Resolvin E1 Inhibits Corneal Allograft Rejection in High-Risk Corneal Transplantation

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PURPOSE. To investigate the effects of Resolvin E1 (RvE1) on corneal allograft rejection in a high-risk corneal allograft transplantation model.

METHODS. High-risk corneal beds were created via placement of intrastromal sutures in the corneas of BALB/c mice for 2 weeks. Allogeneic corneal transplantation was performed by transplanting corneas of C57BL/6 mice onto BALB/c hosts. RvE1 or normal saline (control) was subconjunctivally injected. Allograft survival was observed by slit lamp biomicroscope, and inflammatory cell infiltration was detected by hematoxylin and cosin and immunohistochemistry. The percentage of Th1, Th17, and Treg cells in draining lymph nodes (DLNs) were evaluated by flow cytometric analysis. The levels of Th1, Th2, and Th17-associated cytokines in the grafts were measured by cytoketric bead array and real-time PCR.

RESULTS. RvE1 treatment significantly improved allograft survival compared to the control group. After RvE1 treatment, the infiltration of neutrophils and CD4+ T (Th1/Th17) cells were decreased in corneal grafts, and the percentage of Th1/Th17 cells in DLNs were reduced. In addition, RvE1 treatment significantly reduced the mRNA expression of proinflammatory cytokines in the graft including IL-1α, IL-1β, TNF-α, IL-6, IL-22, INF-γ, IL-17A, IL-17E, IL-21, and IL-22 as well as the protein level of the proinflammatory cytokines, including IL-2, TNE IL-6, IFN-γ, and IL-17. However, RvE1 treatment did not alter the percentage of Treg cells in DLNs and the expression of IL-4, IL-5, and IL-10.

CONCLUSIONS. RvE1 treatment improves allogeneic corneal graft survival in a high-risk corneal transplantation model via inhibiting the Th1/Th17-related inflammation.

Keywords: transplant rejection, Resolvin E1, Th1, Th17

Corneal transplantation is currently the most common treatment for restoring corneal function, but the immune rejection-induced graft failure is a major hurdle for keratoplasty.1 The 5-year survival rate of “low-risk” keratoplasty in the noninflammatory and nonvascularized host beds is approximately 90%.2 However, the survival rate of “high-risk” keratoplasty in an inflamed host bed containing blood and lymphatic vessels is as low as <50% due to immune-mediated rejection.3,4 The graft antigen presenting cells (APCs) induce allosensitization of the recipient primed cells, which are recruited into the graft and produce detrimental effect.5 Both clinical and experimental studies using CD4+ antibodies or CD4+ knockout mice have found that CD4+ T cells play an important role in corneal rejection.6–8 CD4+ T cells are activated by interaction with the major histocompatibility complex (MHC) molecules and subsequently differentiate into four subtypes: Th1, Th2, Th17, and Treg (CD4+CD25+Foxp3+ regulatory T cells). Each subtype produces its own specific cytokines, which contribute to graft inflammation, disorganization, and rejection.9–11

It has been reported that Th1, Th2, Th17, and Treg are involved in acute corneal allograft rejection.12,13 Th1 cells are characterized by the transcription factor Tbet and secrete IFN-γ, which mediates graft rejection by inducing mononuclear cells infiltration into the grafts.14 Th2 cells express the transcription factor GATA-3 and produce IL-4, IL-5, and IL-13, which are associated with transplantation tolerance.13,14 Th2 cells are mainly associated with eosinophil infiltration into the grafts.15,16 Recently, Th17 cells, characterized by their transcription factor RORγt, have been found to play an important role in graft immunity by secreting many cytokines such as IL-17, IL-17F, IL-21, and IL-22.15 Most importantly, IL-17, a strong proinflammatory cytokine, has been reported to induce leukocyte infiltration, promotes chemokine expression, and mediates tissue inflammation in allograft rejection.16–18 Treg cells express the transcription factor Foxp3 and produce IL-10 and TGF-β. Treg cells play an important role in the regulation of immune homeostasis, and promote immunologic tolerance from corneal allografts.19,20

Resolvins are lipid mediators that are biosynthesized from the essential dietary omega-3 polyunsaturated fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Resolvins have been found to reduce acute inflammation and regulate immunity.21 Resolvin E1 (RvE1; 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid) is produced via the oxidation of EPA.22 RvE1 has been shown to possess a variety of mediates anti-inflammatory actions both in vitro and in vivo.23–25 Recently, RvE1 has been reported to suppress immune responses by reducing polymorphonuclear cells (PMNs) infiltration in acute corneal inflammation, downregulating CD4+ T cells, and inhibiting the release of cytokines.26–27 Recently, RvE1 has been found to improve renal...
allograft survival via IL-17 in acute allograft rejection, and resolvin D1 (RvD1) reduces dendritic cell (DC) maturation and suppresses alloimmunity in corneal transplantation.28,29 However, the effect of RvE1 on immune responses in keratoplasty remains unclear.

In this study, we aimed to investigate the effect of RvE1 on corneal allograft rejection in a murine high-risk corneal transplantation model, and to study its role in regulating CD4+ T cells and their cytokines.

**MATERIALS AND METHODS**

**Animals**

Six to eight-week-old male BALB/c (H-2d) and C57Bl/6 (H-2b) mice were obtained from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China). Animals were housed in a specific pathogen-free environment. All experimental procedures and study protocols were approved by the Institutional Animal Care and Use Committee of Chongqing University of Medical Sciences. The use of animals was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**High-Risk Allogeneic Corneal Transplantation**

To create high-risk host beds, three interrupted intrastromal sutures (11–0 nylon, MANI, Inc., Japan) were inserted into the central cornea of recipient BALB/c mice to induce inflammatory lymphangiogenesis and hemangiogenesis. Sutures were placed for 2 weeks, and corneal transplantation performed as previously described.30 Briefly, the central corneas of 2 mm in diameter were excised from donor C57Bl/6 mice, and secured in the recipient high risk graft beds of BALB/c mice with eight interrupted sutures (11–0 nylon, MANI, Inc.). The anterior chamber of the eye was restored with air. Antibiotic ointments were applied to the eyes postoperatively. The corneal allograft transplantation was repeated three times, with 96 mice in each experiment. A total of 32 animals were excluded, which developed complications, consisting of cataract, anterior chamber loss, peripheral anterior synechiae, persistent hyphema, or infection. New models were supplemented.

BALB/c mice with allogeneic corneal transplantation were randomly assigned to two groups: the RvE1 group receiving 10 μL RvE1 (1 μg/10 μL) (Toronto Research Chemicals, Ontario, Toronto, Canada) and the control group receiving the same volume of normal saline. RvE1 or saline were subconjunctivally injected on days 0 and 7 posttransplantation as previously described.29 BALB/c mice receiving corneas from BALB/c mice were used as syngeneic controls, and treated with normal saline.

**Allografts Assessment of Graft Survival**

All grafts were examined at least three times a week for 8 weeks, using slit-lamp biomicroscope. Graft rejection was defined by the presence of opacity and unclear iris (score >2) according to a standardized scale (0 to 5+).31 To exclude primary graft failure, grafts with scores greater than or equal to 2+ at 1 week after transplantation were excluded from this study. Mice with infection, hyphema, and cataract or other surgery-associated complications were excluded.

**RNA Isolation and Reverse Transcriptase (Real-Time) PCR**

At 3 weeks after surgery, six recipient mice in each group were killed. Corneas were carefully removed without containing the conjunctival and iris tissues. Total RNA was isolated from whole-thickness corneas using the TRizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was reverse transcribed into complementary DNA using PrimeScript RT reagent kit (Takara, China). Then, real-time PCR was performed in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primers used in the study were listed in the Table. All primers were obtained from Chong Qing Sheng Gong Company (Chong Qing, China). The relative expression of each target mRNA was analyzed by normalizing the expression of GAPDH using the 2−ΔACT method.

**Flow Cytometry**

The submandibular and cervical lymph nodes from recipient mice (n = 6) of each group were obtained on day 21 after surgery. Single cell suspensions were prepared by pressing the lymph node tissues with the plunger of a 5-mL syringe. Then, cell suspension was passed through a 40-μm cell strainer (BD Pharmingen, San Jose, CA, USA). Cells were then washed,
centrifuged, and resuspended in PBS. Cell concentration was adjusted to 3 × 10^6/mL and used for the following experiments. Cells from each sample were divided into three parts. In one part, cells were incubated with FITC-conjugated mouse anti-CD4 (eBioscience, San Diego, CA, USA) and phycoerythrin (PE)-conjugated mouse anti-CD25 (eBioscience) antibodies for 30 minutes at 4°C in the dark. Cells were then fixed and permeabilized using the Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's instructions, and stained with PE-Cy5 anti-mouse Foxp3 mAb (eBioscience) for at least 30 minutes at 4°C. For the other two parts, cells were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Corp., St. Louis, MO, USA) and 1 μg/mL ionomycin (Sigma-Aldrich Corp.) for 4 hours at 37°C, followed by incubation with 10 μg/mL Brefeldin A (Medchemexpress, NJ, USA) for 1 hour at 37°C. Cells were then incubated with mouse APC-conjugated anti-CD3 (eBioscience) and FITC-conjugated anti-CD8a (eBioscience) antibodies for 30 minutes. Cells were washed, fixed, permeabilized, and stained with PE-conjugated anti-IL-17A and IFN-γ antibodies (eBioscience) for another 30 minutes. Finally, the samples were resuspended with a flow cytometry staining buffer and analyzed in a FACSCalibur flow cytometer (BD Bioscience-Pharmingen, San Diego, CA, USA) by using Flowjo software (Treestar, Inc., Ashland, OR, USA).

**Histological Evaluation and Immunohistochemistry**

At 3 weeks after surgery, eyeballs were enucleated and fixed with 4% paraformaldehyde solution for 24 hours. The tissues were embedded in paraffin. The paraffin-embedded tissue sections (4–6 μm thick) were cut. For histological examination, the sections were stained with hematoxylin and eosin (H&E), and examined under a light microscope. For immunohistochemistry, the sections were deparaffinized and rehydrated, and examined under a light microscope. Sections without endogenous peroxidase activity. Sections were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour. The samples were visualized with diaminobenzidine (DAB) solution and viewed using a light microscope. Sections without incubation with primary antibodies were used as negative controls. The histopathological score data of corneas in each group were calculated by a second independent examiner, who was blinded to the initial score. The positive levels of IL-17 and IFN-γ cells were defined by the percentage of positive cells and the intensities of staining (0 to 7).

**Statistical Analysis**

All analyses were performed using the GraphPad Prism software (GraphPad Prism Software, Inc., San Diego, CA, USA) and SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA). Data are presented as mean ± SEM. The survival probability was estimated by using Kaplan–Meier survival curves and evaluated by the log-rank test. Dunnnett’s multiple comparison tests and ANOVA or Student’s t-test were used to compare the difference among groups. A P value < 0.05 was considered statistically significant.

**RESULTS**

**RvE1 Treatment Promotes the Survival of Corneal Allograft**

To investigate whether RvE1 promoted the survival of transplanted corneas in the high-risk corneal transplantation model, we examined the opacity of the corneal grafts and scored for 8 weeks using slit-lamp biomicroscope. Kaplan–Meier survival curve analysis showed 100% survival was observed in corneal grafts from the syngeneic group at 8 weeks for after transplantation (Fig. 1A). However, on postoperative day 21, the grafts in the control group resulted in a rejection rate of almost 100% with pronounced opacity, edema, and neovascularization (mean survival times [MST], 14.25 ± 5.06 days) and RvE1 treatment significantly improved the survival rate (MST, 30.25 ± 13.79 days, P < 0.001). In the control group, the cornea exhibited pronounced opacity and edema, and numerous new vessels grew into the center of the grafts on postoperative day 21 (Fig. 1B). In the RvE1 and syngeneic groups, only a few small vessels in the graft limbus were observed in the cornea, with mild edema on postoperative day 21 (Fig. 1B).

**RvE1 Treatment Reduces Inflammatory Infiltration and Edema in the Allogeneic Grafts**

Inflammatory cells such as neutrophils, macrophages, CD4+ T cells, and CD8+ T cells were predominant in corneal allografts. We examined whether RvE1 reduced inflammatory cell infiltration in the high-risk corneal transplantation model, using H&E staining. At 3 weeks after corneal transplantation, a large number of inflammatory cells and obvious edema were observed in the corneal stroma in the control group (Fig. 2A). Inflammatory cells and edema were reduced in the corneal stroma in RvE1 group (Fig. 2A). In the syngeneic group, no obvious edema and inflammatory cell infiltration in the cornea were found (Fig. 2A). Moreover, more neovascularization was found in grafts stroma in the control group than in the RvE1 group (Fig. 2A).

**RvE1 Reduces CD4+ T Cell Infiltration in the Corneal Grafts**

We then investigated whether RvE1 treatment reduced CD4+ T cell infiltration in the corneal grafts. The immunohistochemical results showed that massive CD4+ T cells were found in the epithelial and stroma in the control group (Fig. 2B). RvE1 treatment reduced the number of CD4+ T cells in the cornea.
FIGURE 1. RvE1 treatment promoted survival of corneal allograft in the high-risk corneal transplantation model. (A) High-risk graft beds in BALB/c mice were transplanted with cornea from BALB/c mice (syngeneic control), and from C57BL/6 mice treated with vehicle (control) or RvE1. Kaplan-Meier survival curves were plotted to evaluate the survival of the grafts during 8 weeks (*P < 0.001). RvE1 versus control. (B) Slit-lamp biomicroscope showing corneal opacity, edema, and neovascularization of corneal grafts on day 21 postoperation.

FIGURE 2. RvE1 treatment inhibited inflammatory cell infiltration and edema at day 21 after corneal allogeneic transplantation. (A) Representative H&E staining in the corneas from the control, RvE1, and syngeneic groups. Magnification ×400. (B) Representative immunohistochemical staining for CD4 in the corneal from the control, RvE1, and syngeneic groups. (C) Real-time PCR results showed the relative mRNA expression of CD4 in the corneal from the control, RvE1, and syngeneic groups. *P < 0.05, **P < 0.01, ***P < 0.001 versus control. Arrow shows CD4⁺ T cells.
Only few CD4\(^+\) T cells were observed in the corneal in the syngeneic group (Fig. 2B). Real-time PCR results showed on day 21 after transplantation, the CD4 expression in the corneal draft was significantly decreased in the RvE1 group compared with the control group (*\(P < 0.001\); Fig. 2C).

**RvE1 Inhibits Induction of Th1 and Th17 Cells in the Draining Lymph Nodes (DLNs)**

At 21 days after transplantation, we examined whether RvE1 treatment reduced the induction of alloimmunity by quantifying the number of CD3\(^+\)CD8a\(\text{IFN-}\gamma^+\) Th1 cells and CD3\(^+\)CD8a\(\text{IL-17A}^+\) Th17 cells in the isplateral draining lymphoid tissues of the cornea, using flow cytometry (Fig. 3). Compared with the syngeneic group, the percentage of Th1 cells and Th17 cells were significantly higher in the control group (*\(P < 0.01\); Fig. 3). RvE1 treatment significantly reduced the percentage of Th1 cells and Th17 cells (*\(P < 0.001\); Fig. 3).

**RvE1 Suppresses Th1 and Th17 Cells in the Grafts**

At 3 weeks after transplantation, the immunohistochemical staining showed that more IL-17-immunopositive cells and IFN-\(\gamma\)-immunopositive cells were found in the corneal epithelium and stroma in the control group. Less IL-17-immunopositive cells and IFN-\(\gamma\)-immunopositive cells were observed in the cornea in the RvE1 group (Fig. 4). Few IL-17-immunopositive cells and IFN-\(\gamma\)-immunopositive cells were found in the syngeneic group (Fig. 4). The mean histopathological scores of the corneas in different groups showed signs of graft rejection in three groups 21 days after the corneal transplantation.

**RvE1 Reduces the Expression and Secretion of Proinflammatory Cytokines in Corneal Grafts**

At 21 days after transplantation, we measured the expression of Th1-, Th2-, and Th17-related proinflammatory cytokines in corneas from the control, RvE1, and syngeneic groups, using quantitative real-time PCR. Compared with the syngeneic group, the corneal expression of IL-1\(\alpha\), IL-1\(\beta\), TNF-\(\alpha\), IL-2, IFN-\(\gamma\), IL-17A, IL-17E, IL-21, and IL-22 was significantly increased in the control groups (*\(P < 0.05\); Figs. 5A, 5B). RvE1 treatment significantly reduced the expression of these cytokines in the cornea (*\(P < 0.05\); Figs. 5A, 5B). In contrast, RvE1 treatment significantly increased the corneal expression of IL-4 (*\(P < 0.05\)), reduced the corneal expression of IL-6 and IL-13 (*\(P < 0.05\)), and did not alter the expression of IL-5 (*\(P > 0.05\)). Moreover, we examined the production of Th1-, Th2-, and Th17-related cytokines in the corneas in the control, RvE1, and syngeneic groups at 3 weeks using CBA Mouse Th1/Th2/Th17 Kit. The production of IL-2, IL-6, TNF-\(\gamma\), and IL-17A in the control group was significantly increased compared with the syngeneic group (*\(P < 0.05\); Figs. 5A, 5B, 5D). The expression of IL-4 and IL-10 was not significantly different among groups (Fig. 5D).

**RvE1 Does Not Alter the Treg Population After Transplantation**

An increase in FoxP3 expression on CD4\(^+\) T cells in the DLN has been reported to be positively related with the survival of cornea grafts. We investigated whether RvE1 treatment influenced the number of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) Treg cells in
the DLN of transplanted corneas in the high-risk corneal transplantation. Flow cytometric analysis showed that at 3 weeks posttransplantation, there were no significant differences in the percentage of Treg cells (CD4^+CD25^+FoxP3^+) in DLN among the control, RvE1, and syngeneic group (Figs. 6A, 6B). At 3 weeks after transplantation, IL-10 expression and secretion were not significant differences among the three groups (Figs. 5D, 6C), and there were no significant differences in the expression of FoxP3 in the DLN among the three groups (Figs. 6C, 6D).

**DISCUSSION**

Corneal blindness is the second ophthalmopathy that may lead to blindness in the world after cataract, and corneal allotransplantation is the most effective treatment. The success rate of keratoplasty is not as good as expected due to immunological rejection, especially in the high-risk transplantation model. Therefore, it is important to improve the survival rate of corneal grafts in keratoplasty. In this study, we found that RvE1 treatment improved survival of corneal grafts in a high-risk corneal transplantation model. Our study suggests RvE1 may be used for corneal allografting.

RvE1 plays an important role in reducing recruitment of CD4^+ T cells and neutrophils, suppressing vascular inflammation, and inhibiting proinflammatory cytokine secretion. It has been reported that BLT1/ChemR23 regulates the anti-inflammatory functions of RvE1. RvE1 reduces PMN infiltration and transendothelial migration via BLT1, and inhibits APC functions by targeting DC migration via ChemR23. RvE1 reduces IFN-γ production and blocks proinflammatory signals such as TNF-α-driven NF-κB activation via ChemR23. In the present study, we found that RvE1 treatment significantly increased the survival rate of corneal grafts following high-risk keratoplasty. Histological analysis showed that RvE1 decreased neutrophil infiltration, reduced CD4^+ T cells, and alleviated edema in grafts, suggesting that RvE1 decreased infiltration of CD4^+ T cells and thus prevented allograft rejection.

IFN-γ secreting T cells, especially Th1 cells, have been reported to play a proinflammatory role in corneal allograft rejection. Th1 cells suppress the Treg cells expansion, recruit neutrophils and monocytes, and secrete Th1-related cytokines such as IL-17A at the early stage of allograft rejection. Anti-IL-17 antibodies greatly improve the survival rate of corneal grafts and inhibit the production of inflammatory cells and cytokines in corneal grafts. In the T-bet (−/−) mice model, neutralization of IL-17 inhibits cardiac allograft rejection and vasculopathy. These findings indicate that Th1 cells and Th17 cells play a key role in allograft rejection. In this study, we also found that RvE1 inhibited allograft rejection by decreasingTh1/Th17 cell infiltration and reducing secretion of cytokines in corneal grafts, suggesting that RvE1 may reduce allograft rejection by acting on Th1/Th17 cells. Following allosensitization and activation, Th1 and Th17 cells can move from the graft to the regional draining lymphoid tissues. In this study, the flow cytometric and immunohistochemical analyses showed that RvE1 treatment reduced Th1-cells and Th17-cells differentiation in the DLNs, and suppressed Th1/Th17 cell infiltration into the grafts. These findings suggest that RvE1 treatment can postpone the development of corneal immuno-rejection by inhibiting Th1/Th17 cell infiltration and RvE1 has a protective effect on allograft rejection. These results are consistent with other studies showing that RvE1 suppresses Th1/Th17 cells in other diseases such as renal transplantation and herpes simplex keratitis.

To investigate further the mechanism of RvE1 in corneal allografting, we analyzed the mRNA expression and protein level of various cytokines in corneal grafts. We found that RvE1 treatment downregulated the mRNA expression of proinflammatory cytokines including IL-1α, IL-1β, TNF-α, IL-6, IFN-γ, IL-17A, IL-17F, IL-21, and IL-22 at 3 weeks after corneal transplantation, when almost all the grafts in the control group were rejected. Furthermore, RvE1 treatment reduced the protein levels of inflammatory cytokines such as TNF, IL-2, IFN-γ, and IL-17. IL-1 (IL-1α and IL-1β) is a potent proinflammatory cytokine that is produced by monocytes and macrophages, and neutralizing its activity suppresses APC migration in the corneal. IL-1β and TNF-α induce the
FIGURE 5. RvE1 inhibited the expression and secretion of proinflammatory cytokines after corneal allogeneic transplantation. (A, B) RT-PCR results showing the relative expression of Th1/Th17-related cytokines TNF-α, IL-1α, IL-1β, IL-2, and IFN-γ (A), IL-17A, IL-17F, IL-21, and IL-22 (B), and IL-4, IL-5, IL-6, and IL-13 (C) in the corneas from the control, RvE1, and syngeneic groups. (D) CBA results showed the production of proinflammatory cytokines, TNF, IL-2, IL-4, IL-6, IL-10, IFN-γ, and IL-17A in the corneas from the control, RvE1, and syngeneic groups. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.
secretion of IFN-γ by triggering Th1 response. IFN-γ is a proinflammatory cytokine that upregulates the expression of MHC class I and II, and contributes to corneal rejection. IL-2 is a proinflammatory factor produced by Th1 cells. IL-6 is a proinflammatory factor in a variety of irritating eye diseases, and is found to be upstream of IL-17 in the Th17 differentiation pathway. IL-17A recruits T cells and neutrophils, and promotes the Th1-related immunity. Our results suggest that RvE1 treatment improves the survival of corneal grafts via suppressing the Th1/Th17 immune responses.

Th2 and Treg cells contribute to the transplantation tolerance by a wide range of regulatory mechanisms. Although RvE1 has been reported to increase the expression of anti-inflammatory cytokine IL-10, we found that RvE1 treatment did not alter the RNA and protein levels of IL-10. This discrepancy may be attributed to different animal models of corneal transplantation and different experimental conditions between the two studies. In addition, RvE1 treatment did not significantly change the mRNA expression of Foxp3 and IL-5 and the percentage of Treg cells in DLNs. Taken together, our findings suggest that RvE1 did not alter Th2/Treg-related immune responses.

In conclusion, we found that RvE1 treatment significantly improved the survival of corneal grafts and inhibited corneal allograft rejection via inhibiting infiltration of neutrophils and CD4+ CD25+Foxp3+ (Treg) cells into the graft and upregulating proinflammatory cytokines. These findings suggest that RvE1 may be a new treatment for corneal allograft rejection, and further experiments need to be performed to compare with other drugs and the effects of neovascularization and lymphatic vessels.

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FIGURE 6. RvE1 treatment did not affect the CD4+CD25+Foxp3+ (Treg) cells in LN. (A) Representative flow cytometric plots showing the staining of CD25 and Foxp3 on CD4+ cells from the LN in the control, RvE1, and syngeneic groups. (B) The percentage of CD4+CD25+Foxp3+ (Treg) cells. (C, D) RT-PCR results showing the relative IL-10 expression in the cornea and Foxp3 expression in LN. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.


