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Modified *IL-10*-expressing dendritic cells ameliorate TNBS-induced Crohn's disease in mice.

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Abstract

This article investigated the therapeutic effects of immature Dendritic Cells (imDC) expressing modified interleukin-10 (*IL-10*) in a mouse model of Crohn's disease and the possible underlying mechanisms. Forty mice were randomly divided into control group, imDC- treatment group (imDC group), *LacZ* gene-modified imDC treatment group (*LacZ*-imDC group) and *IL-10* gene-modified imDC treatment group (*IL-10*-imDC group). The DAI scores of the imDC group, *LacZ*-imDC group, and *IL-10*-imDC group were significantly lower than that of the control group (P<0.05). Furthermore, the DAI score of the *IL-10*-imDC group was markedly reduced compared to those of the imDC and *LacZ*-imDC groups (P<0.05). Similarly, the TDI scores of the imDC group, *LacZ*-imDC group, and *IL-10*-imDC group were significantly lower than that of the control group (P<0.05). In particular, the TDI score of the *IL-10*-imDC group was significantly lower than those of the imDC and *LacZ*-imDC groups (P<0.05). The percentages of splenic Treg cells in the imDC, *LacZ*-imDC, and *IL-10*-imDC groups were significantly higher than that of the control group (P<0.05). The percentage of Treg cells in the *IL-10*-imDC group was also markedly higher than those in the imDC and *LacZ*-imDC groups (P<0.05). In conclusion, when successfully transfected into imDC, *IL-10* can inhibits the process of DC maturation. Infusion of this *IL-10*-imDC can significantly alleviate the progress of Crohn's disease compared with imDC without *IL-10* modification, and the mechanism of action may be associated with the upregulation of Treg cells.

Keywords: Crohn's disease, Interleukin-10, Immature dendritic cells, Transfection, Regulatory T cells.

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Introduction

Crohn's disease is a chronic, recurrent autoimmune disease characterized by dysregulation of immune function in the gut. Although the exact etiology has not been fully elucidated, it is now recognized that the environment, genetics, infection and immune disorders are all involved in the development of Crohn's disease [1]. Previous studies have demonstrated that the status of antigen-presenting cells is closely associated with changes in the number of regulatory T (Treg) cells [2,3]. As the most important class of antigen-presenting cells, dendritic cells (DCs) play an important role in regulating T cell activation. While mature Dendritic Cells (mDC) induce the differentiation of naive T cells into T helper 1 (Th1) and T helper 17 (Th17) cells to promote immune responses, immature Dendritic Cells (imDC) promote the differentiation of naive T cells into Treg cells to down-regulate immune responses [4]. It has been reported that interleukin-10 (*IL-10*) is an immunosuppressive cytokine that holds DCs in a stable immature stage, and thus plays an important role in facilitating naive T cell differentiation into Treg cells [5]. The goal of the current study was to investigate the use and possible mechanism of *IL-10* gene-modified imDC in treating TNBS-induced Crohn's disease in mice.

Materials and Methods

Materials

Fifty 6-8 week old female Balb/c mice were purchased from the Experimental Animal Center of Wuhan University (license number: SCXK (E) 2014-0004). All animals were housed in the animal facility under SPF conditions in the Remin Hospital of Wuhan University. Ten of these mice were used for the isolation and in vitro culture of dendritic cells.

Five percent (W/V) aqueous 2, 4, 6-Trinitrobenzene Sulfonic Acid (TNBS) was purchased from Sigma-Aldrich, USA. Recombinant mouse Interleukin-4 (rmIL-4) and recombinant

mouse granulocyte Macrophage Colony Stimulating Factor (rmGM-CSF) were purchased from Peprotech, USA. PE-Cy7-labeled anti-mouse CD11c, PE-labelled anti-mouse CD80, APC-labelled anti-mouse CD86 and CD25, FITC-labelled anti-mouse MHC-II and CD4 antibodies were all purchased from BD Biosciences, USA. An adenovirus vector carrying the mouse *IL-10-EGFP* gene and the control vector carrying the *lacZ-EGFP* gene were synthesized and packaged by Wuhan Biobuffer, at a titer of 1×10^7 TU/ml and higher. Flow cytometry was performed on a FACScalibur by BD Biosciences, USA. The IX71 inverted microscope used in this study was manufactured by Olympus, Japan.

Methods

The experimental research methods were approved by the Institutional Ethics Committee of Renmin Hospital of Wuhan University (approval number [2015]28.

Isolation, induction and transfection of mouse bone marrow-derived dendritic cells: Mice were sacrificed by cervical dislocation and cells were prepared according to a modified method reported previously [6]: Bone marrow mononuclear cells were collected and cultured in RPMI 1640 containing 10% fetal bovine serum at 1×10^6 cells/ml, 4 ml/well of a 6-well plate. Each well was supplemented with *rmIL-4* (10 ng/ml) and rmGM-CSF (10 ng/ml), and the cells were cultured in a 37ºC incubator with 5% CO_2. On day 3, 2 ml of the culture medium from each well was transferred to a new 6-well plate and 2 ml fresh cytokine-containing medium was then added to each well of the original and the new plates. DCs were collected on day 5 and washed twice with PBS. A portion of the cells was returned to culture in complete medium without any treatment. The remaining cells were transferred to a 24-well plate at 1×10^6 cells/well in 500 µl, transfected with recombinant adenovirus carrying *LacZ* or *IL-10* at an MOI of 1:100 and cultured in a 37ºC incubator with 5% CO_2. The cells were washed 2 h later, resuspended in complete medium and returned to 37ºC, 5% CO_2 incubator for 24 h. Transfection efficiency was observed under an inverted fluorescence microscope, and the cells were harvested and counted. Three types of cells were produced by the methods described above, namely imDC, *LacZ*-imDC and *IL-10*-imDC.

Detection of DC maturation by flow cytometry: Cells with a density of 1 10^6 cells/ml were collected and resuspended in 100 µl medium containing anti-mouse PE-Cy7-CD11c, PE-CD80, APC-CD86 and FITC-MHC-II antibodies. The cells were incubated in the dark for half an hour and washed twice with PBS for flow cytometry analysis.

Generation of animal model and treatment groups: The mouse model was established according to a previous study [7] with modifications: after 7 days of acclimation, mice were allowed only drinking water for 24 h. The animals were stimulated to promote defecation before TNBS enema. A 3.5 F catheter (smeared with medical paraffin oil) was inserted into the intestine via the anus to a depth of about 5 cm. Each animal was slowly injected with 0.1 ml of TNBS/50% ethanol solution of (1:1 mixture of TNBS solution and anhydrous ethanol). The mice were kept in head-down position for 1 minute after catheter withdrawal. After successful TNBS administration, the 40 mice were randomly divided into control group, immature dendritic cell treatment group (imDC group), *LacZ* gene modified immature dendritic cell treatment group (*LacZ*-imDC group) and *IL-10* gene modified immature dendritic cell treatment group (*IL-10*-imDC group), with 10 mice per group. At 24 h after disease induction, mice were given intravenous injections of 100 µL of PBS for control group, untransfected imDC for imDC group, *LacZ* adenoviral transfected imDC for *LacZ*-imDC group, and *IL-10* adenoviral transfected imDC for *IL-10*-imDC group, at approximately $1 \times 10_6$ cells per mouse.

Disease severity rating: The psychological condition, stool consistency, presence or absence of bloody stools and body weight of the animals were observed and recorded daily. Mice were sacrificed on day 7 after disease induction for further analysis of other relevant indicators. Disease Activity Index (DAI) was scored according to published standards [8].

0: normal stool consistency, no bloody stool or occult blood, no body weight decline.

1: soft stools, occult blood weakly positive, body weight loss>0-5%.

2: soft stools, occult blood positive, body weight loss>5-10%.

3: watery stools, visible faecal blood, body weight loss>10-15%.

4: watery stools, a large amount of visible faecal blood, body weight loss>15%.

DAI scores were the combined scores of stool consistency plus stool blood plus body weight change.

Intestinal tissues of the affected regions were harvested under a dissecting microscope, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned for HE staining. Pathological scoring was conducted by two researchers of the study based on the standards from the literature [9].

For inflammatory cell infiltration, sections were scored as follows: 0 points for no infiltration; 1 point for mild infiltration; 2 points for moderate infiltration; 3 points for heavy infiltration. For infiltration depth: 0 points for no infiltration; 1 point for epithelial infiltration; 2 points for mucosal infiltration; 3 points for full-thickness colon infiltration. For ulcer depth: 0 points for no ulcer; 1 point for ulceration of the epithelium; 2 points for ulceration of the mucosa; 3 points for ulceration of the muscularis mucosa. TDI scores were the combined scores of inflammatory cell infiltration plus infiltration depth plus ulcer depth.

Detection of the percentage of splenic Treg cells by flow cytometer: Mouse spleens were collected and ground on a 200 mesh screen with a syringe plunger while being rinsed with PBS. After centrifugation, the cells were resuspended in 4 ml PBS and slowly laid over 8 ml lymphocyte separation solution along the wall of the test tube. The tubes were centrifuged at 2000 rpm (rotor radius: 10 cm) for 20 min in order to isolate cells from the middle cloud-like layer. After washing with 10

volumes PBS by centrifugation, cells were resuspended in PBS, counted and adjusted to 1×10^6/ml. FITC-CD4 and APC-CD25 monoclonal antibodies were added to 100 µl cell aliquots, incubated in the dark for half an hour, washed twice with PBS and fixed in 4% paraformaldehyde for flow cytometry analysis.

Statistical analysis

SPSS19.0 statistical software was used for data analysis. Normally distributed measurement data were presented as ($\bar{x} \pm S$). Comparisons between multiple groups were performed using ANOVA, while pair-wise comparisons were conducted using the LSD-t test. $P<0.05$ was considered statistically significant.

Results

Isolation, culture, identification and recombinant adenoviral transfection of imDC

Dendritic cells were successfully differentiated from bone marrow cells by rmIL-4 and rmGM-CSF induction. They were transfected with at an MOI of 1:100 recombinant *IL-10* adenovirus or control recombinant *LacZ* adenovirus, with a transfection efficiency of about 70%. Increased expression of EGFP in transfected cells was observed under a fluorescence microscope (Figures 1 and 2).

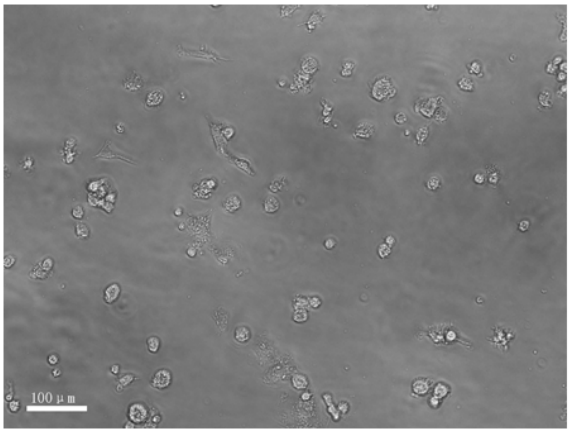

Figure 1. *Observation of immature dendritic cells at 24 h post-transfection under an inverted microscope at low magnification.*

Phenotypic identification of IL-10-transfected imDC

The dendritic cell surface marker CD11c was highly expressed (about 85% expression) on imDCs, *LacZ*-imDCs and *IL-10*-imDCs. Non-transfected imDCs expressed low levels of MHC-II (58.2 ± 6.1%), and co-stimulatory molecules CD80 (28.5 ± 4.7%) and CD86 (33.2 ± 3.6%), which were consistent with the characteristics of imDCs [10]. *LacZ* adenovirus transfection had no significant effect on the phenotypes of imDC, as the expression levels of MHC-II (59.3 ± 6.7%), CD80 (28.2 ± 5.1%) and CD86 (33.5 ± 4.1%) were similar to those in the non-transfected imDC. In contrast, imDC transfected with *IL-10* adenovirus showed significantly reduced expression of MHC-II (35.1 ± 4.4%), CD80 (14.6 ± 3.2%), CD86 (15.5 ± 3.6%), (t=7.14, 9.12, 9.54 respectively, $P<0.05$) (Figure 3).

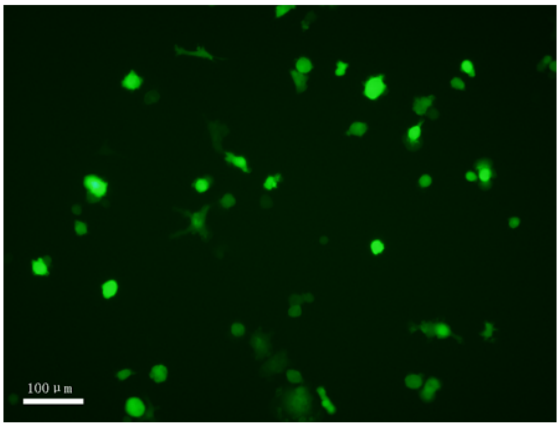

Figure 2. *Observation of increased EGFP expression (green fluorescence) in immature dendritic cells at 24 h post-transfection under an inverted fluorescence microscope at low magnification.*

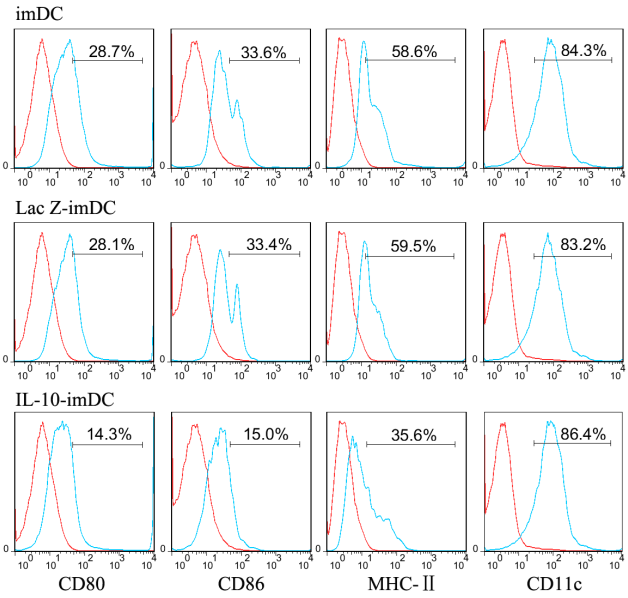

Figure 3. *Surface expression levels of CD80, CD86, MHC-II, and CD11c of different imDC groups. The horizontal axis represents fluorescence intensity and vertical axis represents the number of cells.*

Pathological changes and disease severity evaluation

Pathological analysis of the colon tissues from each group of mice indicated that while TNBS-treated control mice had severe inflammatory cell infiltration throughout the mucosal layers, severe mucosal damage and disruption, disappearance of glandular structure and deep ulceration into the muscularis mucosa, these pathological changes were slightly improved in the imDC group and *LacZ*-imDC group, with distinguishable epithelial cell outlines and the presence of a smaller number of glands. Although there was significant inflammatory cell infiltration, it did not reach muscularis mucosa, and there was no obvious deep ulceration. In contrast, the pathological changes in *IL-10*-imDC group were much more subdued than

the other groups. The mucosa, submucosa, muscularis mucosa and serosa membrane were clearly visible without significant damage or disruption of the mucosal epithelium. There was little damage to the intestinal glands, and inflammatory cell infiltration was significantly reduced compared with the other 3 groups.

The DAI scores of the imDC group (3.98 ± 0.84), *LacZ*-imDC group (4.02 ± 0.83), and *IL-10*-imDC group (2.91 ± 0.72) were significantly lower than that of the control group (5.07 ± 0.91) (F=11.56, t=4.164, 4.011, 8.251, respectively, P<0.05) (Figure 4). Furthermore, the DAI of *IL-10*-imDC group was markedly lower than those of imDC and *LacZ*-imDC groups (F=6.217, t=4.237, 4.396, respectively, P<0.05) (Figure 5).

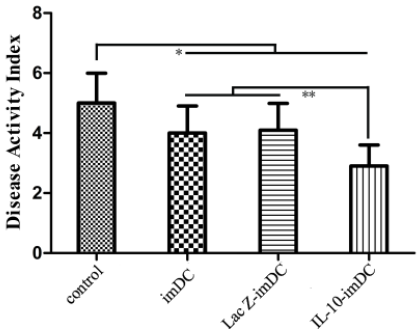

Figure 4. *Disease Activity Index (DAI) scores of mice each group. Note:* *P<0.05.*

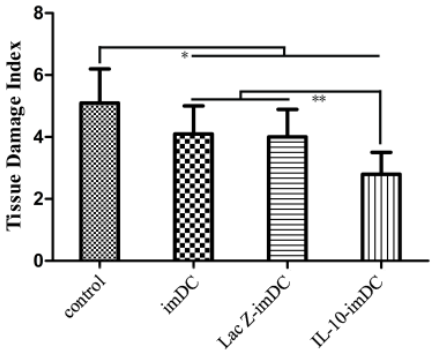

Figure 5. *Pathological index (TDI) scores of in mice colon tissues in each group. Note:* *P<0.05.*

Similarly, the TDI scores of the imDC group (3.84 ± 0.90), *LacZ*-imDC group (4.00 ± 0.82) and *IL-10*-imDC group (2.85 ± 0.75) were significantly lower than that of the control group (5.13 ± 0.15) (F=17.15, t=5.674, 4.970, 10.029, respectively, P<0.05). In particular, the TDI of *IL-10*-imDC group was also significantly lower than those of the imDC and *LacZ*-imDC groups (F=5.784, t=3.792, 4.405, respectively, P<0.05) (Figure 6).

Percentage of mouse splenic CD4+CD25 + Treg cells in each group

The percentages of splenic $CD4^+CD25^+$ Treg cell sin imDC group (4.19 ± 0.54%), *LacZ*-imDC group (4.30 ± 0.49%) and *IL-10*-imDC group (6.15 ± 0.73%) were significantly higher than the control group (3.08 ± 0.31%) (F=55.78, t=6.516, 7.161, 18.022, respectively, P<0.05). There was also a marked increase of $CD4^+CD25^+$ Treg cells in the *IL-10*-imDC group when compared with the imDC and *LacZ*-imDC groups (F=34.17, t=10.405, 9.820, respectively, P<0.05).

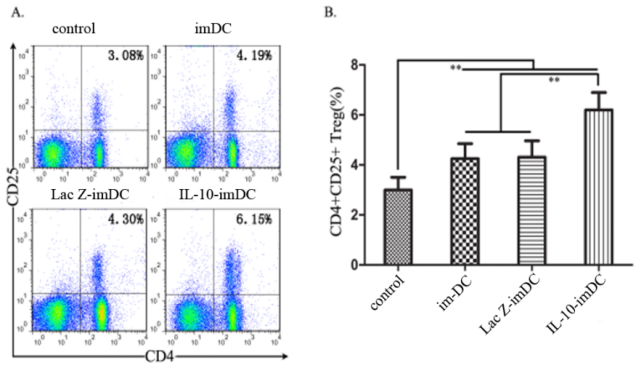

Figure 6. *Changes of mouse splenic $CD4^+CD25^+$ Treg cells in different groups. A. Representative flow cytometry dot plots; B. Bar graph of the data;* *P<0.05.*

Discussion

Immunoregulatory roles of dendritic cells in vivo

Dendritic cells are the most important antigen-presenting cells in the body and play important roles in T cell activation [11]. Studies have shown that mDC express high levels of MHC-II and co-stimulatory molecules CD80 and CD86 and promote the differentiation of naive T cells into functionally mature, pro-inflammatory T cells such as Th1 and Th17 cells [12]. On the other hand, imDCs express low levels of the aforementioned surface molecules, and thus are unable to provide an effective 'second signal' necessary for T cell activation, resulting in the generation of immune tolerance [13]. Past studies have shown that imDC may have certain therapeutic effects for treating autoimmune diseases, but the efficacies were far from satisfactory [14]. One of the reasons could be that the complex *in vivo* microenvironment can induce the maturation of the infused imDC into mDC, thereby switching their function from immunosuppression to immune activation.

Effect of IL-10 on dendritic cells

It has been reported that *IL-10* cytokine-treated or *IL-10* gene-modified imDCs has a stronger tendency to stay in their immature states [15,16]. *IL-10* is a potent immunosuppressive molecule, and plays a vital role in immune regulation. *IL-10* can inhibit DC maturation and their ability to function as antigen-presenting cells by reducing the surface expression of MHC-II and co-stimulatory molecules CD80 and CD86 [12]. In this study, *IL-10* was successfully transfected into DCs via an adenovirus vector with high transfection efficiency and the modified DCs were examined by flow cytometry. In addition, transfection of the control adenoviral vector carrying the *lacZ* gene had little effect on DC phenotype and maturation, suggesting that the adenoviral vectors have very low cytoxicity

on the DCs [17]. However, *IL-10* gene-modified DCs demonstrated significantly reduced surface expression of MHC-II, CD80 and CD86. When DCs remain at the immature stage due to inhibited expression of these molecules, they are unable to provide co-stimulatory signals for T cell activation, and thereby may induce T cell energy and immune tolerance.

Therapeutic effects and possible mechanisms of IL-10 gene-modified DCs in mouse model of Crohn's disease

We found that administration of *IL-10*-transfected DCs via tail vein injection significantly ameliorated Crohn's disease in mice, as alleviation of the disease could be observed by both the DAI scores and pathological changes. Although the disease was also reduced in the two groups that received unmodified imDCs or control vector transfected imDCs, the effect was not as profound as that seen in mice given *IL-10*-imDC, suggesting that *IL-10*-transfected imDCs may have a greater ability to maintain their immature state in order to sustain their immunotolerant function. Previous studies have shown that in addition to inhibiting effector T cells, another important activity of *IL-10* is the induction of $CD4^+CD25^+$ Treg cells [18]. To further investigate the specific mechanisms of *IL-10*-expressing imDCs in this study, changes in mouse splenic $CD4^+CD25^+$ Treg cells [19] in each treatment group were examined by flow cytometry. It was found that the percentage of $CD4^+CD25^+$ Treg cells in the *IL-10*-imDC group was significantly higher than those in the other groups. Given that $CD4^+CD25^+$ Treg cells are immunosuppressive cells that have an important role in peripheral immune tolerance; previous studies indicated that reduction of $CD4^+CD25^+$ Treg cells could be one of the causes for the development of Crohn's disease [20]. In this study, we found that splenic Treg cells were significantly increased in mice given *IL-10*-imDC, which in turn suggested that increased Treg cells could be one reason for the imDC-mediated alleviation of Crohn's disease.

In summary, we have successfully generated *IL-10* gene-modified imDCs, and confirmed reduced levels of cell surface molecule expression by flow cytometry. In a TNBS–induced mouse model of Crohn's disease, inoculation of these cells resulted in significant improvement of disease progression. The underlying mechanisms may be associated with the immune tolerance-inducing function of imDC and upregulation of peripheral $CD4^+CD25^+$ Treg cells. These findings may offer better methods and ideas for clinical research of Crohn's disease and shed new light on the prospects of imDC application.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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