Tocilizumab promotes corneal allograft survival in ratsby modulating Treg-Th17 balance

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Abstract

AIM: To examine the therapeutic effects of tocilizumab on experimental corneal transplantation and its effect on Treg/Th17 balance.

METHODS: Allograft corneal graft was performed between host Sprague Dawley and Wistar donor rats. The rats were randomly divided into four groups, normal, autograft, allograft, and allograft treated with tocilizumab. Kaplan-Meier was performed to draw the survival curve. The protein levels of IL-17A (Interleuki-17A), VEGF(vascular endothelial growth factor), and Foxp3 (Forkhead box protein 3) were measured by immunohistochemistry. The mRNA levels of IL-17A, VEGF, RORγt (Retinoid-related orphan receptor gammat), IL-6 (Interleuki-6) and Foxp3 were detected by real-time polymerase chain reaction (RT-PCR). The Treg and Th17 cells were investigated by flow cytometry.

RESULTS: The survival time of tocilizumab group was (24 ± 1.27)d longer than that of allograft group (10 ± 0.55)d. Moreover, immunohistochemical examination revealed that IL-17A and VEGF protein levels in the allograft group were significantly higher than that of tocilizumab group $(P<0.01)$, while Foxp3 levels in the allograft group was significantly lower than that of the tocilizumab treated group $(P<0.001)$. Flow cytometry showed that the number of Th17 (T helper 17) cells in allograft group was significantly higher than that in tocilizumab group $(P<0.001)$. Meanwhile, the number of Tregs was significantly lower than in tocilizumab group $(P<0.001)$. Simultaneously, Foxp3 mRNA expression level in corneal tissues of tocilizumab treated group was significantly higher than other groups $(P<0.001)$.

CONCLUSION: These findings suggest that tocilizumab may promote corneal allograft survival, possibly by modulating Treg-Th17 balance.

KEYWORDS: Tocilizumab; Corneal Transplantation; Th17/Treg

INTRODUCTION

Corneal transplantation is the most commonlyused treatment for end-stage corneal diseases. Despite cornealimmune privilege and anterior chamber-associated immune deviation, immune rejection is still the major limiting factor for corneal grafting. This is specifically seen in patients with high-risk corneal transplantation during corneal vascularization, repeated transplantation, alkali burn, which had almost less than 54.2% long-term survival rate[1-3]. Therefore, prevention and treatment of corneal graft rejection is still an ongoing and important issue in clinical research. Clinically, medicines that suppress the rejection have been applied to improve corneal transplantation success rate and long-term survival rate of the graft, but are still limited due to drug tolerance in certain patients, reappearance of rejection, high costs and also severe toxic and side effects. Thus, there is a pressing need to screen for immunosuppressive agents with high efficiency and low toxicity. Recently, interleukin-6 (IL-6) has been considered as a key mediator in the pathogenesis of graft-versus-host diseases(GVHD)[4, 5]. The pathway of IL-6/IL-6 receptor (IL-6/IL-6R) signaling regulates various biological process, including cell growth and differentiation, as well as immune and hematopoietic systems[6]. Tocilizumab is a recombinant monoclonal antibody specific to IL-6R, and is originally used for the treatment of rheumatoid arthritis[7]. According to previous studies, tocilizumab could reduce Th17 cell proportion, and increase Treg cell proportion, thus changing the Th17/Treg balance in patients with rheumatoid arthritis[8-10]. Recent reports have demonstrated that tocilizumab may reduce the severity of GVHD in steroid-refractory GVHD[12, 13]. A previous studydemonstrated that tocilizumab could prolong the survival time of islet transplantation and protect the function of islet after transplantation[14]. Some ophthalmological studies also reported that tocilizumab could reduce the formation of corneal neovascularization[15, 16]. However, whether tocilizumab could affect the immune mediated corneal graft rejection is unknown. Hence, this study was conducted.

MATERIALS AND METHODS

Animals and material Wistar rats served as hosts, and accepted corneal grafts from either Sprague-Dawley (SD) rats (autograft) or Wistar rats (allograft).Female rats, aged 6-8 weeks and weighing 180-220 g, were purchased from the Experimental Animal Centre of Southern Medical University.

There are 9 wistar rats in normal group. In each of the other 3 groups, there are 24 wistar rats in each group respectively.

All rats were housed in a specific pathogen-free (SPF) environment. Animal experiments were approved by the Nanfang Hospital Animal Ethics Committee. Anesthesia was carried out by injecting 3% thioethamyl (1.5ml/kg). Tropicamide, a compound of mydriatics, eye drops and 10-0 nylon line was purchased from Ethicon (USA). Tocilizumab was purchased from Roche (Switzerland).The antibodies for immunohistochemistry were purchased from Abcam (UK) and Santa Cruz (USA).The antibodies for flow cytometry were purchased from Ebioscience (USA). RNA extraction, reverse transcription and qPCR kits were purchased from Takara (Japan).

Corneal transplantation and post-transplant therapies Penetrating orthotopic corneal transplantationwas performed as previously described[17]. Briefly, a 3.5-mm central area of the cornea was excised from the donor and secured in the host graft bed of 3.0-mm diameter with 8-10 interrupted 10-0 nylon sutures.The occurrence of hyphema, synechia and cataract in rats during the operation were regarded as failed cases. The failed experimental animals were removed and supplemented with new ones.Four groups were included: normal, autograft, allograft, allograft treated with tocilizumab by tail vein injection intravenously, 2mg(0.1ml) (asGroup Tocilizumab). In the allograft rats group, same amount of 0.9% saline was injected (as Group Allograft). In Wistar rats group, there was no intervention conducted, and served as normal controls. Specific groupingand schematic representation of the experiment wereshown in Table 1.

Rejection observation and judgment standard After the operation, 15 grafts from each group were randomly selected for clinical evaluation of rejection. According to the scoring method of Larkin[18], the corneal rejection score was recorded. Rejection was defined by a total score of not less than 5 points or an opacity score of over 3 points. Long-term survival was defined by no sign of rejection for more than 100 days.

Hematoxylin-eosin staining (H&E) and immunohistochemistry The eyeballs (3 rats for each group)with transplantation were taken out after the rats were sacrificed. These were then fixed with 4% polyformaldehyde solution, a gradient dehydrated with alcohol, followed by embedding in paraffin, and then were cut into 4 um slices. Some slices were selected for staining by hematoxylin-eosin (HE) and the corneal thickness and inflammatory cell infiltration under microscopy were observed. Some slices were used for immunohistochemistry by using rabbit anti-rat IL-17A antibody (sc7927, Santa Cruz), mouse anti-rat VEGF antibody (ab22510, abcam), mouse anti-rat Foxp3 antibody (ab22510, abcam) as primary antibodies. Goat anti-mouse antibody (ab6788, abcam), goat anti-rabbit antibody (ab97049,abcam) were used as secondary antibodies. Experiments that used PBS, instead of primary antibody, were setas negative controls. Using SP three step method, antigen repair by heat was performed. The specimens were observed under microscopy after BAD staining and mounting, and then photographs were taken by using the 400X field of vision(Olympus digital camera, Japan). Using Imagepro-Plus software, the cumulative integrated optical density [IOD(sum)] and corneal tissue area were calculated by the formula: IOD:IOD(mean)=IOD(sum)/Area.

Quantitative reverse transcription-PCR Corneal grafts (3 rats for each group) were taken out after the rats were sacrificed, then total RNA were extracted from the grafts by Trizol and RNA concentration was measure by D260/D280 value using Nanodrop. RNA were then used for cDNA synthesis using Reverse Transcriptase Kit (Cat:RR037A, Takara). Rat GAPDH was used as endogenous control.. The primer pair sequences used for the PCRs were: 5'-ACCACAGTCCATGCCATCAC-3', and 5'-TCCACCACCCTGTTGCTGAT-3' for rat GAPDH, 5'-TGCTGCTACTGAACCTGGAG-3', and 5'-GCGTTTGGACACACTGAACT-3' for rat IL-17A, 5'-GACAGGGCCCCACAGAGA-3', 5'-TTTGTGAGGTGTGGGTCTTCTTT-3' for rat RORγt, 5'-AGTGGCAGGGAAGGAGTGTC-3', and 5'-TTCCAAGTCTCGTGTGAAGGC-3' for rat Foxp3, 5'-GGCCTCTGAAACCATGAACT-3', and 5'-TGAACTTCACCACTTGGCAT-3' for rat VEGF, 5'-ATTCTGTCTCGAGCCCACCA-3', and 5'-GGAAGGCAGTGGCTGTCAAC-3' for rat IL-6. The PCR reaction solution was prepared according to the instructions of the qPCR Kit (Cat:RR420A, Takara) and PCR analysis were carried out with 7500 PCR instrument (ABI, USA). Transcript quantification was performed in triplicate for each sample.

Flow cytometric analysis Fourteen days after operation, 3 rats were randomly selected from each group, anaesthetized by 3% thioethamyl (1.5ml/kg) intraperitoneally, and then 5 ml blood sample was collected from the heart by blood collection tube containing heparin. Lymphocyte was separated in the ultra clean cabinet by lymphocyte separation medium(LTS1083, TBD, China). Each lymphocyte was evenly divided into 2 samples (sample A and sample B). Sample A was placed in a 1640 culture medium containing 10% fetal bovine serum and 4uL(2uL /mL) Cell Stimulation Cocktail (plus protein transport inhibitors) (85-00-4975-93, eBioscience, USA). The lymphocyte was cultured in Cell incubation box at 37 ℃ with 5% CO_2 for 6h. Subsequently, the lymphocytes were collected by centrifugation at 2000 rpm and were divided into 3 tubes (experimental tube lymphocyte, ISO control tube lymphocyte and empty tube lymphocyte), followed by the addition of anti-rat CD4 FITC (empty tube lymphocyte were added nothing) and then placing at 4 ℃ environment for 30min in dark. After that, Foxp3/Transcription Factor Staining Buffer (85-00-5523-00,eBioscience,USA)was added to the lymphocytes. After maintaining for 30 min, the Anti-Mouse/Rat IL-17A PE were added in the experimental tube lymphocytes, Rat IgG2a K Isotype Control PE were added in the ISO control tube, and nothing in the empty tube lymphocytes.

After 30 minutes, the unbinding antibodies were washed by PBS. Finally, the percentage of Th17 (CD4+IL-17A+) in CD4+ cells was tested by BD FACSCalibur (BD, USA). Sample B was also divided into 3 tubes (experimental tube lymphocyte, ISO control tube lymphocyte and empty tube lymphocyte) and no stimulation and culturing were performed. Anti-Rat CD4 FITC was added into experimental tube lymphocytes and Anti-Rat CD4 FITC and Mouse IgG1 K Isotype Control APC were added into Anti-Rat CD25 APC, ISO control tube lymphocytes, and nothing in the empty tube lymphocytes. All the tubes in sample B were maintained at 4℃ for 30min in dark. Then, Foxp3/Transcription Factor Staining Buffer was added to the lymphocytes. After 30 min, t Anti-Mouse/Rat Foxp3 PE were added in the experimental tube lymphocytes, Rat IgG2a K Isotype Control PE were added in the ISO control tube lymphocytes, and nothing in the empty tube lymphocytes. After 30min, the unbinding antibodies were washed away by PBS. Finally, the percentage of Treg (CD4+CD25+Foxp3+) in CD4+ cells was measured by BD FACSCalibur. All antibodies in flow cytometric analysis were purchased from eBioscience company (USA).

Statistical analysis The experimental data were analyzed by IBM SPSS Statistics 20 and GraphPad Prism software. One-way ANOVA was performed to test the variation of corneal neovascularization area, mRNA and protein expression levels,as well as the ratio of Th17/Treg in each group.

RESULTS

Clinical observation of rejection and draw survival curve

After operation, all rats were examined by slit-lamp microscopy on alternate days to calculate the rejective indices (RIs) according to opacity, edema, and neovascularization of grafts.According to thesurvival curve (Figure 2C), the survival time of tocilizumab group was (24 ± 1.27) d,while the survival time of allograft was (10 ± 0.55)d.After mydriasis (Figure 2A), the results showed that the neovascularization area in autograft and allograft groups were 2.21 ± 0.19 mm^2 and 3.31 ± 0.66 mm^2, respectively with Imagepro-Plus software, and were significantly higher than that of tocilizumab group, 0.33 ± 0.17mm^2 ($P<0.01$), (Figure 2B). On day 14 after operation, the intact cornea was cut from the limbus by puncturing the limbus with a bayonet, and stored at -80 ℃.
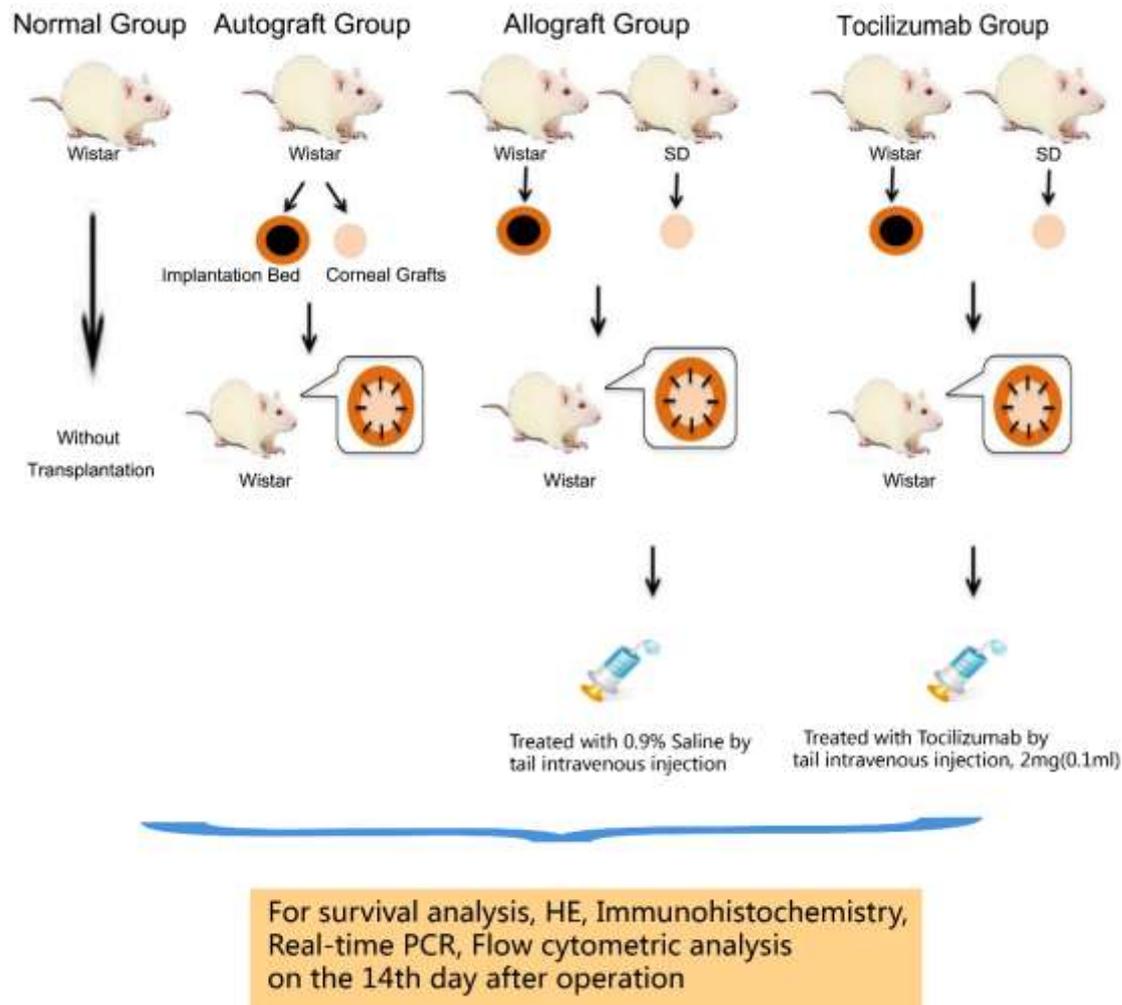
Figure 1. Schematic representation of the experiment.
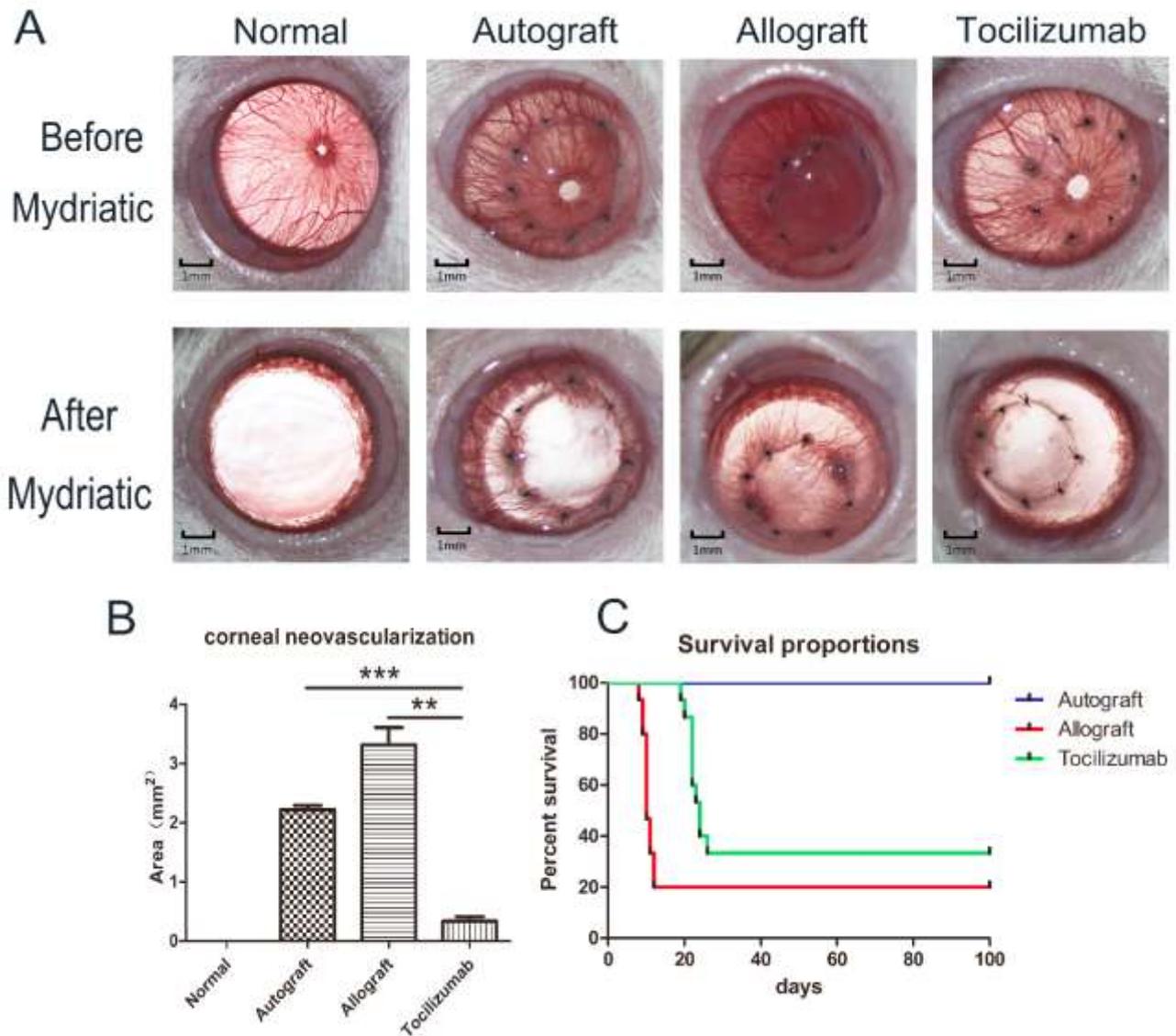
Figure 2. Clinical observation of corneal graft after corneal transplantation (A) The appearance of corneal grafts after transplantation. Fourteen days after transplantation, the opacity, area of neovascularization and edema were observed under the microscopy before and after mydriasis.(B) Corneal neovascularization area in each group after mydriasis (n=15). **$P<0.01$, ***$P<0.001$. (C) Survival percentages after surgery. On postoperative day 100, the corneal graft and the rejection score were recorded for drawing the survival curves (n=15).

Histopathological and immunohistochemical analysis

In the normal group, the structure of the cornea was clear, and there was no blood vessel and lymphocyte infiltration. As shown in figure 3A, 14 days after surgery, the corneal structures in the autograft group were clear, but showed a few new blood vessels and slight inflammatory cell infiltration in the stroma. In the allograft group, the corneal graft was thickened with a large number of lymphocytes infiltrated, and new blood vessels were observed in the stroma. More importantly, the corneal structures in the Tocilizumab group were regular with individual lymphocytes, but without any obvious vascular cavity. Immunohistochemistry revealed that IL-17A and VEGF were mainly expressed in the corneal epithelial and stromal layers, and Foxp3 was predominantly expressed in the nucleus of stromal layer cells. The expressions of IL-17A and VEGF in the cornea of allograft group were significantly higher than that of thetocilizumab group, while the expression of Foxp3 was lower than that of the tocilizumab group. The corneal grafts of autograft group also exhibited high levels of VEGF expression. The results of mean IOD detected by Imagepro-Plus software were presented in Figures3B, 3C, 3D, showing variations in similar expression pattern by visual observation. The mean IL-17A IOD of the allograft group and tocilizumab group were 0.026 ± 0.002 and 0.005 ± 0.001, respectively ($P<0.05$). Moreover, the mean VEGF IOD of autograft group and allograft group were 0.022 ± 0.001 and 0.021 ± 0.002, respectively, while that of the Tocilizumab group was 0.009 ± 0.001 ($P<0.05$). The mean Foxp3 IOD of allograft group was 0.006 ± 0.006, while that of Tocilizumab group was as high as 0.112 ± 0.032 ($P<0.05$).
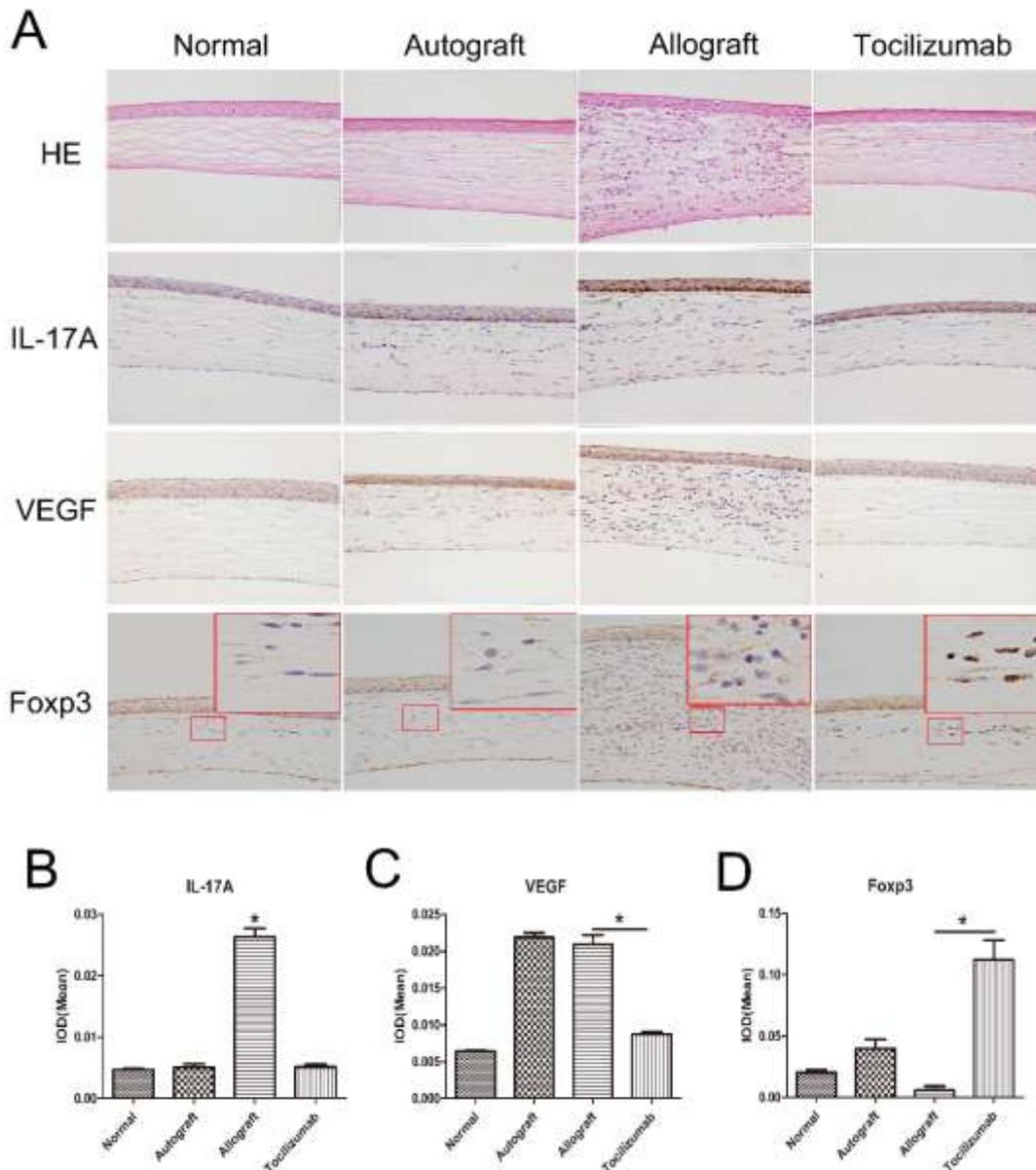
Figure 3 H&E stainingand immunohistochemistry analysis of gene expression (A) H&Estaining and immunohistochemistry paraffin sections under 400 x microscopy. The red box in Foxp3 immunohistochemistry means that the local areas were enlarged by ten times,showing better nuclear staining. (B-D) The cartogram of mean IOD values of IL-17A, VEGF and Foxp3 expressions (n=3).*P<0.05.

The relative mRNA levels of IL-17A, RORγt, VEGF, IL-6 and Foxp3 in corneal grafts.
The expression levels of IL-17A, RORγt, VEGF and IL-6 in the corneal grafts of allograft group were significantly elevated when compared with those of the normal group, and autograft group also showed VEGF accumulation in the cornea. Compared to allograft group, the expressions of IL-17A, RORγt, VEGF and IL-6 in tocilizumab group were all decreased to different extents. The expression of Foxp3 gene in Tocilizumab group was remarkably higher than that of the other three groups(P< 0.001).
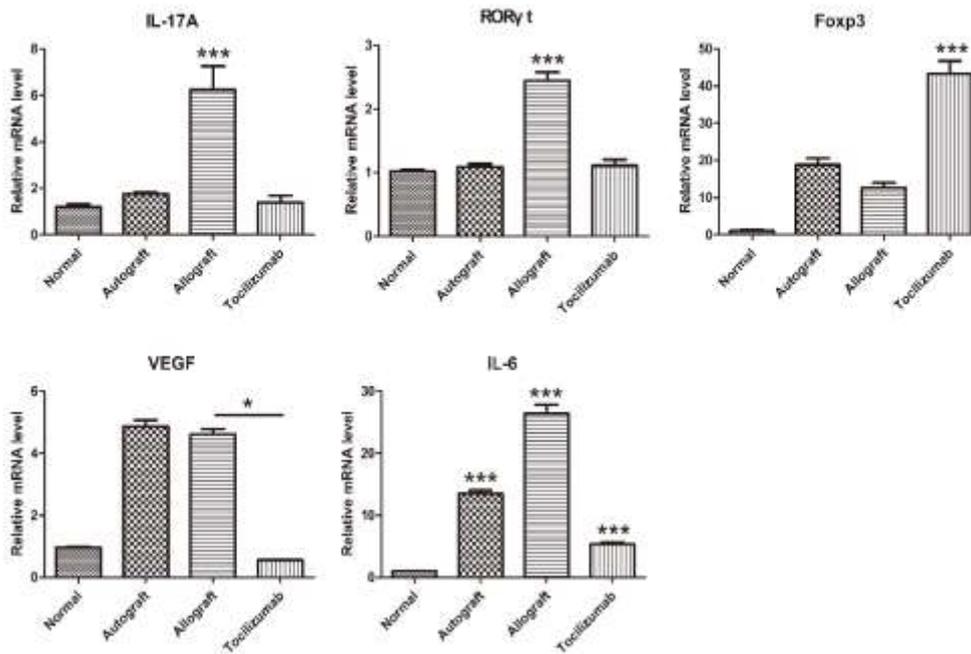
Figure 4 Expression levels of IL-17A, RORγt, Foxp3, VEGF and IL-6 in corneal graft. Fourteen days after transplantation, the relative expression levels of IL-17A, RORγt, Foxp3, VEGF and IL-6 genes in each corneal graft group were shown here (n=3). *$P<0.05$, ***$P< 0.001$.

Flow cytometry analysis

On day 14 after transplantation, the percentages of Th17 cells in CD4+ cells of rat blood between the normal group and autograft group showed no significant differences, which were 1.20±0.19% and 1.40±0.66% respectively. In the allograft group, it was found to be 7.32±1.33%, and was significantly higher than that in normal group ($P<0.001$), while it was only 2.05±0.29% in tocilizumab group, showing no significant elevation ($P = 0.21$). The tocilizumab group had the highest percentage of Treg cells in CD4+ cells of rat blood (7.27±0.21%), followed by normal group (6.96±0.47%) and autograft group (5.80±0.95%). The allograft group had the lowest percentage (4.60±0.20%), showing significant differences when compared with the tocilizumab group ($P<0.001$). The percentages of CD4+CD25highFoxp3+ cells in CD4+CD25+Foxp3+ cells were also different from each group with the tocilizumab group, exhibiting the highest (84.40±1.91%), and are higher than those in the normal group (80.83±0.89%, $P< 0.05$) and the allograft group (76.30±0.91%, $P< 0.001$).
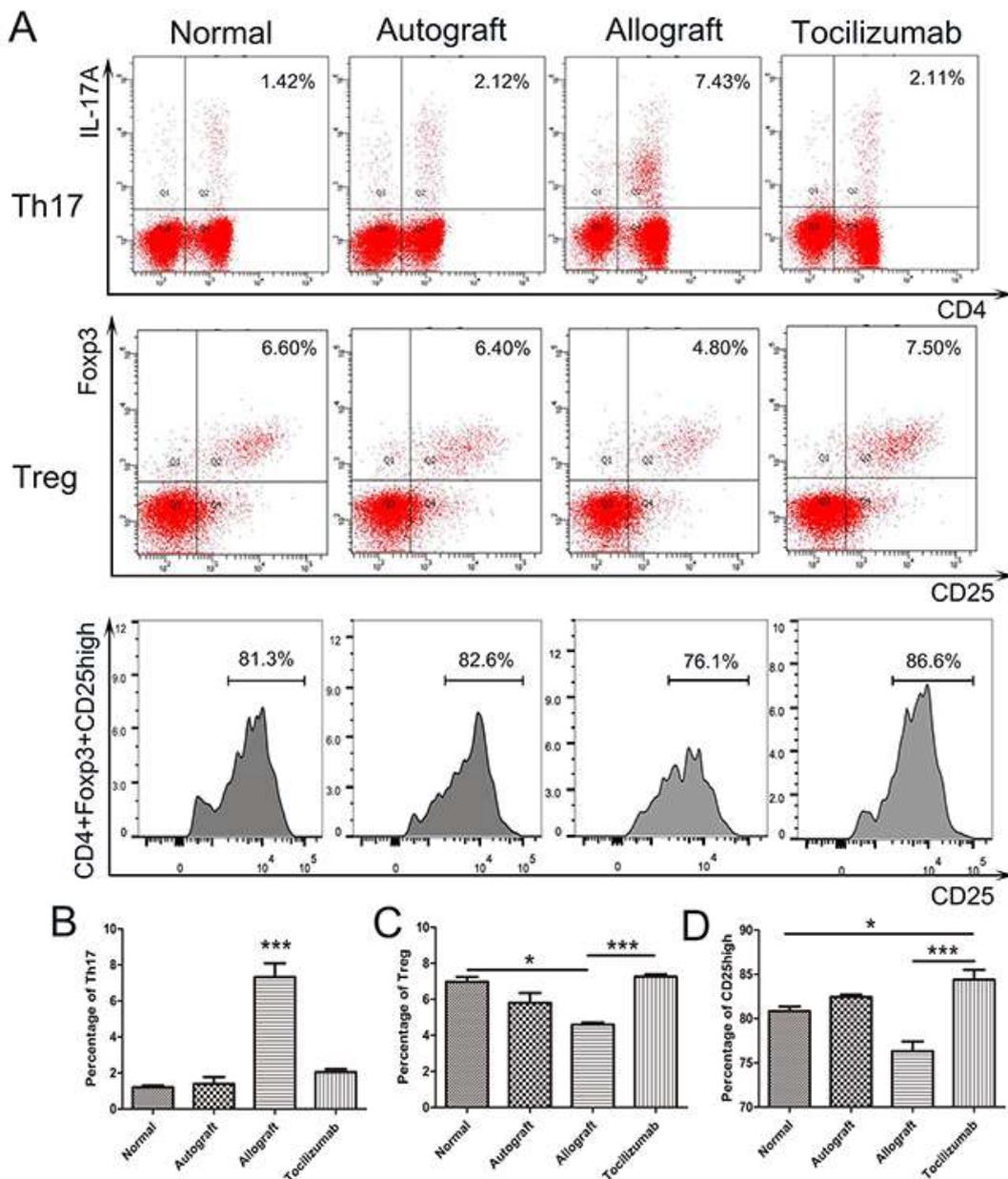
Figure 5 Analysis of blood lymphocytes by flow cytometry on postoperative day 14 (A) Flow type scatter plot of Th17 (CD4+IL-17A+) and Treg (CD4+CD25+Foxp3+) and the cell count of CD4+CD25highFoxp3+ Tregs. (B) Percentages of CD4+IL-17A+ Th17 cells in CD4+ cells (n=3). (C) Percentages of CD4+CD25+Foxp3+ Treg cells in CD4+ cells (n=3). (D) Percentages of CD4+CD25highFoxp3+ Treg cells in CD4+CD25+Foxp3+ Treg cells (n=3). *$P<0.05$,***$P< 0.001$.

DISCUSSION

The immune response following corneal transplantation is a complicated process, and infiltration of inflammatory cells and neovascularization at the implantation site after transplantation are major risk factors of corneal graft rejection[3]. Common immunosuppressive drugs mayeffectively inhibit rejection, but are associated with side effects such as renal and liver toxicities. Tocilizumab is a recombinant humanized monoclonal antibody against IL-6R that specifically binds with IL-6R and blocks signal transduction of IL-6R to STAT3. More interestingly, it is accompanied with less toxic side effects, no immunogenicity and no induction of immune response[7].

The present study found that tocilizumab could significantly prolong the survival time of corneal grafts, shifting the Th17/Treg balance. Th17 cells, which are a subset of CD4 T cells with a role in autoimmunity, have been implicated as main players in the acute phase of allograft rejection[24,29]. Conversely, CD4+CD25+Foxp3+Regulatory T cells (Treg), which maintain immune homeostasis, play a vital role in protecting grafts from immune rejection[27].Studies have shown that IL-6 and TGF-β regulate the differentiation of T helper cell precursors (Thp) into Th17. When IL-6 levels are low, TGF-β induces dphfferentiation of Thp into Treg.[19]. Meanwhile, due to blockage of IL-6 signaling pathway and unaffected TGF-βlevels, Thp cells were inclined to differentiate into Tregs[19, 24].Therefore, we hypothesized that tocilizumab might induce the biological activity loss of IL-6R by specifically binding with IL-6R, thus blocking the IL-6 signaling pathway. It

has beenobserved that interruption of signalling transduction might induce downstream phosphorylation of STAT3, decrease in RORγt expression, eventually decreasing IL-17A expression and reducing Th17 cell number and activity[20-23]. Therefore, tocilizumab could prolong the survival time of the graft by shifting the balance of Th17/Treg cells[24].

Corneal neovascularization is an important risk factor of rejection after corneal transplantation[25]. According to a previous study, IL-17A played an important role in the formation of corneal neovascularization, as it could promote the growth of corneal neovascularization by destroying the VEGF-A/sVEGFR-1 balance in the cornea, and blocking of IL-17A could suppress corneal neovascularization and inflammatory cell infiltration as well[26]. Another study showed thattocilizumab could affect the expression of MMPs and bFGF by reducing the phosphorylation of STAT3, and then by down-regulating the content of VEGF in the cornea, thus suppressing the formation of corneal neovascularization[15]. Therefore, the use of tocilizumab also prevents corneal graft rejection by reducing corneal neovascularization.

Interestingly, we observed that the expression of corneal IL-6 was also reduced when treated with tocilizumab to block IL-6R in this experiment. This may be due to that the Th17 cells could secrete IL-6, and so tocilizumab could reduce the secretion of IL-6 by inhibiting Th17[19]. The reduction of IL-6 in turn reduces the rejection of graft and formation of corneal neovascularization. The results showed that tocilizumab can prolong corneal allograft survival by increasing the proportion of CD4+CD25highFoxp3+Treg cells.

It has been widely acknowledged that IL-17A secreted by Th17 plays a partial role in rejection of liver, kidney and other organs transplantation[28-32]. But in corneal transplantation, the role of IL-17A still remainscontroversial. Our study observed that IL-17A expression was increased in allograft group, while it decreased in tocilizumab group. Therefore, we supposed that IL-17A could promote corneal graft rejection. Some previous studies have claimed that IL-17A promotes transplant rejection, andanti-IL-17 therapy restricts and reverses late-term corneal allo-rejection[24, 33-35]. However, some studies have demonstrated that IL-17A could promote graft survival via promoting immune privilege and anterior chamber associated immune deviation[36-37]. Since tocilizumab can effect numerous cytokines except IL-17A in this study, thus further studies are required toclarify its mechanism.

In conclusion, our findings provide experimental evidences for potential clinical application of tocilizumabin corneal graft. However, the effects of tocilizumab on Th17/Treg balance in vitro were not tested, which should done in future studies.

In summary, tocilizumab may promote corneal allograft survival, possibly by modulating Treg-Th17 balance. This may be a novel approach for inhibiting transplant rejection.

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