DCs) carry major histocompatibility complex classes I (MHC I) and II (MHC II) and T-cell-costimulatory molecules on their surface, suggesting that they play important roles in immune regulation [5]. Depending on the type and stage of maturation of the DCs, DC-derived exosomes have been shown to be immunostimulatory or suppressive. Mature DC-derived exosomes carrying tumor
antigens have been used to effectively induce anti-tumor immunity in mice and in human Phase-I clinical trials [6,7]. Meanwhile, immature DC-derived exosomes exhibit immunosuppressive ability and are involved in T-cell immunosuppression to induce peripheral tolerance [8].

IBD, which mainly refers to Crohn’s disease and ulcerative colitis, is characterized by chronic inflammation of the gastrointestinal tract [9]. Although the etiology of these diseases remains unknown, inappropriate and sustained activation of the central and peripheral immune systems seem crucial to IBD development [10]. Colonic inflammation, induced by intrarectal administration of a hapten, trinitrobenzene sulfonic acid (TNBS), is Th1 cell-mediated and used widely as a human IBD model. Moreover, idiointercolitis develops in IL-10-deficient (IL-10-/-) mice between 7 and 11 weeks of age, accompanied by anemia and weight loss [11]. Recently, it was demonstrated that exosomes derived from DCs treated with IL-10 or transfected with the gene for IL-10 can suppress CIA [12]. Due to the essential role of IL-10 in the development of normal mucosal immunity, we investigated whether exosomes derived from IL-10-treated DCs (also known as IL-10-exosomes) can suppress TNBS-induced colitis.

Methods

Animals

Inbred male Wistar rats, aged 12–14 weeks and weighing 180–200 g, were purchased from the Experimental Animal Center of the Shanghai Institute for Biological Sciences. The animals were maintained under SPF conditions and had free access to standard chow and tap water.

DC generation

We used the methods described by Beriou et al. [13] to generate immature bone marrow-derived DCs (BMDCs) from bone marrow progenitor cells. Briefly, bone marrow cells were collected from rat tibias and femurs. The cells then were cultured for 24 h in complete medium [14] to remove adherent macrophages. For exosome production, the complete medium was depleted of contaminating vesicles and protein aggregates by overnight centrifugation at 110,000 g. Then non-adherent cells were placed in fresh growth medium, consisting of complete medium containing 5 ng/mL recombinant rat granulocyte macrophage colony-stimulating factor (rGM-CSF; Peprotech). Cells were then cultured for five days, followed by harvesting and incubation of 1 × 10^6 DCs in 10 ng/mL of recombinant rat IL-10 (rIL-10; Peprotech) in fresh growth medium. After incubation for two days, the culture supernatant was collected for exosome purification.

Exosome purification and FACS analysis

Exosomes were prepared from the culture supernatant of the DCs by differential centrifugation, as described by Kim et al. [14]. Briefly, the culture supernatant was subjected to three successive centrifugations at 300 × g (5 min), 1200 × g (20 min), and 10,000 × g (30 min) to eliminate cells and debris, followed by centrifugation for 1 h at 100,000 × g. To remove excess serum proteins, the exosome pellet was washed with a large volume of phosphate buffered saline (PBS), centrifuged at 100,000 × g for 1 h, and resuspended in 120 µL of PBS for further studies. Exosome batch protein content was quantified and standardized using a micro Bradford protein assay (Bio-Rad). For fluorescence-activated cell sorting (FACS) analysis, 30 µg of exosomes were incubated with 10 µL of 4-µm-diameter aldehyde/sulfate latex beads (Interfacial Dynamics) for 15 min at room temperature in a final volume of 100 µL, followed by 2 h of gentle shaking in 1 mL of PBS. The exosome-coated beads were washed three times in FACS wash (Becton Dickinson) and resuspended in 500 µL of FACS wash. Then, 10 µL of coated beads and DCs were incubated for 1 h with PE-coupled anti-IL-10 (Caltag), PE-coupled anti-MHC II, PE-coupled anti-CD86, PE-coupled anti-CD80, or PE-coupled anti-CD40 (eBioscience), followed by washing and analysis using a FACS Calibur (Becton Dickinson).

Induction of experimental TNBS-colitis and treatment

The haptenating agent TNBS (Sigma-Aldrich) was used at a concentration of 2% in 45% ethanol. TNBS was administered at 100 mg/kg (body weight (BW)) to slightly anesthetized rats through a 3.5-F catheter carefully placed into the rectum. The catheter tip was inserted 8 cm proximal to the anal verge. To ensure proper distribution of TNBS throughout the entire colon, rats were maintained in a vertical position for 1 min after instillation of the TNBS enema. Control animals (n = 10) received 45% ethanol alone using the same technique. We implemented the methods and dosage described by Kim et al. and Duchmann et al. [14,15] to treat colitis in the rats. Three days later, TNBS-treated rats were assigned to three groups, according to the immunosuppressive regimen: IL-10-exosome-treated rats (n = 10) received
an intraperitoneal dose of 10 μg of IL-10-exosomes in a volume of 1 mL. Exosome-treated rats (n = 10) received an intraperitoneal dose of 10 μg of exosomes from immature DCs in a volume of 1 mL. Untreated rats (n = 10) received an intraperitoneal saline in a volume of 1 mL. All rats were killed on Day 8 post-TNBS administration.

**Assessment of inflammation and colitis severity**

**Clinical activity score of colitis.** To assess the clinical severity of the induced colitis, BW and stool consistency were recorded daily using a scoring system described previously [16]. Briefly, the loss of BW was scored as follows: 0, no weight loss; 1, weight loss of 1–5%; 2, weight loss of 5–10%; 3, weight loss of 10–20%; and 4, weight loss of >20%. Stool was assessed using the following scale: 0, normally formed pellets; 2, pasty and semi-formed pellets; and 4, liquid stool. The values were then added, resulting in a total clinical score ranging from 0 (healthy) to 12 (maximal illness/colitis activity).

**Macroscopic scoring system.** Assessment of macroscopic colon damage was performed using the scoring system of Wallace and Keenan [17]. The criteria for evaluating macroscopic damage were based on a semi-quantitative scoring system. Features were graded as follows: 0, no ulcer or inflammation; 1, no ulcer and local hyperemia; 2, ulceration without hyperemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; and 5, ulceration extending >2 cm.

**Histological analysis of the colon.** For histological examination, a sample of colon tissue located precisely 3 cm above the anal canal was obtained from the rats of all treatment groups. Tissues were graded semi-quantitatively from 0 to 5, where 0 indicated no changes and 5 was characterized by marked transmural inflammation with severe ulceration and loss of intestinal glands. Grading was performed in a blinded fashion according to previously described criteria [18,19].

**Measurement of MPO activity**

Myeloperoxidase (MPO) activity was assessed as a marker of neutrophil leukocyte infiltration into and accumulation in the inflamed colon tissue. The MPO activity assay was performed using a modification of the method described by Bradley et al. [20]. Enzyme activity was determined photometrically as the MPO-catalyzed change in absorbance occurring during the redox reaction of 3,3,5,5-tetramethylbenzidine dihydrochloride (Sigma-Aldrich) at 650 nm. MPO (Sigma-Aldrich) was used as an internal standard. Values are expressed as MPO units per gram of wet tissue.

**Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)**

Analysis of the expression level of selected genes was performed by qRT-PCR. Total RNA levels were obtained from small colon pieces (50 mg). qRT-PCR was performed using an Applied Biosystems GenAmp 7300 Sequence Detection System with SYBR Green PCR Reagent (Applied Biosystems). A constant amount of cDNA corresponding to the reverse transcription of 100 ng of total RNA was amplified as Voisine et al. described [21]. The results are expressed as $2^{-\Delta\Delta C_t}$, where $\Delta C_t = (C_t_{Target} - C_t_{\beta\text{-actin}})Q - (C_t_{Target} - C_t_{\beta\text{-actin}})C_B$.

Rat primers were as follows:

- $\beta\text{-actin}: AAGTCCCTCACCCTCCTCCAAAAG$ and $AAGCAATGTGTGCACCTTCCC$;
- IL-10: $TGCTATGTGCCGTGCTTTACTG$ and $TCAAATGCCTTGGATTTCG$;
- IL-2: $CCTTGTCCACACCGCACC$ and $GCTTTGACAGATGGCTATCC$;
- IFN-γ: $TGGATGACTGCGAAAGAAAAG$ and $GATTCTGGTGACAGCTTGC$;
- TGF-β: $CTGAACGCTGGACAGCTG$ and $ACGATCGTGGACAACTGCT$;
- IL-6: $TCCTACCCGACTCTCAATGC$ and $TTGGATGTCCTTGCTTACGCC$;
- TNF-α: $GTTGCCTCCCTTTCTT$ and $CCTGGTCAACAAATCAGCATT$.

**Characterization of Colonic lamina propria (LP) CD4+CD25+Foxp3+ Tregs**

Colonic LP cells were isolated using a modification of the technique described by van der Heijden and Stok [22]. Briefly, colonic samples were washed in cold Ca/Mg-free HBSS and were cut into 0.5-cm pieces. The epithelium was removed from the LP by incubation with 2 mM DTT and 1 mM EDTA in HBSS for 2 x 20 min. Tissues were digested using collagenase V (150 U/mL, Sigma-Aldrich) in complete medium for 2 h. Then LP cells were harvested from the supernatant and resuspended at a density of $1 \times 10^6$ cells/mL. The cells were stained with PE-coupled anti-CD25 and FITC-coupled anti-CD4 monoclonal antibodies for 30 min. After surface staining, the cells were resuspended in
fixation/permeabilization solution, intracellularly stained with APC-labeled anti-Foxp3 (BD Cytofix/Cytoperm kit) according to the manufacturer's protocol, and analyzed using a FACS Calibur. Corresponding rat isotype controls were included in the analyses.

**Statistical analysis**

All values are expressed as the mean ± SEM per group. Comparisons of more than two groups were conducted by one-way analysis of variance.
(ANOVA). Differences were considered statistically significant if the p-value was < 0.05.

Results

Phenotypic characteristics of IL-10-treated DCs and IL-10-treated DCs derived exosomes

The levels of MHC II and costimulatory molecules on DCs before and after treatment with IL-10 were examined by flow cytometry. As shown in Figure 1A, IL-10 treatment of DCs resulted in highly decreased level of MHC II, as well as lowered levels of CD80, CD86 and CD40. These observations are consistent with published reports regarding the ability of IL-10 treatment to down-regulate levels of MHC II and costimulatory molecules on DCs [23,24]. To examine their composition, exosomes were isolated from immature DCs and IL-10-treated DCs and were also analyzed by flow cytometry. We observed a significant decrease in the levels of MHC II, CD80 and CD40 on exosomes secreted by IL-10-treated DCs (IL-10-exosomes). Interestingly, IL-10-exosomes expressed moderate levels of CD86 than exosomes obtained from immature DCs (Figure 1B). We next analyzed the membrane-associated IL-10 of exosomes by flow cytometry. IL-10-exosomes expressed a higher level of IL-10, while no membrane-associated IL-10 was present in exosomes from immature DCs. To exclude rIL-10 contamination in the exosomes solution, rIL-10 was analyzed by ELISA on the
supernatant of the two populations of exosomes and no rIL-10 protein was detected. These results confirm that an immunosuppressive phenotype of exosomes accompanied with membrane-associated IL-10 can be generated from DCs treated by rIL-10.

**Exosomes derived from IL-10-treated DCs can suppress acute TNBS-induced colitis**

TNBS-treated rats developed acute colitis coupled with extensive wasting. To investigate the
anti-inflammatory capability of IL-10-exosomes in vivo, TNBS-treated rats were administered IL-10-exosomes, exosomes from immature DCs or salines at Day 3 post-TNBS enema. Treatment with IL-10-exosomes significantly slowed disease development and led to improvement, as assessed by animal weight change and clinical examinations of the colitis (Figure 2A and 2B, \( p < 0.05 \) versus untreated TNBS-induced colitis rats). Macroscopic analysis of colons obtained 8 days after TNBS administration showed striking hyperemia, necrosis, and inflammation compared with those of ethanol-treated control groups, which showed almost no signs of inflammation. However, IL-10-exosome administration significantly improved macroscopic scores compared with the untreated TNBS-induced colitis rats (Figure 2C, \( p < 0.05 \)). The severity of colon inflammation and ulceration was further evaluated by histological examination. By Day 8, transmural inflammation characterized by the infiltration of inflammatory cells, predominantly neutrophils and lymphocytes, was associated with ulcerations, loss of goblet cells, and fibrosis throughout the colon in untreated TNBS-induced colitis rats. Administration of IL-10-exosomes restored the histological appearance of the mucosa and submucosa (Figure 2D, \( p < 0.05 \) and Figure 2E). The results of these experiments demonstrate that administration of IL-10-exosomes effectively attenuates TNBS-induced colitis development. In contrast to the IL-10-exosomes, exosomes from immature DCs were unable to inhibit the colitis response.

**Exosomes derived from IL-10-treated DCs down-regulate the inflammatory response in TNBS-induced colitis**

Rat TNBS-induced colitis is thought to be a model of Th1-mediated disease. This colitis is characterized by dense infiltration of lymphocytes and macrophages into the lamina propria and thickening of the colon wall. To further analyze the anti-inflammatory effect of IL-10-exosomes in vivo, MPO and cytokine mRNA expression was measured in the colons of TNBS-induced colitis rats treated with IL-10-exosomes, exosomes from immature DCs or salines. As shown in Figure 3A, Colonic MPO levels were significantly down-regulated in IL-10-exosome-treated rats in comparison to untreated rats. Consistent with this result, administration of IL-10-exosomes significantly decreased colonic expression of pro-inflammatory cytokines mRNA, including IFN-\( \gamma \), IL-2, and TNF-\( \alpha \) (Figure 3B–D, \( p < 0.05 \) versus untreated TNBS-induced colitis rats), and increased colonic expression of anti-inflammatory cytokine IL-10 mRNA (Figure 3E, \( p < 0.05 \) versus untreated TNBS-induced colitis rats). We also measured the TGF-\( \beta \) and IL-6 mRNA expression in the colon tissue. but found no difference between

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**Figure 4. IL-10-exosome treatment leads to induction of CD4+CD25+Foxp3+ T-cell proliferation in colon LP.** (A) Representative flow cytometric analysis of LP cells harvested from control rats, untreated TNBS-induced colitis rats, and TNBS-induced colitis rats treated with IL-10-exosomes or exosomes derived from immature DCs on Day 8. Cells are gated on CD4+ T cells and isotype-matched control antibodies were used to set the gate. (B) Percentage of Foxp3+ or CD25+ T cells within the CD4+ T-cell population from each group. The results are expressed as the mean ± SEM from five rats per group (\( n = 10 \)). *,**, \( p < 0.05 \) vs. untreated TNBS-induced colitis rats.
IL-10-exosome-treated and untreated TNBS-induced colitis rats (Figure 3F and G). These results demonstrate that IL-10-exosomes suppress TNBS-induced colitis, as indicated by a reduction in MPO and Th1-type cytokine levels. However, exosomes from immature DCs cannot effectively reduce the inflammatory response.

Exosomes derived from IL-10-treated DCs increase mucosal Treg numbers in acute TNBS-induced colitis

The higher levels of colonic IL-10 mRNA expression in the IL-10-exosome-treated rats prompted us to assess the Treg-stimulating capacity of the IL-10-exosome. Tregs play a pivotal role in autoimmunity and transplantation tolerance. After inducing colitis using TNBS, we isolated colonic LP cells from untreated rats and rats treated with IL-10-exosomes or exosomes derived from immature DCs. IL-10-exosome treatment resulted in a considerable up-regulation of CD4+CD25+Foxp3+ T cells in colon LP. In addition, the populations of CD4+CD25+ and CD4+Foxp3+ T cells also increased in colonic LP of IL-10-exosome-treated rats (Figure 4B, p < 0.05 versus untreated TNBS-induced colitis rats). These results demonstrate that LP Tregs may play an important role in the rats TNBS-colitis treatment with IL-10-exosomes. However, the colonic LP Tregs up-regulated was not observed in the rats treatment with immature DC-derived exosomes.

Discussion

Exosomes have recently been shown to be involved in regulating certain biological processes. Given the ability of DC-derived exosomes to stimulate immune responses in culture and in vivo, we were interested in determining whether exosomes derived from immunosuppressive DCs were able to inhibit inflammation. Previous reports have shown that intravenous injection of DC-derived exosomes genetically modified to express IL-4 or IL-10 can reverse established CIA [12,14]. In this study, we have demonstrated the ability of exosomes derived from IL-10-treated DCs to suppress colon inflammation in a rat TNBS-induced colitis model.

Intestinal inflammation induced by TNBS is a Th1-mediated model of IBD. In the rat TNBS-induced colitis model, inflammation and T-cell-mediated immune dysfunction develop in response to hapten-modified autologous proteins and luminal antigens [25]. Using this model, we provided evidence that IL-10-exosomes protect against the development of colon inflammation. More specifically, administering IL-10-exosomes to rats effectively attenuated the severity of wasting, diarrhea, and colon inflammation, as assessed by measuring the macroscopic- and microscopic-damage scores. Furthermore, while IL-10-exosome treatment significantly attenuated the TNBS-induced increase in expression of Th1 type cytokines as IFN-γ, IL-2, and TNF-α in the colon, these effects were not reproduced by treatment with exosomes derived from immature DCs. IL-10 treatment of murine BMDCs can generate immunosuppressive DCs [26]. To determine the composition of the exosomes derived from IL-10-treated DCs, in contrast to exosomes from immature DCs, we performed flow cytometric analysis on the different exosome fractions. We observed that exosomes produced by IL-10-treated DCs exhibited more immunosuppressive phenotype and higher levels of membrane-associated IL-10 than exosomes derived from immature DCs. Kim et al. [14] reported that treatment of BMDC with anti-inflammatory cytokines, such as rIL-10, rIL-4 and rTGF-β, can result in an immunosuppressive phenotype of DC-derived exosomes that were able to suppress the CIA and DTH. Moreover, they further demonstrated that exosomes from rIL-4-treated DCs can express high levels of membrane-bound form of IL-4, which is important for the suppression of the DTH response. In this study, we also observed that exosomes from IL-10-treated DCs can express high levels of membrane-bound IL-10, which may critical to suppress inflammation in rat models of TNBS-induced colitis. Recently, novel methodologies of exosome display technology are developed to modify the molecular composition of exosomes by delivering soluble Ag or membrane proteins that are not detected in naturally generated exosomes [27]. Using these genetic engineering methods, we can generate recombinant exosomes to carry more membrane-associated immune-regulatory molecules to suppress inflammation.

The mechanism of colitis pathogenesis is currently unknown, but it can be controlled by the suppressor cytokine IL-10 [28]. Consistent with this information, we found that IL-10 mRNA levels increased in the colons of IL-10-exosome-treated rats. As several lines of evidence have demonstrated, IL-10 has a distinct role in the development and function of CD4+CD25+ Tregs [29]. Our finding and this past study prompted us to assess the Treg responses after IL-10-exosome treatment. Isolation of LP cells from the colon revealed a significant increase in the number of CD4+CD25+Foxp3+ T in IL-10-exosome-treated rats. This increase was also noted for CD4+CD25+ T cells and CD4+Foxp3+ T cells in the same rats. The importance of CD4+CD25+Tregs in mediating the
inhibition of colitis has also been documented in SCID mice. In that study, CD4+ T cells were sorted into CD25+ and CD25- fractions and tested for their ability to suppress colitis [30]. Our results also confirmed that CD4+CD25+Foxp3+ T cells probably play an important role in suppressing experimental colitis. In accordance with the observations of the present study, these findings underscore the potential of IL-10-exosomes to cure Th1-mediated experimental colitis in rats by increasing the numbers of LP of IL-10-exosomes to cure Th1-mediated experimental colitis. In accordance with the observations of the present study, these findings underscore the potential of IL-10-exosomes to cure Th1-mediated experimental colitis in rats by increasing the numbers of LP of IL-10-exosomes to cure Th1-mediated experimental colitis.

In this report, we demonstrated the ability of exosomes derived from IL-10-treated DCs to reverse TNBS-induced colitis in rats. However, unlike DCs, which can undergo phenotypic changes in vivo, exosomes derived from the DCs presumably reflect the cells' phenotype at the time of isolation. Thus, the use of exosomes derived from immunosuppressive DCs for the treatment of autoimmune diseases, such as IBD, may be safer and more effective than administering DCs themselves. Although warranting further investigation, these findings strongly support the potential of an IL-10-exosome-based therapeutic option for the treatment of IBD in the clinic.

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References

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