

Original Article

IL-35 induces immune tolerance in renal transplantation rats by modulating CD4+CD25+ regulatory T-cells

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Abstract: Objective: The aim of the current study was to unravel the underlying mechanisms of interleukin-35 (IL-35) in inducing immune tolerance in renal transplantation rats by modulating CD4+CD25+ regulatory T-cells. Methods: From a total of 60 Sprague-Dawley (SD) rats, 40 were selected, randomly, for preparation of allograft renal transplantation. They were divided into two groups, the donor group and recipient group. The remaining rats were assigned to the control group. Test group rats were further assigned to test groups A and B, while the control group rats were assigned to control groups A and B. Intravenous infusion of IL-35 was used as the intervention for rats in group A. Rats in group B received intravenous infusion of normal saline. Eight days later, the rats were sacrificed and peripheral blood samples were collected for measurement of serum levels of creatinine (Cr) and blood urea nitrogen (BUN). Serum levels of IL-10, IL-17, and IL-23 were detected using enzyme-linked immunosorbent assays. Percentages of CD4+CD35+ regulatory T-cells were measured using flow cytometry. Moreover, mRNA expression of Foxp3 in renal tissues was determined by polymerase chain reaction. Results: In the experimental group and control group A, significant ($P<0.05$) decreases were observed in levels of Cr, BUN, IL-10, IL-17, and IL-23, compared with levels in the experimental group and control group B, respectively. Of the four groups, the highest levels of Cr, BUN, IL-10, IL-17, and IL-23 were found in rats of test group B. The lowest levels were found in control group A. There were no significant ($P>0.05$) differences between the two control groups concerning ratios of CD4+CD25+ regulatory T-cells. However, the ratio in test group A was significantly ($P<0.05$) higher than that in test group B. Furthermore, expression levels of Foxp3 in test group A and control group A were significantly ($P<0.05$) higher than expression levels in test group B and control group B, respectively. Foxp3 expression in test group B was significantly ($P<0.05$) higher than that in control group A. The highest expression of Foxp3 was recorded in test group A, while the lowest expression was recorded in control group B. Conclusion: IL-35 can affect renal function in renal transplantation rats, as well as expression of IL-6, IL-17, and IL-23. Underlying mechanisms involve IL-35 regulating CD4+CD25+ regulatory T-cells to induce immune tolerance by targeting Foxp3 in renal transplantation rats.

Keywords: IL-35, regulatory T-cells, renal transplantation rat, immune tolerance

Introduction

Interleukin-35 (IL-35) is a member of the recently discovered family of interleukin-12 (IL-12). It is a dimer consisting of two subunits [1, 2]. Initially, IL-35 was deemed as a cytokine that can affect regulatory T-cells and secretion of other cytokines, inducing persistent immune tolerance. Therefore, it is believed that IL-35 plays a critical role in the immune tolerance period after a viral infection [3]. Thus, IL-35 is an area of active research in clinical immunology. In China and other countries,

research concerning immune function has been focused on regulatory T-cells [4]. Regulatory T-cells are a subgroup of T-cells that regulate the function of immune cells. They are involved in autoimmune diseases, allergy, malignancies, infections, and graft rejection [5, 6]. Currently, there is no experimental evidence for the roles of IL-35 in regulatory T-cells. It was hypothesized that the mechanisms of IL-35 affecting the immune function of regulatory T-cells might be a key component in the development of targeted therapy for major immune diseases.

Renal disease is a common chronic disease, with prominent features of immunopathology. Patients have to endure difficulties in treatment and excessively long treatment durations. Renal transplantation is the best choice for end-stage renal disease patients [7, 8]. However, patients still suffer from poor prognosis due to allograft rejection after the transplantation [9].

The current study established a rat model of renal transplantation. This study demonstrated that, through the intervention of IL-35, changes in CD4⁺CD25⁺ regulatory T-cells in the peripheral blood occurred. This study focused on the effects of IL-35 on regulatory T-cells in renal transplantation rats, examining the associated mechanisms of IL-35 in inducing immune tolerance in rats after the renal allograft. Present findings are expected to have clinical application for renal transplantation.

Materials and methods

Animals

A total of 60 Sprague-Dawley rats, aged 8-10 weeks and weighing between 300 g and 380 g, were provided by the Wuhan Hualianke Biotechnology Co., Ltd. (Wuhan, China). The rats were fed under the following conditions: Room temperature (26°C), relative humidity (75%), 10 rats/cage, and a 12-hour light/dark cycle.

Model establishment

A total of 40 rats were randomly selected for preparing the renal transplantation model. Rats of the transplantation model were further divided into two groups, including the donor group and recipient group. The remaining 20 rats were enrolled into the control group, which received no treatment. Renal transplantation was carried out, according to the methods of Shrestha et al. [10]. For all donor rats, the left kidney was selected for transplantation. Surgery procedures for the donor: The rats were first anesthetized by intraperitoneal infusion with 0.3% pentobarbital sodium. An incision was made at the middle of the abdomen to expose the vessels and vena cava above the iliac vessels. The abdominal aorta and vena cava were lifted, followed by ligation and detachment of the left adrenal veins. In the abdominal aorta of the distal end of renal pedi-

cle, a stoma was made for intubation. Pre-cooled heparin sodium (4°C) was infused, while the left renal aorta was blocked. The left renal vein was cut off. The left kidney of the donor was extracted and preserved in heparin sodium at 4°C. A similar protocol was followed for left kidney removal in the recipients. The initial part of ureter was freed and ligated, then the left aorta and vein were blocked. The incision was closed using the 10.0 atraumatic suture. Veins of donor kidneys were connected to the recipient renal veins through the cuff cannula. Urinary tracts were reconstructed by inserting the ureter into the bladder. Every procedure was approved by the Animal Care and Use Committee of the Second Affiliated Hospital of Guangzhou University of Chinese Medicine and was in conformity with the guidelines of National Institute of Health (No. 81004).

After surgery, the rats were returned to the cages. Penicillin was given through muscular injections (1,000,000 units/kg) for 3 days consecutively. Animals in the test group and control group were further divided randomly into test groups A and B and control groups A and B, respectively. For rats in group A, IL-35 was given for treatment. For those in group B, IL-35 was substituted with normal saline. Drugs were given for 7 days. On the 8th day, the rats were sacrificed by cervical dislocation.

Outcome measures

Renal function: A total of 5 mL venous blood was collected from the rats and placed at room temperature for 30 minutes. This was followed by centrifugation at 4,000 rpm for 10 minutes to collect the supernatant. The serum was analyzed in an automatic biochemical analyzer, detecting levels of creatinine and blood urea nitrogen. Cytokines were detected using the enzyme-linked immunosorbent assay kits of IL-6 (VAL604, Beinuo Life Science, Shanghai, China), IL-17 (VAL604, Beinuo Life Science, Shanghai, China), and IL-23 (69-21102, Wuhan Mosak Biotechnology Co., Ltd., Wuhan, China). All procedures were in strict accordance with manufacturer instructions. CD4⁺CD25⁺ regulatory T-cells were detected through flow cytometry. Moreover, mRNA expression of Foxp3 was measured using the fluorescent Real-time Quantitative PCR (qPCR). Total RNA was extracted from the cells using TRIzol Reagent (15596026, Chengdu East Creative Technology

Table 1. Primer sequences

	F	R
GAPDH	5'-CCTCGTCTCATAGACAAGATGGT-3'	5'-GGGTAGAGTCATACTGGAACATG-3'
Foxp3	5'-CTGCTTGGCAGTGCTTGAGAA-3'	5'-CCCAGGAAAGACAGCAACCTT-3'

Foxp3 mRNA expression levels in the kidney tissues were detected using the qRT-PCR kit (YS-04382P, Shanghai Caiyou Industry Co., Ltd.,

Shanghai, China). Relative expression of Foxp3 was expressed using the $2^{-\Delta\Delta Ct}$ method, with GAPDH was as used the internal control. Primer sequences are shown in **Table 1**.

Statistical methods

SPSS 24.0 statistical software (SPSS Inc., Chicago, IL, USA) was utilized for data analysis and processing. Results are expressed as mean \pm standard deviation. Analysis of variance (ANOVA) for repeated measurements was adopted for comparisons between different groups, while Student's t-test was used for pairwise comparisons. $P < 0.05$ suggests statistical significance.

Results

Models

During surgery, one rat from the donor group and two rats from the recipient group did not survive. All rats survived in control group. Finally, there were 9 rats in test group A, 9 rats in test group B, 10 rats in control group A, and 10 rats in control group B.

Renal function

Differences in the levels, as tested by ANOVA, of Cr and BUN between the four groups were statistically significant ($P < 0.001$). In test group B and control group B, the rats had significantly ($P < 0.05$) higher levels of Cr and BUN, compared to levels in test group A and control group A, respectively. Furthermore, Cr and BUN levels were elevated significantly ($P < 0.05$) in test group B, compared to levels in control group B. Of the four groups, the lowest and highest levels of Cr and BUN in rats were found in control group A and test group B, respectively (**Table 2**, **Figures 1** and **2**).

Levels of cytokines

Differences in expression levels, as tested by ANOVA, of IL-6, IL-17, and IL-23 between the four groups were statistically significant ($P <$

Table 2. Comparison of liver function

	Cr ($\mu\text{mol/L}$)	BUN (mmol/L)
Test group A	92.41 \pm 5.15	5.84 \pm 0.60
Test group B	117.92 \pm 8.07	7.19 \pm 0.86
Control group A	26.52 \pm 4.12	1.62 \pm 0.35
Control group B	42.14 \pm 6.27	3.14 \pm 0.42
F	472.513	179.326
P	<0.001	<0.001

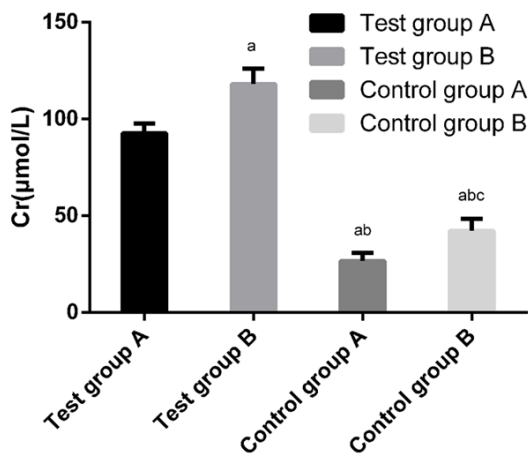


Figure 1. Measurement of serum creatinine levels in the four groups of rats. Kidney transplantation affects serum creatinine levels in rats. Serum levels of creatinine were the highest in kidney transplanted rats treated with IL-35 (test group B). ^a $P < 0.05$ vs. the level of Cr in test group A; ^b $P < 0.05$ vs. the level of Cr in test group B; ^c $P < 0.05$ vs. the level of Cr in control group A.

Co., Ltd., Chengdu, China). Purity and concentration levels of the total RNA was determined using a spectrophotometer, with an $\text{OD}_{260}/\text{OD}_{280}$ ratio between 1.8 and 2.0. From the total RNA, 2 μL aliquot was mixed with 1 μL oligo dT in a water bath (72°C) for 2 minutes. This was followed by centrifugation at 4,000 rpm for 10 minutes. Next, the mixture was collected for the following cDNA preparation protocol: 2 μL 10X RT buffer, 1 μL dNTPs, 0.5 μL RNase, 100 u M-MLV, and 2 μL H_2O . The samples were incubated for 1.5 hours at 42°C, followed by inactivation of the reverse transcriptase at 95°C for 5 minutes. The cDNA was preserved at -20°C.

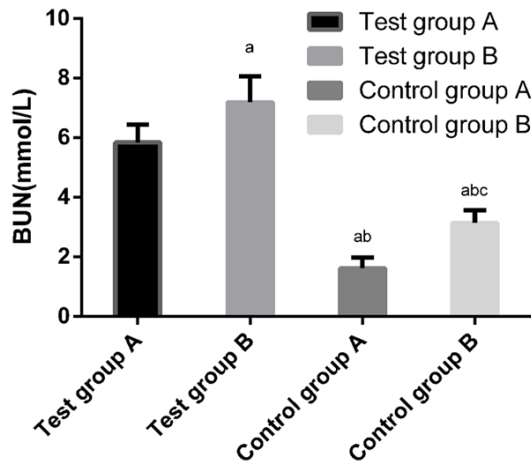


Figure 2. Measurement of serum Blood Urea Nitrogen (BUN) levels in the four groups of rats. Kidney transplantation affects serum BUN levels in rats. Serum levels of BUN were highest in kidney transplanted rats treated with IL-35 (test group B). ^a $P < 0.05$ vs. the level of BUN in test group A; ^{ab} $P < 0.05$ vs. the level of BUN in test group B; ^{abc} $P < 0.05$ vs. the level of BUN in control group A.

0.001). Levels of IL-6 in test group A, test group B, control group A, and control group B were 36.59 ± 4.14 , 121.77 ± 5.02 , 20.71 ± 3.87 , and 89.86 ± 2.52 pg/mL, respectively. Levels of IL-6 in test group A and control group A were significantly ($P < 0.05$) higher than levels in the other two groups. Levels of IL-6 in test group B were also significantly ($P < 0.05$) higher than levels in control group B. Of the four groups, rats in test group B had the highest and control group A had the lowest levels of IL-6. Levels of IL-17 in test group A, test group B, control group A, and control group B were 72.78 ± 3.05 , 179.24 ± 16.33 , 21.33 ± 1.69 , and 36.76 ± 5.27 pg/mL, respectively. Similarly, levels of IL-17 in test group A and control group A were significantly ($P < 0.05$) higher than levels in test group B and control group B. Levels of IL-17 in test group B were also significantly ($P < 0.05$) lower than levels in control group B. The highest and lowest levels of IL-17 were found in test group B and control group A, respectively. Levels of IL-23 in test group A, test group B, control group A, and control group B were 329.84 ± 4.88 , 409.68 ± 5.69 , 208.52 ± 10.57 , and 262.84 ± 8.04 pg/mL, respectively. Furthermore, levels of IL-23 in test group A and control group A were also significantly ($P < 0.05$) lower than levels in test group B and control group B, respectively. Levels of IL-23 in test group B were significantly ($P < 0.05$) higher than levels in control group B.

Of the four groups, test group B and control group A had the highest and lowest levels of IL-23, respectively (Table 3, Figures 3-5).

Levels of CD4+CD25+ regulatory T-cells

Differences, as analyzed by ANOVA, in ratios of CD4+CD25+ regulatory T-cells between the four groups were statistically significant ($P < 0.001$). Ratios in test group A, test group B, control group A, and control group B were $8.16 \pm 1.86\%$, $4.52 \pm 0.75\%$, $5.19 \pm 0.50\%$, and $5.22 \pm 0.59\%$, respectively. There was no statistical significance ($P > 0.05$) in the ratios between control group A and control group B. The ratio in test group B was significantly ($P < 0.05$) lower than that in test group A, control group A, and control group B. The ratio in test group A was significantly ($P < 0.05$) higher than that in control group A and control group B (Figure 6).

Foxp3 expression

Differences in Foxp3 expression, as analyzed by ANOVA, between the four groups were statistically significant ($P < 0.001$). Expression of Foxp3 (relative to GAPDH) in test group A, test group B, control group A, and control group B was 62.73 ± 8.64 , 5.16 ± 0.71 , 24.77 ± 3.8 , and 1.21 ± 0.14 , respectively. Furthermore, expression levels of Foxp3 in test group A and control group A were significantly ($P < 0.05$) higher than expression levels in test group B and control group B, respectively. Expression levels of Foxp3 in test group B were also significantly ($P < 0.05$) higher than expression levels in control group A. Of the four groups, the highest and lowest expression of Foxp3 was found in test group A and control group B, respectively (Figure 7).

Discussion

Renal transplantation is currently the most efficient method for treatment of advanced renal diseases. The success rate of renal transplantation has improved along with advances in medical technology and clinical practice [11]. However, immune tolerance caused by renal transplantation is considered a critical factor responsible for post-transplantation survival and renal function levels of patients [12]. Generally, aiding immune tolerance, adjuvant treatment is carried out using immunosuppressants during renal transplantation. Immuno-

Table 3. Comparison of cytokine levels (pg/mL)

	IL-6	IL-17	IL-23
Test group A	36.59±4.14	72.78±3.05	329.84±4.88
Test group B	121.77±5.02	179.24±16.33	409.68±5.69
Control group A	20.71±3.87	21.33±1.69	208.52±10.57
Control group B	89.86±2.52	36.76±5.27	262.84±8.04
F	1323.428	393.287	1189.084
P	<0.001	<0.001	<0.001

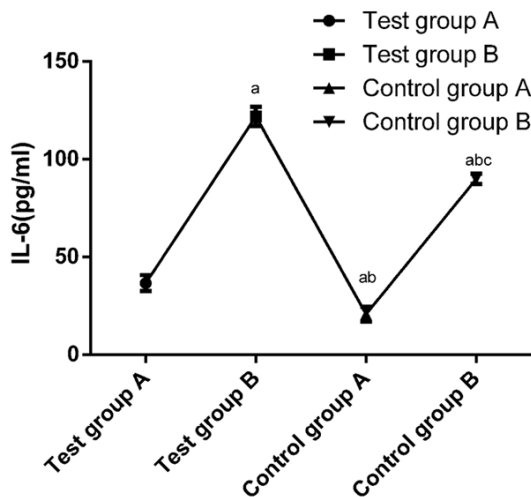


Figure 3. Measurement of serum IL-6 levels in the four groups of rats. Kidney transplantation affects serum IL-6 levels in rats. Serum levels of IL-6 were highest in kidney transplanted rats treated with IL-35 (test group B). ^a*P*<0.05 vs. the level of IL-6 in test group A; ^b*P*<0.05 vs. the level of IL-6 in test group B; ^c*P*<0.05 vs. the level of IL-6 in control group A.

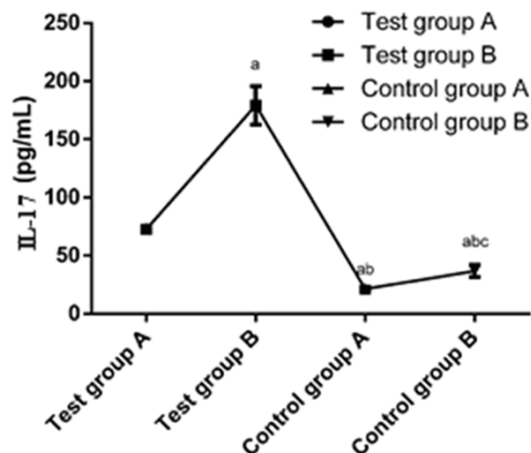


Figure 4. Measurement of serum IL-17 levels in the four groups of rats. Kidney transplantation affects serum IL-17 levels in rats. Serum levels of IL-17 were the highest in kidney transplanted rats treated with IL-35 (test group B). ^a*P*<0.05 vs. the level of IL-17 in test group A; ^b*P*<0.05 vs. the level of IL-17 in test group B; ^c*P*<0.05 vs. the level of IL-17 in control group A.

suppressants mainly target the following pathways: 1) Blocking activation of T-cells; 2) Inhibiting the generation of T-cells; and 3) Inducing the apoptosis of T-cells [6, 13, 14]. Inducing specific immune tolerance in patients has been regarded as a major goal in improving the efficiency of renal transplantation or organ donation in general [15].

However, current knowledge remains insufficient. Mechanisms employed to induce immune tolerance remain unknown. Through a review of the literature [16-18], it was hypothesized that IL-35 may have a role in regulating the activation of T-cells and levels of cytokines. IL-35 can be an effective potential target of immune suppression in organ transplantation. The current study established a renal transplantation rat model, involving IL-35 treatment. Changes in ratios of CD4+CD25+ regulatory T-cells in these rats were determined through flow cytometry. This study also measured levels of Foxp3 and cytokines in the blood of rats using q-PCR. The aim of this study was to determine the effects of IL-35 on regulatory T-cells in rats after renal transplantation. This study further aimed to unravel the mechanisms of IL-35-induced immune tolerance in rats after renal transplantation.

Results of this study demonstrated that IL-35-treated rats had lower levels of Cr and BUN, compared to levels in the non-treated rats. Additionally, rats subjected to renal transplantation showed higher levels of Cr and BUN, compared to levels in control groups. This is consistent with the results of Hueper et al. [19]. Cr and BUN, indicators of renal function, are significantly elevated after renal transplantation. This results from allogenic antigen attack and consequent damage of the kidneys [20, 21]. In the current study, lower levels of Cr and BUN in test group A, compared to levels in test group B, suggested that, after renal transplantation, IL-35 may reverse damaged renal function to protect the kidneys. This indicates a possible correlation between IL-35 and immune suppression. Next, this study determined levels of IL-6, IL-17, and IL-23 in rats of the four groups. Results showed that IL-35 treatment resulted in decreased levels of IL-6 and IL-23, but increased levels of IL-17, compared to levels in the IL-35-untreated group. Hence, results suggest that IL-35 may ameliorate immune suppression of rats after renal transplantation. IL-6

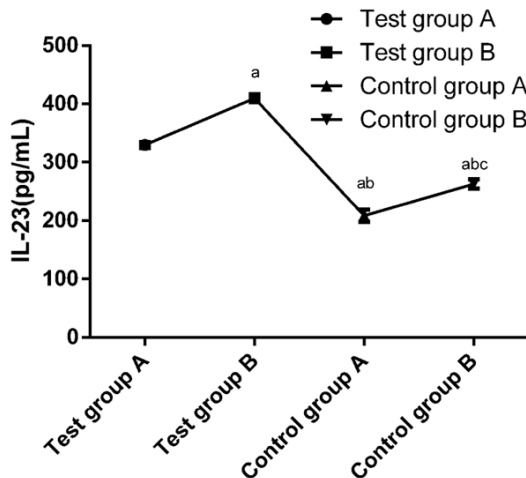


Figure 5. Measurement of IL-23 levels in the four groups of rats. Kidney transplantation affects serum IL-23 levels in rats. Serum levels of IL-23 were highest in kidney transplanted rats treated with IL-35 (test group B). ^aP<0.05 vs. the level of IL-23 in test group A; ^bP<0.05 vs. the level of IL-23 in test group B; ^cP<0.05 vs. the level of IL-23 in control group A.

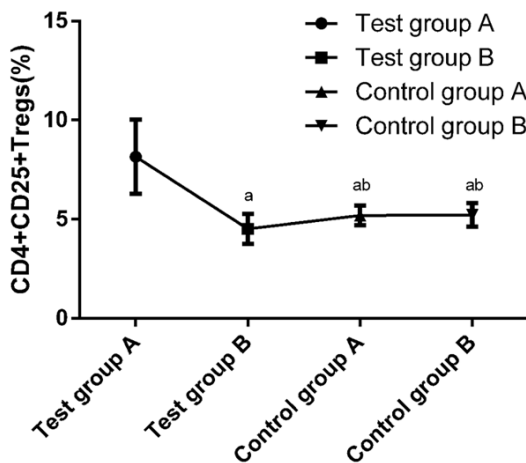


Figure 6. Comparison of ratios of CD4+CD25+ regulatory T-cells in the four groups of rats. Kidney transplantation affects serum CD4+CD25+ regulatory T-cells in rats. The ratios of CD4+CD25+ regulatory T-cells were highest in kidney transplanted rats treated with IL-35 (test group B). ^aP<0.05 vs. the ratios of CD4+CD25+ regulatory T-cells in test group A; ^bP<0.05 vs. the ratios of CD4+CD25+ regulatory T-cells in test group B; ^cP<0.05 vs. the ratios of CD4+CD25+ regulatory T-cells in control group A.

and IL-23 are known factors involved in T-cell-mediated transplant rejection. They could activate T-cells to attack the exogenous cells and induce the maturation of B cells, further exacerbating organ damage [22, 23]. Inflammation

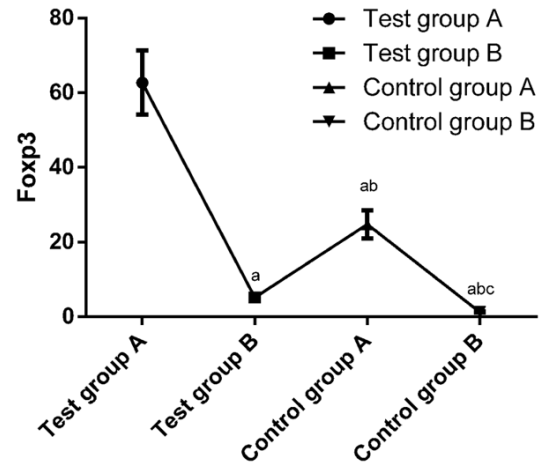


Figure 7. Measurement of Fcpx3 expression levels in the four groups of rats. Kidney transplantation affects Fcpx3 mRNA expression levels in rats. Expression levels of Fcpx3 were highest in kidney transplanted rats treated with IL-35 (test group B). GAPDH was used as the normalizing gene for quantification of Fcpx3. ^aP<0.05 vs. the Fcpx3 expression in test group A; ^bP<0.05 vs. the Fcpx3 expression in test group B; ^cP<0.05 vs. the Fcpx3 expression in control group A.

can also be induced by IL-17-secreting CD4+ T-cells, which further activates the T-cells to produce pro-inflammatory factors [24]. Results of this study implied that IL-35 downregulates levels of IL-6, IL-17, and IL-23, decreasing activation and proliferation of T-cells, thereby protecting the transplant and increasing immune tolerance. The current study also measured ratios of CD4+CD25+ regulatory T-cells of rats in the four groups. Results showed that IL-35-treated rats showed higher ratios, compared to the ratios of untreated rats. This indicates the significance of IL-35 in inducing immune tolerance and maintaining immune balance after transplantation. CD4+CD25+ regulatory T-cells can suppress immune response, through Fcpx3, by inducing immune tolerance and inhibiting acute rejection [25]. Current Fcpx3 quantification showed that IL-35 treatment resulted in the upregulation of Fcpx3 mRNA. Results indicate that IL-35-related immune tolerance is mediated by Fcpx3 in rats after renal transplantation. Fcpx3 is an important factor of regulatory T-cells [26], the induction of which leads to immunosuppression of regulatory T-cells. Hence, IL-35 enhances immune tolerance through upregulation of expression of Fcpx3. This, in turn, facilitates the generation of CD4+CD25+ regulatory T-cells to inhibit the responses of

effector T-cells and other targeted cells. Results showed significant differences in the ratios of CD4+CD25+ regulatory T-cells between the two control groups. It is believed that this may be due to IL-35 suppression in the normal tissues, which is a subject of ongoing study.

The current study aimed to investigate the effects of IL-35 on CD4+CD25+ regulatory T-cells in rats after renal transplant. One limitation of this study is that the effects of IL-35 on other cytokines (IL-2, IL-10, etc.) were not tested. Due to the small sample size, the current study could not use statistical testing to analyze data. The conclusions of this study have to be validated in humans through clinical research.

In conclusion, IL-35 can affect the renal function of renal transplantation rats, as well as expression levels of IL-6, IL-17, and IL-23. Furthermore, potential mechanisms underlying immune tolerance involve IL-35 regulation of CD4+CD25+ regulatory T-cells by targeting Foxp3 in renal transplantation rats.

Disclosure of conflict of interest

None.

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