Study on the effects of regulatory T cells on renal function of IgAN rat model


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Abstract. – OBJECTIVE: CD4+CD25+ regulatory T cells (Tregs) plays a key role in maintaining immune tolerance. IgAN has a close relationship with the immune response. However the significance of CD4+CD25+ T cells to improve renal function of IgAN patients is not clear. The purpose of this study is to evaluate renal function of experimental IgAN rats treated by CD4+CD25+ Tregs cells.

MATERIALS AND METHODS: CD4+CD25+ Tregs were separated from the blood of SD rats by immunomagnetic beads, and amplified in vitro. The amplified in vitro and cultured 2*10⁶ CD4+CD25+ Tregs were infused intravenously into IgAN rat model, 3 times every other day. The serum creatinine, urea nitrogen, urinary protein and red blood cells were detected in the fourth and eighth week. The glomerular damage was evaluated by pathological analysis.

RESULTS: Tregs cells can be amplified largely in vitro. After CD4+CD25+ T cells were infused into IgAN rat model, urine protein and red blood cells were improved. The glomerular injury can be improved by pathological analysis.

CONCLUSIONS: CD4+CD25+ regulatory T cells can significantly improve the symptoms of immunoglobulin A nephropathy (IgAN) rat model, and have clinical application prospect.

Key Words: Immunoglobulin A nephropathy (IgAN), Regulatory T cells (Tregs), Immune suppression.

Introduction

Immunoglobulin A nephropathy (IgAN) is the most common worldwide primary glomerulonephritis. The mesangial IgA deposition is its characteristic. However its pathogenesis is still unclear. The increased serum poly IgA1 (pIgA1) and abnormal structure of low glycosylation are considered as the important pathogenic factors of IgAN at present. Poly IgA1 is generated by polyclonal active B cells. The process of B cells secreting IgA is regulated by T cells1. Therefore, the immune regulatory function disorder of T cells may lead to out-of-control B cells to produce excessive and abnormal IgA, which plays an important role in the pathogenesis of IgAN. CD4*CD25* regulatory T cells (Treg) is a kind of subgroup of inhibitory T cell, and plays an important negative regulatory role in the immune system. It plays an important role in autoimmunity, immune defense and immune surveillance by directly or indirectly inhibiting the proliferations of T cells, B cells, dendritic cells (DC), NK cells and monocytes/macrophages, immune activity and intercellular contact2. In this study, Tregs by mass culture were transfused into IgAN rat model to investigate the regulatory role of IgAN rat model.

Materials and Methods

Isolation and Culture of Natural Tregs

SD rats were purchased from the Animal Center of Henan Province, Zhengzhou, China. The treatment of animal conformed to the animal processing operation manual. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA) Eighth Edition, 2010. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of People's Hospital of Zhengzhou. After the rats were anesthetized with pentobarbital, and the heart was exposed. The blood was extracted from the heart, anticoagulated by heparin. PBMCs were obtained by lymphocyte separation medium density gradient centrifugation method. The magnetic bead separation method was that CD4*CD25* Treg and CD4*CD25+T cells were obtained by two-step separation namely CD4 negative selection and CD25 positive selection. The specific steps were in strict accordance with the instruction of the reagent KitMACS (Bergisch Glad-
Detection of Urine Index
The urine in 24 h was collected after treatment at the end of 4 and 8 weeks respectively. After centrifugation, the supernatant was extracted. The urinary protein in 24 h was determined by Coomassie brilliant blue method. The red cell count in urine sediment was determined under optical microscope (Fuji Photo Film Co. Ltd, Tokyo, Japan).

Pathological Detection of Renal Tissue
The fixed tissue was embedded by paraffin, sectioned and underwent HE staining. The morphological changes of renal tissues were observed under light microscope. The immunofluorescence staining was performed using the direct method, and IgA deposition was observed.

Statistical Analysis
The experimental data were statistically analyzed by SPSS 18.0 software (SPSS Inc, Chicago, IL, USA). The measurement data of normal distribution were represented with mean±standard deviation (x±s). The means in two groups were compared with \( t \) test. The means in multi groups were compared with single factor analysis of variance (One-way ANOVA). Least-significant Difference (LSD) or Dunnett’s \( t \) test was used for two-two comparison. \( p < 0.05 \) indicated the difference had statistical significance.

Results

CD4+CD25+ Treg Can be Rapidly Amplified and the activity in vitro Can be Saved
According to the method of our laboratory, the growth of Treg was selectively stimulated using anti CD3 and anti CD28 coated magnetic beads, IL-2 and rapamycin. After it was cultured for 3 weeks in vitro, Treg rapidly amplified \((1.0*10^9 + 0.15*10^9)\). The number was nearly 2000 times of fresh sorted Treg \((5.23*10^5 + 1.52*10^5)\). Trypan blue staining showed that the cell survival rate was >94%.

High-purity Preservation and Phenotypic Marker of Amplified CD4+CD25+ Treg in vitro
In the cell end product amplified for 3 weeks, the purity of CD4+CD25+FoxP3+ cells was \(93.22 + 5.7\)%, which maintained similar phenotypic features with the freshly sorted Treg. The purity of the latter was \(95.4 + 3.5\)%.

Preparation and Treatment of Rat IgAN Model
From the first day, 400 mg/kg BSA (distilled water into water solution of 4 ml/kg) was used for intragastric infusion every other day, a total of 8 weeks; Castor oil was used for subcutaneous injection 0.3 ml +CCl4 0.1 ml, once a week, a total of 9 weeks; 0.05 mg/ lipopolysaccharide was injected from rat tail vein in the sixth week (0.5 ml solution prepared with 0.9% NaCl). Pathological examination and immunohistochemical analysis confirmed the model as the IgAN rat model. The IgAN rat model was grouped: IgAN untreated group (IgAN by pathological identification, but without treatment), Tregs \((2*10^6\) cells) treatment group (every other day, continuous infusion for 3 times) and control group (without any treatment). There were 8 rats in each group.

Serological Detection
After blood collection, the serum was centrifuged for separation. The blood urea nitrogen (BUN), creatinine (Scr) and other indicators were determined using automatic biochemical analyzer (Hitachi Ltd, Tokyo, Japan).
Determination of Blood Biochemical Index

The rats were executed after treated for 8 weeks. There were no statistically significant differences in blood biochemical indexes between model group and Treg-treated group and that in normal control group ($p > 0.05$) (Table I).

Determination of Urine Red Blood Cell and Urine Protein

The urine red blood cell count and urinary protein quantification in Treg-treated group after treated for 4 weeks were significantly lower than that before treatment, there was significant compared with that in model group ($p < 0.01$), but still increased significantly than that in control group ($p < 0.01$). After treated for 8 weeks, Treg-treated group continued to improve, urine red blood cell count and urinary protein continued to decline, but still in the abnormal range (Table II).

Morphological Change of Kidney Tissue

The light microscope showed that glomerular mesangial cells and substrate were normal in normal control group. No glomerular atrophy, the renal tubular and interstitial structure were normal. The capillary loops opened well. The glomerular mesangial region was slightly broadened in model group and presented to be lobulated. The mesangial cell proliferated and mesangial substrate increased. Partial glomerulus atrophy. There was plenty of inflammatory cell infiltration in renal tubulointerstitial. The renal tubular epithelial cells swelled and degenerated, and lumen was occluded. There was mild hyperplasia in glomerular mesangial cells and mesangial substrate, and renal tubular epithelial cell degeneration in Tregs-treated group. The lumen was partially open. The interstitial inflammatory cell infiltration in Tregs-treated group was slighter than that in model group (Figure 1).

The fluorescence microscope did not show IgA deposition in glomerular mesangial region in normal control group; There was yellow green crumby strong IgA fluorescence in glomerular mesangial area in model group; IgA in glomerular mesangial area displayed yellow green granular fluorescence in IgAN-Tregs treated group, and the intensity was weak compared with that in model group (Figure 1).

Discussion

The autoimmune nature of IgAN has been revealed in the past 10 years. The recruitment of T cells into the kidney is the main feature of glomerular nephritis. CD4+ T helper cells have an important role in regulating immune response. Th17 and Tregs are the new subgroups of CD4+ T cells. Tregs inhibit autoreactive T cells and maintain immune tolerance. Huang et al have found that the number of CD4+CD25+ cells in IgAN

Table I. Comparison of blood biochemical indexes of rats in all groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>BUN</th>
<th>Scr</th>
<th>ALT</th>
<th>AST</th>
<th>TP</th>
<th>ALB</th>
<th>TG</th>
<th>CHOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8</td>
<td>7.67 ± 0.54</td>
<td>29.8 ± 4.23</td>
<td>53.4 ± 18.3</td>
<td>145.2 ± 23.46</td>
<td>62.8 ± 15.02</td>
<td>35.3 ± 2.05</td>
<td>1.35 ± 0.23</td>
<td>1.44 ± 0.18</td>
</tr>
<tr>
<td>IgAN models</td>
<td>8</td>
<td>7.21 ± 0.35</td>
<td>29.3 ± 5.21</td>
<td>59.2 ± 20.1</td>
<td>139.2 ± 25.6</td>
<td>63.5 ± 13.34</td>
<td>30.3 ± 1.82</td>
<td>1.16 ± 0.21</td>
<td>1.26 ± 0.20</td>
</tr>
<tr>
<td>Treg-treated</td>
<td>8</td>
<td>7.20 ± 0.28</td>
<td>29.5 ± 3.52</td>
<td>57.8 ± 18.7</td>
<td>138.4 ± 25.2</td>
<td>58.4 ± 14.38</td>
<td>32.8 ± 2.35</td>
<td>1.20 ± 0.19</td>
<td>1.32 ± 0.23</td>
</tr>
</tbody>
</table>

Table II. Urine red blood cell count and urinary protein quantitative comparison of rats in all groups.

<table>
<thead>
<tr>
<th>Items</th>
<th>Groups</th>
<th>n</th>
<th>Week 0</th>
<th>Week 4</th>
<th>Week 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red cells in urine</td>
<td>Normal</td>
<td>8</td>
<td>27.80 ± 6.82</td>
<td>29.22 ± 6.24</td>
<td>28.45 ± 7.32</td>
</tr>
<tr>
<td></td>
<td>IgAN models</td>
<td>8</td>
<td>181.23 ± 5.68&quot;</td>
<td>245.83 ± 40.35&quot;</td>
<td>238.20 ± 45.32&quot;</td>
</tr>
<tr>
<td></td>
<td>Treg-treated</td>
<td>8</td>
<td>178.34 ± 7.12&quot;</td>
<td>92.78 ± 19.35&quot;</td>
<td>58.45 ± 7.23&quot;</td>
</tr>
<tr>
<td>Urine protein</td>
<td>Normal</td>
<td>8</td>
<td>4.65 ± 1.24</td>
<td>4.09 ± 1.08</td>
<td>4.26 ± 1.36</td>
</tr>
<tr>
<td></td>
<td>IgAN models</td>
<td>8</td>
<td>12.45 ± 3.35&quot;</td>
<td>11.87 ± 2.68&quot;</td>
<td>12.03 ± 2.04&quot;</td>
</tr>
<tr>
<td></td>
<td>Treg-treated</td>
<td>8</td>
<td>11.35 ± 2.36&quot;</td>
<td>8.35 ± 1.52&quot;</td>
<td>7.25 ± 1.24&quot;</td>
</tr>
</tbody>
</table>

Note: "$p < 0.01.$
Study on the effects of regulatory T cells on renal function of IgAN rat model

Patients was less than that in normal people. Wang et al. studied the mice infected by group A streptococcus nasal injection, and confirmed that Th17 was the main T cell induced by infection. The correlation of Th17 reaction and gastrointestinal infection revealed human detrimental autoimmune reaction. Treg and Th17 balance can influence the pathology or results of autoimmune diseases.

Regulatory T cells can be roughly divided into three categories: regulatory T cells (tTregs) derived from thymus, which mainly plays an inhibitory effect on self antigen and is in favour of clonal selection of T cells; inducible regulatory T cells (iTregs), which is mainly produced due to the induction of external stimulation of antigen and plays the immune tolerance against antigen; naturally regulatory T cells (nTregs), which is circulating in the peripheral blood, can interact with effector cells or other cells, and plays an immune inhibitory effect in vivo. The phenotype of inducible Treg and function state are instable. Therefore, the current study focused on natural Treg. NTreg belongs to the CD4+ T cells. The cell surface can highly express IL-2 receptor α chain. The transcription factor FoxP3 is currently recognized specific intracellular marker of nTreg, which plays an important role in the development and function of Treg. Immune suppression is that Treg plays a key role in immune tolerance induction and maintenance by T cell receptor (TCR) mediated signal activation to inhibit other CD4+ and CD8+ T cell activation and proliferation. Therefore, it is the study hotspot of organ transplantation and autoimmune diseases. However, the content of nTreg was very few in human body, and not easy to be amplified, which greatly limited the clinical research and application of Treg. In this study, Tregs were used for sorting by MACS immunomagnetic beads. The high-purity CD4+CD25+Treg was obtained. The activity analysis showed that sorted cell viability by this sorting method was more than 95%. In addition, anti CD3 and anti CD28 monoclonal antibody, IL-2 and rapamycin were added in Treg culture system. Among them, rapamycin can selectively inhibit CD4+CD25+ T cells, avoid the amplification problem in vitro of T effector cells (Teff), ensure the purity of Treg, and induce the inhibitory function of Treg. After cultured for 3 weeks, Treg largely amplified. The cell activity is good, and the main phenotypic marker is kept. This provides an important guarantee for the subsequent animal experiments.

The possibility of cultivated Tregs in vitro to treat IgAN experimental rat model was evaluated in this experiment. After treated by amplified Tregs cells in vitro for 4 weeks, urinary protein and urinary red cells in IgAN rats were significantly improved compared with that in untreated rats. The renal pathological examination showed that renal tissue was improving. The immunohistochemical analysis of IgA showed that the deposition of IgA in the mesangial cells was less than that in IgAN rats. The above results further tended to be normal after IgAN rats were treated by Tregs for 8 weeks. These results implied that the lesion extents of urinary red blood cells, urinary protein and renal tissue could be improved by Tregs in varying degree. The main mechanism was that Tregs migrated to the renal tissue, inhibited T effector cells, reduced the damage of inflammation to kidney, and gradually recovered the renal function, which also further proved that IgAN was an autoimmune disease.

Conclusions

The natural Tregs cells can be largely cultured and amplified in vitro, and renal function of IgAN rat can be restored by its immunosuppressive function. For some autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, Tregs may also have the roles to treat or improve clinical symptoms.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References


