

# NF- $\kappa$ B *c-Rel* Is a Potential Therapeutic Target for Acute Corneal Transplant Rejection

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**Received:** August 27, 2023

**Accepted:** October 21, 2023

**Published:** November 14, 2023

Citation: Zheng Q, Liu R, Jiang B, Sun J, Wang T, Ruan Q. NF- $\kappa$ B *c-Rel* is a potential therapeutic target for acute corneal transplant rejection.

*Invest Ophthalmol Vis Sci.* 2023;64(14):16.

<https://doi.org/10.1167/iovs.64.14.16>

**PURPOSE.** The purpose of this study was to determine the role of nuclear factor kappa B (NF- $\kappa$ B) *c-Rel* during acute corneal transplant rejection and whether targeting *c-Rel* can reduce corneal transplant rejection.

**METHODS.** Allogeneic corneal transplantation was performed in wild-type and *c-Rel*-deficient mice. Corneal graft survival rate, opacity, neovascularization, and edema were evaluated by slit-lamp microscopy. Adeno-associated virus 6 (AAV6) expressing *c-Rel*-specific small hairpin RNA (AAV6-shRel) and the small-molecule compound pentoxifylline (PTXF) were used to reduce *c-Rel* expression. Enzyme-linked immunosorbent assay was used to determine the expression of inflammatory cytokines. *c-Rel* expression was determined by quantitative RT-PCR and western blot. The effect of *c-Rel* inhibition on corneal transplant rejection was examined using a mouse model of acute allogeneic corneal transplantation. Tear production and corneal sensitivity were measured to determine the potential toxicity of AAV6-shRel and PTXF.

**RESULTS.** The expression of *c-Rel* and its inflammatory targets was increased in both mice and patients with corneal transplant rejection. Loss of *c-Rel* reduced corneal transplant rejection in mouse. Both AAV6-shRel and PTXF were able to downregulate the expression of *c-Rel* and its inflammatory targets in vitro. Treatment with AAV6-shRel or PTXF reduced corneal transplant rejection in mouse and downregulated the expression of inflammatory cytokines in peripheral blood mononuclear cells from patients with corneal transplant rejection. Treatment with AAV6-shRel or PTXF displayed no side effects on tear production or corneal sensitivity.

**CONCLUSIONS.** Increased expression of *c-Rel* is a risk factor for acute corneal transplant rejection, and targeting *c-Rel* can efficiently reduce corneal transplant rejection.

**Keywords:** corneal transplantation, inflammation, shRNA, adeno-associated virus, transcription factors

Corneal transplantation is essential for treating irreversible corneal blindness caused by severe infection and chemical injury. However, even though cornea is an immune-privileged tissue, corneal graft rejection remains the major complication following corneal transplantation.<sup>1</sup> The survival of corneal grafts varied from ~95% at 1 year to ~73% at 5 years.<sup>2-4</sup> The 2-year survival of grafts placed on “high-risk” recipients was less than 50%.<sup>5-7</sup> In inflamed corneas, human leukocyte antigens (HLAs) are expressed on epithelial, stromal, and endothelial cells.<sup>8,9</sup> After corneal transplantation, the expression of pro-inflammatory cytokines, chemokines, cellular adhesion molecules, and angiogenic factors is upregulated.<sup>10-13</sup> Effector T cells produce pro-inflammatory cytokines, resulting in cornea opacity, increased neovascularity, and destruction of corneal epithelial cells.<sup>10,11,14-16</sup> Although a number of risk

factors contribute to loss of the immune privilege of the cornea, inflammation remains the most important high-risk factor for corneal transplant rejection.<sup>10,17,18</sup>

The primary therapy strategy for treating corneal transplant rejection is the use of anti-inflammatory and immunosuppressive drugs, including corticosteroid and cyclosporine A.<sup>7,19</sup> However, although those immunosuppressive therapies have demonstrated benefits in preventing acute graft rejection, the risk of corneal graft rejection remains as high as 40% to 90% in high-risk recipients.<sup>20</sup> Additionally, significant side effects are often associated with the use of those immunosuppressants. In recent years, gene therapy has been widely used to treat various diseases, including corneal transplant rejection.

*c-Rel* is a member of nuclear factor kappa B (NF- $\kappa$ B) transcription factor family. Unlike other NF- $\kappa$ B proteins that

are expressed in a variety of cell types, *c-Rel* is preferentially expressed in activated lymphocytes and monocytes and promotes the expression of various pro-inflammatory cytokines such as interleukin (IL)-23,<sup>21</sup> tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ),<sup>22</sup> IL-17A,<sup>23</sup> IL-2,<sup>24</sup> IL-6,<sup>25</sup> IL-1 $\beta$ ,<sup>26</sup> and interferon- $\gamma$  (IFN- $\gamma$ ).<sup>27</sup> In addition to playing important roles in normal immune cell function, the *c-Rel* gene is a susceptibility locus for certain autoimmune diseases. Because *c-Rel*-deficient mice are resistant to the development of a variety of inflammatory diseases and do not suffer from developmental problems or infectious diseases, *c-Rel* has become an attractive drug target for the treatment of inflammatory disease. Indeed, we have previously reported that targeting *c-Rel* using small interfering RNA (siRNA) can efficiently treat psoriasis,<sup>28</sup> rheumatoid arthritis (RA),<sup>29</sup> and experimental autoimmune encephalomyelitis (EAE).<sup>30</sup>

In the current study, we determined that the expression of *c-Rel* and its inflammatory targets was positively correlated with corneal transplant rejection in both human and mouse. Targeting *c-Rel* can efficiently reduce corneal transplant rejection in mouse.

## MATERIALS AND METHODS

### Ethics Statement

For the human studies, this study abided by all relevant ethical regulations for work with human participants and was approved by the Human Organ Donation Ethics Committee of the Affiliated Qingdao Eye Hospital of Shandong First Medical University. For the animal studies, this study abided by all relevant ethical regulations regarding the use of research animals and was approved by the Animal Care and Use Committee of Shandong Eye Institute, Shandong First Medical University and Shandong Academy of Medical Sciences.

### Organ Procurement Statement

Informed consent was obtained from the donors. No study participant received compensation. None of the organs was procured from executed prisoners.

### Human Sample Collection

Human blood samples were obtained from normal subjects without corneal transplantation and from patients 14 months to 3 years after penetrating keratoplasty (PKP). The main criteria for the determination of graft rejection are as follows: (1) congestion of the conjunctiva, (2) swelling and thickening of the graft, (3) vision loss, (4) anterior chamber reaction, (5) growth of new blood vessels in the graft, and (6) presence of keratic precipitates.

### Corneal Transplantation

Allogeneic and syngeneic corneal transplantations were performed as described previously.<sup>31</sup> Briefly, 2.5-mm-diameter donor corneal grafts from C57BL/6 mice (allogeneic) or BALB/c mice (syngeneic) were transplanted into 2-mm-diameter host beds of BALB/c mice via eight interrupted sutures (11-0 nylon; Mani, Takenzawa, Japan). Alternatively, donor corneal grafts from BALB/c mice were transplanted to *c-Rel*<sup>-/-</sup> or wide-type littermate control mice on the C57BL/6 background. The host eye lids were closed with one 10-0 suture in order to promote healing. Seven days after

surgery, the corneal sutures were removed. The corneal graft survival rate was evaluated using slit-lamp microscopy, and opacity, neovascularization, and edema were scored twice a week as described previously.<sup>32</sup> The rejection index was determined using a 0 to 10 index system. Detailed criteria to determine the rejection index are provided in the Supplementary Materials.

### Treatment With Adeno-Associated Virus 6 or Pentoxifylline

For in vitro study, bone marrow-derived dendritic cells (BMDCs), bone marrow-derived macrophages (BMDMs), RAW264.7 cells, Jurkat cells, and mouse and human CD4<sup>+</sup> T cells (pretreated overnight with anti-CD3, 5 mg/mL, and anti-CD28, 5 mg/mL; eBioscience, San Diego, CA, USA) were treated with adeno-associated virus 6 (AAV6)-shNC (negative control) or AAV6 expressing *c-Rel*-specific small hairpin shRNA (AAV6-shRel) ( $1 \times 10^{11}$  vector genomes [vg] per 1 million cells) for 48 hours. The transduction efficiency (number of green fluorescent protein [GFP]<sup>+</sup> cells/number of total cells) was determined using both regular and fluorescent microscopes and analyzed by Image Pro Plus 6.0. For *c-Rel* mRNA detection, transduced cells were stimulated with lipopolysaccharide (LPS, 200 ng/mL) (for BMDC, BMDM, and RAW264.7 cells) or anti-CD3 (5 mg/mL) plus anti-CD28 (5 mg/mL) (for Jurkat and human CD4<sup>+</sup> T cells) for 6 hours. Alternatively, cells were treated for 24 hours to detect the protein expression of *c-Rel* and inflammatory cytokines. With regard to treatment with PTXF (#6493-05-6, P2050; TCI Chemicals, Zwijndrecht Belgium), BMDC, BMDM, RAW264.7, Jurkat, and mouse and human CD4<sup>+</sup> T cells were pretreated with PTXF (500  $\mu$ g/mL) for 30 minutes. Cells were then stimulated and analyzed as mentioned above.

For in vivo study, the recipient mice were injected subconjunctivally with 5  $\mu$ L AAV6-shNC or AAV6-shRel ( $1 \times 10^{13}$  vg/mL) 1 week before the surgery. The same treatment was repeated immediately after corneal transplantation and every 3 days thereafter. Alternatively, immediately after corneal allogeneic transplantation, recipient mice received eye drop of 5  $\mu$ L PBS or PTXF (20 mg/mL) three times a day for 1 week and once a day thereafter.

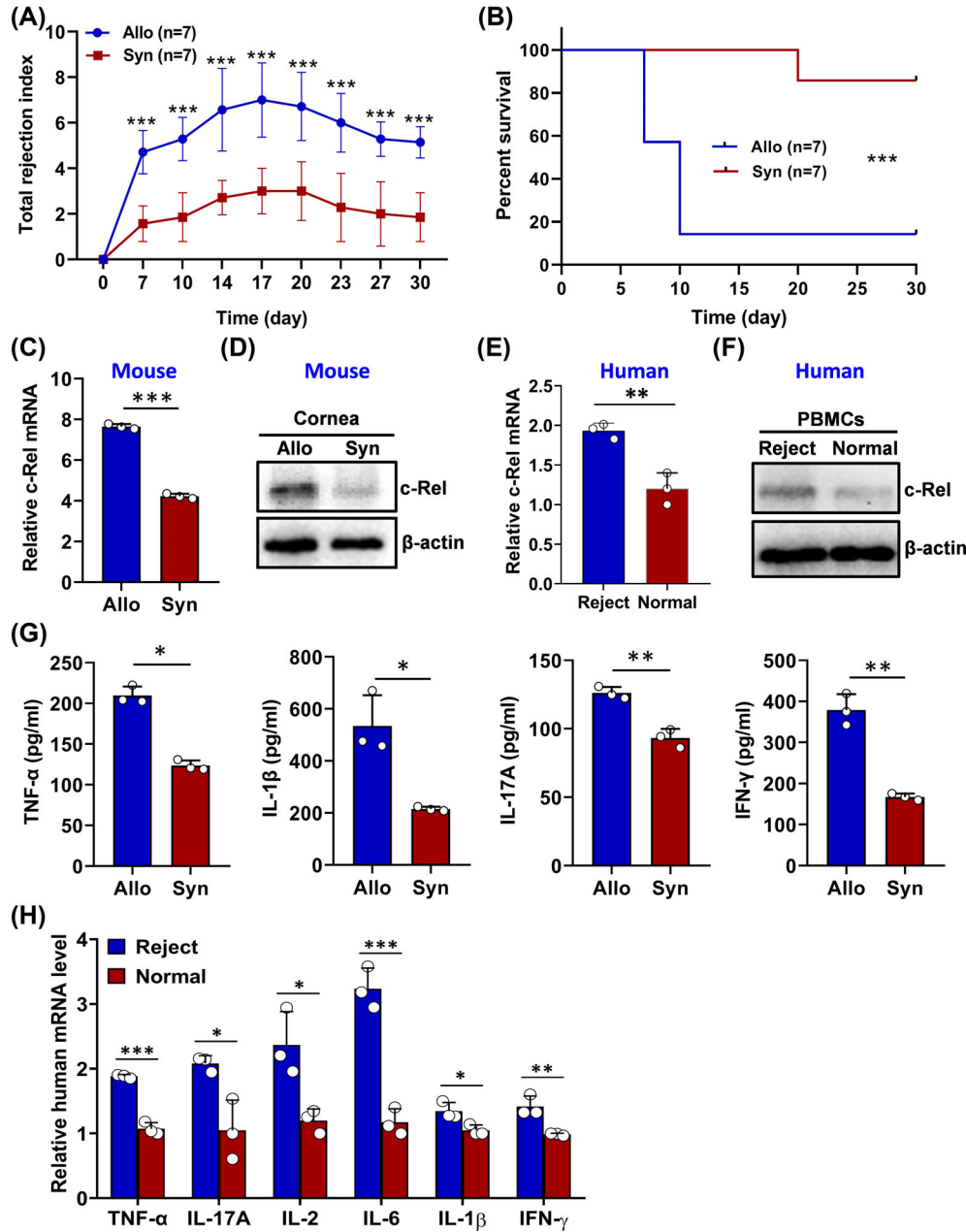
### Western Blotting

Western blot was used to detect *c-Rel* protein expression. Briefly, total protein from corneal and various types of cells was prepared using radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors (#1861280; Thermo Fisher Scientific, Waltham, MA, USA), and a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China) was used for protein quantification. Protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene fluoride (PVDF) membrane (#IPVH00010; Merck Millipore, Burlington, MA, USA). After blocking with 5% skim milk, membranes were incubated overnight with anti-*c-Rel* (#AF2699, 1:400 dilution in blocking buffer; R&D Systems, Minneapolis, MN, USA) or anti- $\beta$ -actin (#20536-1-AP, 1:2000 dilution in blocking buffer; Proteintech, Rosemont, IL, USA). Membranes were then incubated for 1 hour at room temperature with HRP-Conjugated Affinipure Goat Anti-Mouse IGG (H+L) antibody (#SA00001-1, 1:5000 dilution in blocking buffer; Protein-

tech) or HRP-Conjugated Affinipure Goat Anti-Rabbit IgG (H+L) antibody (#SA00001-2, 1:5000 dilution in blocking buffer; Proteintech). Signals were detected by chemiluminescence assay using ChemiDoc Touch (Bio-Rad Laboratories, Hercules, CA, USA) and Image Lab Touch 1.2.0.12 software (Bio-Rad Laboratories).

### Enzyme-Linked Immunosorbent Assay

For the preparation of tissue extract, corneas were homogenized in PBS containing protease and phosphatase inhibitor cocktail (#78442; Thermo Fisher Scientific) using TissueLysor II per the manufacturer's instructions (QIAGEN,



**FIGURE 1.** The expression of *c-Rel* and its inflammatory targets was increased in mice that received allogeneic transplants and in patients with corneal transplant rejection. (A–D) Corneal transplantation was performed with C57BL/6 (allogeneic) or BALB/c (syngeneic) mice as the donors and the BALB/c mice as the recipients. The total rejection index (A) and the graft survival rates (B) were determined as described in the Materials and Methods section. Results shown are combined from two independent experiments. Mice that received syngeneic and allogeneic transplantation were sacrificed at day 14 after surgery. Corneas from each group ( $n = 3$ ) were homogenized, and total RNA and protein extracts were prepared. The expression of *c-Rel* mRNA (C) and protein (D) was determined by quantitative RT-PCR and western blot, respectively. (E, F) Corneal transplantation was performed on patients; PBMCs from patients with corneal transplant rejection ( $n = 3$ ) were isolated, and the expression of *c-Rel* mRNA (E) and protein (F) was determined by quantitative RT-PCR and western blot, respectively. PBMCs isolated from normal subjects ( $n = 3$ ) without corneal transplantation were used as control. (G) The concentrations of inflammatory cytokines in cornea extracts from mice that received allogeneic or syngeneic transplantation were determined by ELISA. (H) Relative mRNA expression of inflammatory cytokines in the PBMCs from normal subjects and patients with corneal transplant rejection was determined by quantitative RT-PCR. Results shown are representative of two independent experiments. Syn, syngeneic; allo, allogeneic. Data are presented as means  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Hilden, Germany). The concentration of inflammatory cytokines in the corneal extract was determined by enzyme-linked immunosorbent assay (ELISA) per the manufacturer's instructions (eBioscience). To determine the concentration of inflammatory cytokines in the culture supernatant, BMDM, BMDM, RAW264.7, Jurkat, and mouse and human CD4<sup>+</sup> T cells were treated as mentioned above, and the concentrations of the inflammatory cytokines IL-6 (mouse, 88-7064-88; eBioscience), IL-1 $\beta$  (mouse, 88-7013-88; eBioscience), IL-17A (mouse, 88-7371-88; eBioscience; human, 216167; Abcam), IL-12 (mouse, 88-7126-88; eBioscience), IL-23 (mouse, 88-7237-88; eBioscience), TNF- $\alpha$  (mouse, 88-7324-88; eBioscience; human, 181421; Abcam), IFN- $\gamma$  (mouse, 88-7314-88; eBioscience), and IL-2 (human, 270883; Abcam) in the culture supernatant were determined by ELISA per the manufacturer's instructions.

### RNA Isolation and Reverse Transcription–Polymerase Chain Reaction

Total RNA from the cornea or various types of cells was isolated using TRIzol Reagent following the manufacturer's instructions (#T9424; Sigma-Aldrich, St. Louis, MO, USA). RNA samples were reversely transcribed using the PrimeScript RT Reagent Kit (#RR047A; Takara, Shiga, Japan). The expression of *c-Rel* and inflammatory cytokines was determined by quantitative reverse transcription–polymerase chain reaction (RT-PCR) using specific primers and the 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Relative levels of expression were determined using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control. The primers for the detection of all genes were synthesized by Invitrogen (Shanghai, China). Primer sequences are provided in the Supplementary Materials.

### Statistical Analysis

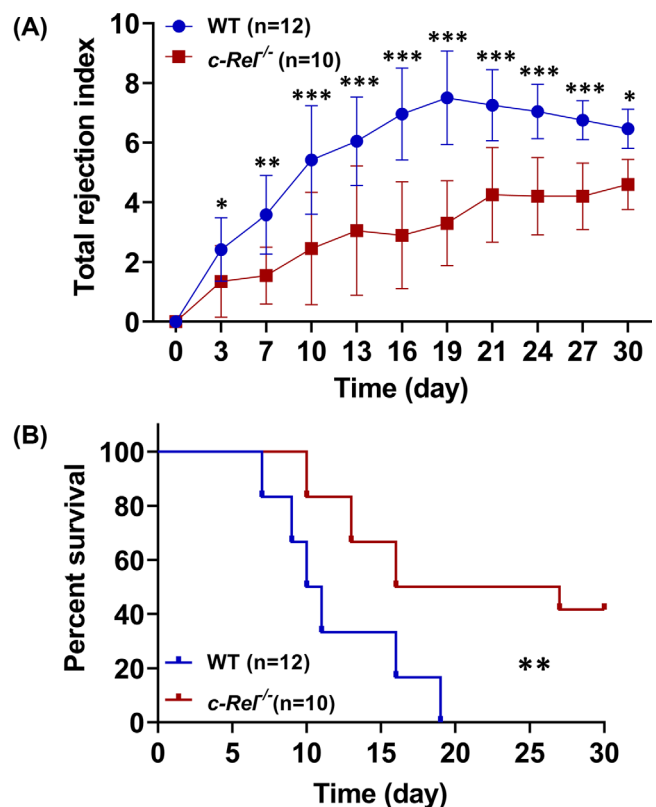
Data were analyzed using Prism 9.2.0 (GraphPad, San Diego, CA, USA) and SPSS Statistics 23.0 (IBM, Chicago, IL, USA) and are expressed as mean  $\pm$  SD. The Student's *t*-test was used to compare the significance of the difference between two groups. The Mantel–Cox survival analysis was used for statistical analysis of the differences in graft survival. For total rejection index comparison, statistical significance was analyzed using two-way ANOVA with Bonferroni correction. Differences were considered statistically significant at \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.

## RESULTS

### Expressions of *c-Rel* and Its Inflammatory Targets Are Increased in Both Mice and Patients With Corneal Transplant Rejection

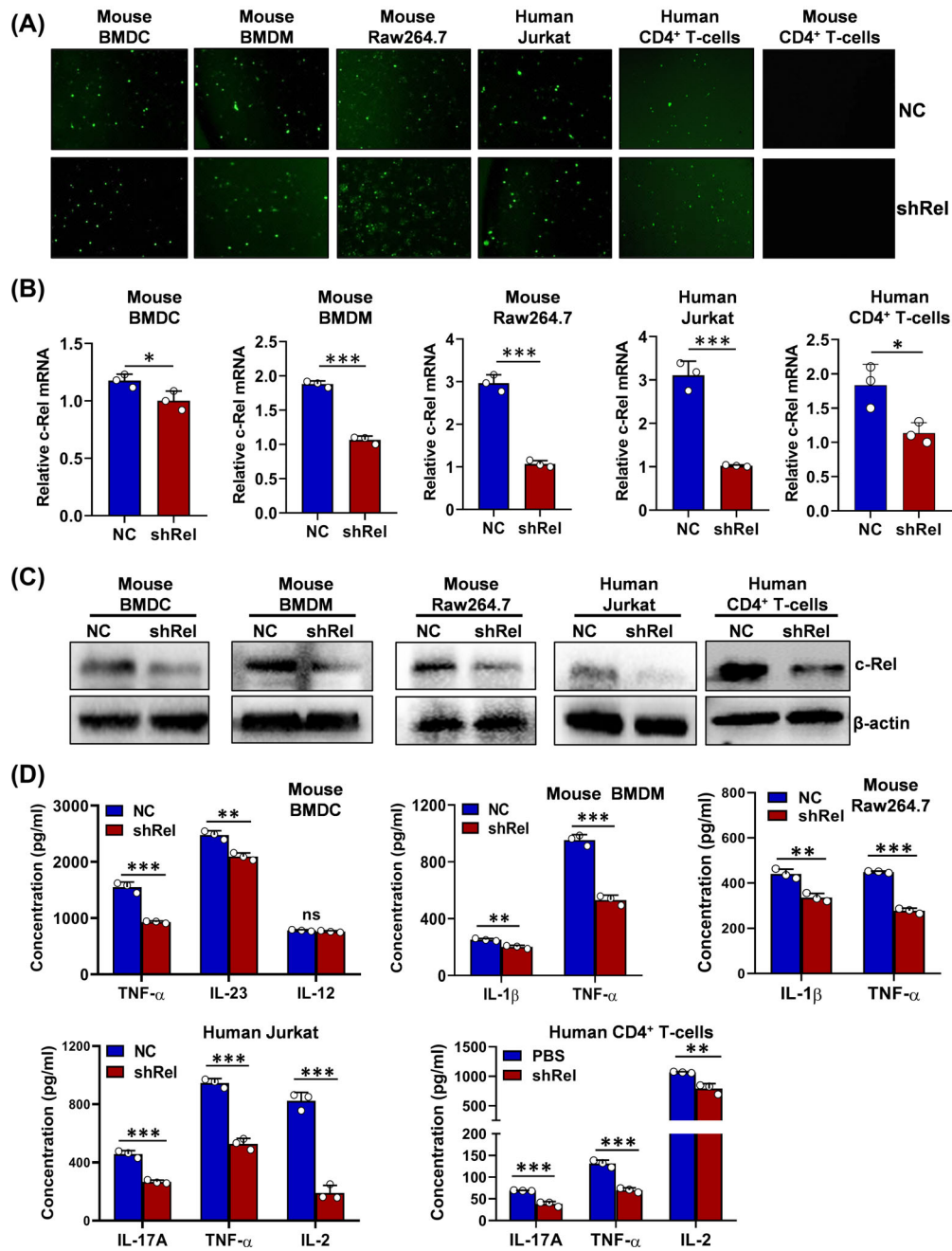
Corneal transplant rejection involves an allo-specific immune response, which is mediated by a variety of immune cells and inflammatory cytokines. To investigate whether altered expression of *c-Rel* is a risk factor for corneal transplant rejection, we established mouse models of syngeneic and allogeneic corneal transplantation and examined the expression of *c-Rel* mRNA and protein in the cornea. As expected, we found that mice that received allogeneic corneal transplants displayed an increased rejection index (Fig. 1A) compared with mice that received syngeneic

corneal transplants. About 30 days after transplantation, ~80% of the grafts were rejected in mice that received allogeneic corneal transplants, whereas most of the grafts survived in the mice that received syngeneic corneal transplants (Fig. 1B). Next, we examined *c-Rel* expression in the cornea and found that the expression of both *c-Rel* mRNA (Fig. 1C) and protein (Fig. 1D) was significantly increased in the cornea from mice that received allogeneic corneal transplants. We further showed that the expression of *c-Rel* mRNA (Fig. 1E) and protein (Fig. 1F) was also increased in the peripheral blood mononuclear cells (PBMCs) from patients with corneal transplant rejection. To investigate whether increased *c-Rel* expression correlates with elevated expression of its targets, we examined the production of *c-Rel* inflammatory targets in the cornea extracts. Our results showed that protein expression of the inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , IL-17A, and IFN- $\gamma$  was significantly increased in the cornea from mice that received allogeneic corneal transplants (Fig. 1G). In addition, we also found that mRNA expression of the inflammatory cytokines TNF- $\alpha$ , IL-17A, IL-2, IL-6, IL-1 $\beta$ , and IFN- $\gamma$  was significantly increased in the PBMCs from patients with corneal transplant rejection (Fig. 1H). Taken together, these results indicate that *c-Rel* may aggravate corneal transplant rejection by promoting the expression of its inflammatory targets.



**FIGURE 2.** *c-Rel*-deficient mice exhibited a reduced corneal transplant rejection index and increased graft survival. Allogeneic corneal transplantation was performed with BABL/c mice as the donors and wild type or *c-Rel*<sup>-/-</sup> mice on the C57BL/6 background as the recipients. The total rejection index (A) and graft survival rates (B) were determined as described in the Materials and Methods section. Results shown are combined from two independent experiments. The bars on the line graphs are the means and standard errors. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



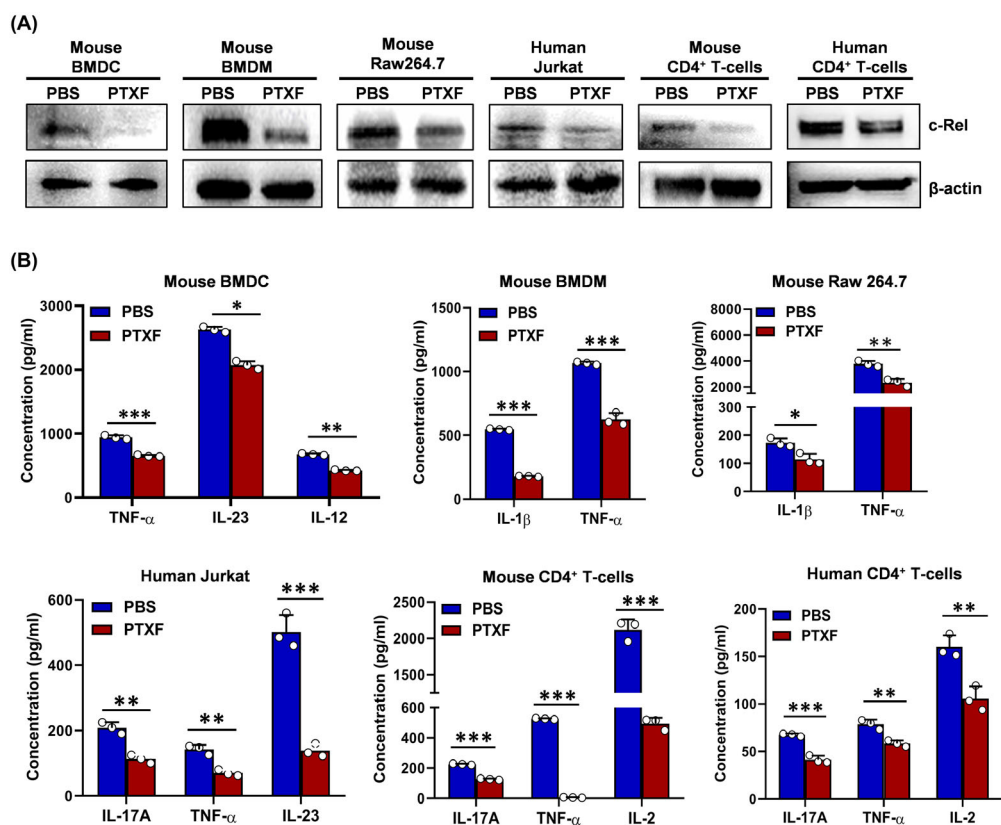


**FIGURE 3.** AAV6 expressing *c-Rel*-specific shRNA downregulates the expression of *c-Rel* and its inflammatory targets in vitro. Mouse BMDCs and BMDMs, RAW264.7 cells, Jurkat cells, and mouse and human primary CD4<sup>+</sup> T cells (CD4<sup>+</sup> T cells were pre-activated overnight with anti-CD3, 5 mg/mL, and anti-CD28, 5 mg/mL) were transduced with AAV6-shNC (NC) or AAV6-shRel (shRel). (A–D) After 48 hours, the transduction efficiency was determined using both regular and fluorescent microscopes (A). Transduced BMDMs, BMDMs, and RAW264.7 cells were then stimulated with LPS (200 ng/mL), and transduced Jurkat and human CD4<sup>+</sup> T cells were treated with anti-CD3 (5 mg/mL) plus anti-CD28 (5 mg/mL). After 24 hours, relative *c-Rel* mRNA was determined by quantitative RT-PCR (B), and *c-Rel* protein expression was determined by western blot (C). The concentration of inflammatory cytokines in the culture supernatant was determined by ELISA (D). Results shown are representative of two independent experiments. Data are presented as mean ± SD. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

**c-Rel-Deficient Mice Exhibit Reduced Corneal Transplant Rejection**

To provide direct evidence that increased *c-Rel* expression is a potential risk factor for corneal transplant rejection, we performed allogeneic corneal transplantation using *c-Rel*-deficient and wild-type littermate control mice as the recipi-

ents. Our results showed that *c-Rel*-deficient mice displayed a decreased rejection index (Fig. 2A) compared with littermate controls. In addition, the percentage of graft survival was increased in *c-Rel*-deficient mice (Fig. 2B). These results indicate that *c-Rel* indeed aggravates corneal transplant rejection and could be a potential therapeutic target for corneal transplant rejection.



**FIGURE 4.** Treatment with PTXF downregulates the expression of *c-Rel* and its inflammatory targets in vitro. Mouse BMDCs and BMDMs, RAW264.7 cells, Jurkat cells, and mouse and human primary CD4<sup>+</sup> T cells were pretreated with PBS or PTXF (500  $\mu$ g/mL) for 30 minutes. BMDCs, BMDMs, and RAW264.7 cells were then stimulated with LPS (200 ng/mL). Jurkat cells and mouse and human CD4<sup>+</sup> T cells were treated with anti-CD3 (5 mg/mL) plus anti-CD28 (5 mg/mL). (A, B) After 24 hours, *c-Rel* protein expression was determined by western blot (A), and the concentration of inflammatory cytokines in the culture supernatant was determined by ELISA (B). Results shown are representative of two independent experiments. Data are presented as mean  $\pm$  SD. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.

### AAV6 Expressing *c-Rel*-Specific shRNA and PTXF Efficiently Downregulates the Expression of *c-Rel* and Its Inflammatory Targets In Vitro

We have previously shown that nanoparticles loaded with *c-Rel*-specific siRNA (siRel) can efficiently downregulate the expression of *c-Rel* and its inflammatory targets in vitro. In the current study, we evaluated the suppressive efficiency of AAV6 expressing *c-Rel*-specific shRNA which targets the same sequence as siRel. We first showed that AAV6 expressing either control shRNA (AAV6-shNC) or *c-Rel*-specific shRNA (AAV6-shRel) can efficiently transduce mouse dendritic cells (BMDCs), mouse macrophages (including both BMDM and RAW264.7 cells), the human T-cell line (Jurkat), and human primary CD4<sup>+</sup> T cells, but not mouse primary CD4<sup>+</sup> T cells (Fig. 3A). The transduction efficiency is  $\sim$ 60% for BMDCs,  $\sim$ 50% for BMDMs,  $\sim$ 90% for RAW264.7,  $\sim$ 70% for Jurkat cells, and  $\sim$ 45% for human CD4<sup>+</sup> T cells.

Next, we examined *c-Rel* mRNA and protein expression in transduced BMDC, BMDM, RAW264.7, Jurkat, and human CD4<sup>+</sup> T cells. Our results show that *c-Rel* mRNA expression was significantly reduced in cells treated with AAV-shRel compared with those treated with AAV-shNC (Fig. 3B). *c-Rel* protein expression was also decreased following transduction with AAV-shRel, as determined by western blot analyses (Fig. 3C). Consistent with the results for *c-Rel* downregulation, we found that the production of *c-Rel* inflammatory

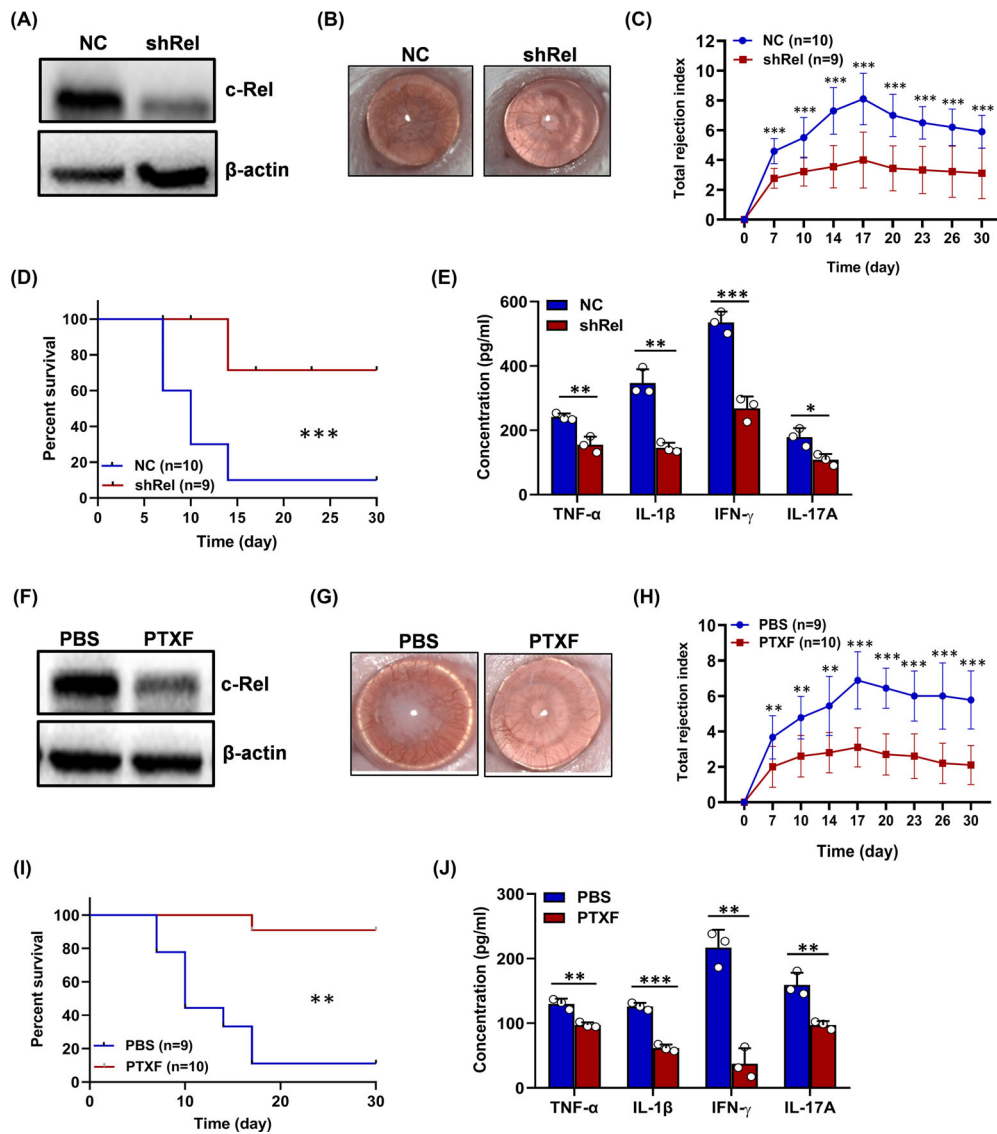
targets was significantly decreased following transduction with AAV-shRel (Fig. 3D).

Next, we evaluated the suppressive efficiency of PTXF, a known small-molecule *c-Rel* inhibitor. Our results indicate that treatment with PTXF can efficiently downregulate *c-Rel* protein expression in BMDC, BMDM, RAW264.7, Jurkat, and both mouse and human CD4<sup>+</sup> T cells (Fig. 4A). We did not examine *c-Rel* mRNA expression because it has been reported that PTXF affects only *c-Rel* expression through a posttranslational mechanism.<sup>33</sup> As expected, the production of *c-Rel* inflammatory targets was significantly decreased following treatment with PTXF (Fig. 4B).

Taken together, these results indicate that both AAV6-shRel and small-molecule *c-Rel* inhibitor PTXF can efficiently downregulate the expression of *c-Rel* and its inflammatory targets in vitro.

### Treatment With AAV6-shRel or PTXF Reduces Corneal Transplant Rejection

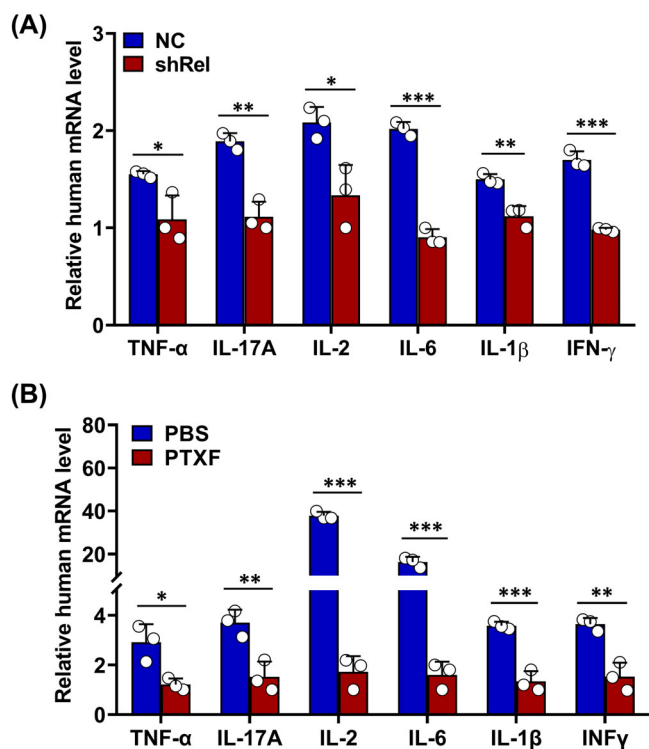
To determine the extent to which inhibiting *c-Rel* mRNA or protein expression reduces corneal transplant rejection, an acute mouse corneal allogeneic transplantation model was used. Mice were subconjunctivally injected with AAV6-shNC or AAV6-shRel 1 week before transplantation. The same treatment was repeated immediately after allogeneic corneal transplantation and every 3 days thereafter. After 30 days,



**FIGURE 5.** AAV expressing *c-Rel*-specific shRNA and PTXF are able to reduce corneal transplant rejection in mice. (A–E) Allogeneic corneal transplantation was performed with BABL/c mice as the recipients and C57BL/6 mice as the donors. AAV6-shNC (NC) or AAV6-shRel (shRel) was injected subconjunctivally 1 week before the transplantation. The same treatment was repeated immediately after transplantation and every 3 days thereafter. Corneas were removed, and the protein level of *c-Rel* was determined by western blot 14 days after transplantation (A), and representative photographs of corneal grafts 21 days after transplantation are shown (B). The total rejection index (C) and graft survival rates (D) were determined as described in the Materials and Methods section. Results shown are combined from two independent experiments. The concentration of cytokines in the corneal extracts ( $n = 3$ , with each sample pooled from 2 or 3 mice) was determined by ELISA 14 days after transplantation (E). (F–J) Allogeneic corneal transplantation was performed with BABL/c mice as the recipients and C57BL/6 mice as the donors. Immediately after transplantation, mice received eye drops of PBS or PTXF 3 times a day for 1 week and once a day thereafter. Corneas were removed, and the protein level of *c-Rel* was determined by western blot 14 days after transplantation (F). The representative photographs of corneal grafts 21 days after transplantation are shown (G). The total rejection index (H) and graft survival rates (I) were determined as described in the Materials and Methods section. Results shown are combined from two independent experiments. The concentration of cytokines in the corneal extracts ( $n = 3$ , with each sample pooled from 2 or 3 mice) was determined by ELISA 14 days after transplantation (J). The bars on the line graphs are means and standard errors. Cytokine concentrations are presented as mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

we found that *c-Rel* protein expression in the cornea from mice treated with AAV6-shRel was significantly decreased (Fig. 5A). More importantly, we found that mice treated with AAV6-shRel exhibited decreased graft opacity (Fig. 5B) and graft rejection index (Fig. 5C) and an increased graft survival rate (Fig. 5D). In addition, the production of *c-Rel* inflammatory targets in the cornea was significantly decreased (Fig. 5E).

We also examined the potential of small molecule *c-Rel* inhibitor PTXF in reducing corneal transplant rejection. Immediately after allogeneic corneal transplantation, mice were treated with PBS or PTXF eye drops three times a day for 1 week and once a day thereafter. After 30 days, we found that PTXF-treated mice exhibited decreased *c-Rel* protein expression (Fig. 5F), decreased graft opacity (Fig. 5G) and graft rejection index (Fig. 5H), and increased graft survival



**FIGURE 6.** AAV6 expressing c-Rel-specific shRNA and PTXF are able to downregulate the expression of inflammatory cytokines in PBMCs from patients with corneal transplant rejection. PBMCs were isolated from patients ( $n = 3$ ) with corneal transplant rejection. (A, B) Cells were then treated in vitro with AAV6 expressing c-Rel-specific shRNA (shRel) or control shRNA (NC) for 24 hours (A). Alternatively, cells were treated with PTXF or PBS for 12 h (B). Relative mRNA expression of inflammatory cytokines was determined by quantitative RT-PCR. Results shown are representative of two independent experiments. Data are presented as mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

rate (Fig. 5I). The production of c-Rel inflammatory targets in the cornea from PTXF treated mice was also significantly decreased (Fig. 5J).

To test whether AAV6-shRel and PTXF can downregulate the expression of inflammatory cytokines, we isolated PBMCs from patients with corneal transplant rejection and treated them in vitro with AAV6-shRel or PTXF, together with AAV6-shNC or PBS as negative controls. Our results showed that the mRNA expression of inflammatory cytokines was significantly reduced after treatment with either AAV6-shRel (Fig. 6A) or PTXF (Fig. 6B).

Taken together, these results indicate that targeting c-Rel can efficiently reduce corneal transplant rejection and the production of c-Rel inflammatory targets.

### Treatment With AAV6-shRel and PTXF Display No Side Effects on Tear Production and Corneal Sensitivity

To determine the potential side effects of AAV6-shRel and PTXF, we conducted preliminary study of their effects on tear production and corneal sensitivity. Our results showed that, although mice that received cornea allograft display increased tear production and decreased cornea sensitivity, mice treated with AAV6-shNC or AAV6-shRel produced similar amounts of tear production (Fig. 7A). There was also no

significant difference in corneal sensitivity between the two groups (Fig. 7B). Similar results were obtained when mice were treated with PBS or PTXF, as evidenced by the results that no significant difference in tear production (Fig. 7C) and corneal sensitivity (Fig. 7D) was found between PBS and PTXF-treated mice.

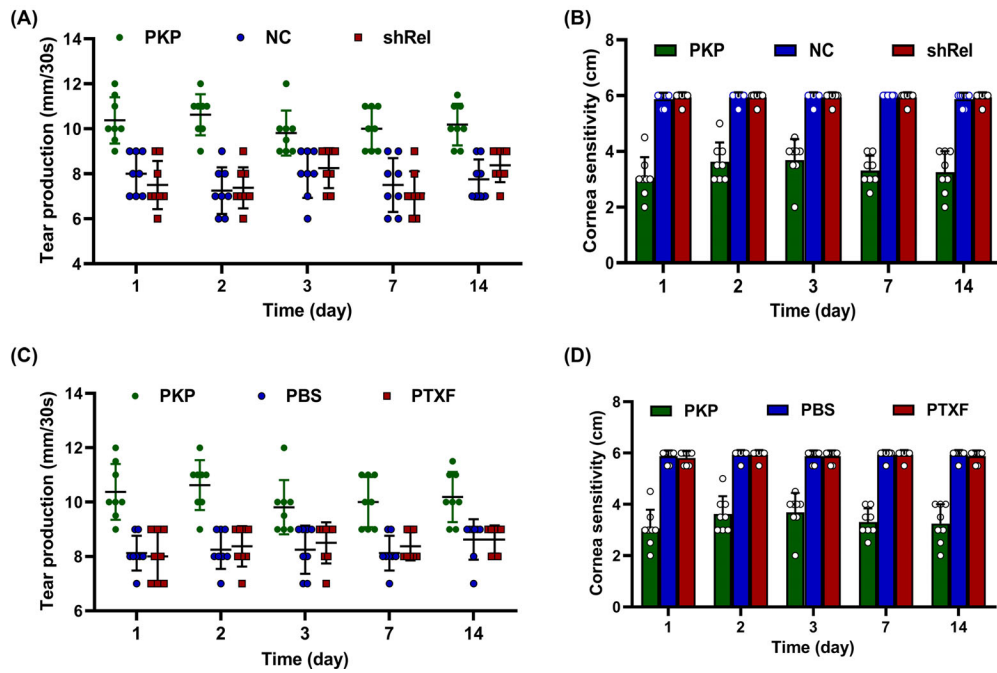
## DISCUSSION

We have previously shown that targeting c-Rel mRNA in regulatory T cells (Tregs) can improve their potential to reduce the risk of corneal transplant rejection.<sup>32</sup> However, because c-Rel is preferentially expressed in activated lymphocytes and monocytes, it remains unknown whether targeting c-Rel mRNA or protein systemically can also improve the survival of corneal grafts after transplantation. Our current study demonstrated that topical administration of c-Rel inhibitor, from either the mRNA or protein level, efficiently reduced corneal transplant rejection in the mouse (Fig. 8).

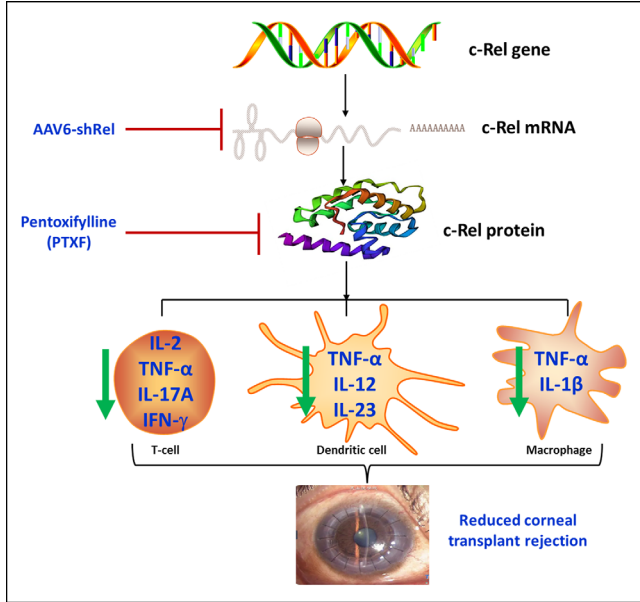
We have previously shown that c-Rel-deficient mice are defective in Treg development.<sup>34</sup> Tregs play an important role in reducing corneal transplant rejection<sup>32</sup>; yet, in our current study, c-Rel-deficient mice exhibited reduced corneal transplant rejection. We believe that, because c-Rel plays a more important pro-inflammatory role in acute inflammatory disease models such as EAE, psoriasis, RA, experimental autoimmune uveitis (EAU), and corneal transplantation, c-Rel-deficient mice display less severe symptoms even though Treg development has been compromised.

Several strategies can be used to target c-Rel, including small-molecule inhibitors and nucleic acid-based drugs.<sup>28,33,35</sup> Nucleic acid-based siRNA drugs have been utilized as therapeutic agents against various diseases.<sup>36</sup> To facilitate the delivery of siRNA drugs, a variety of non-viral and viral vectors have been developed over the past few decades. We have previously shown that nanopolymers loaded with c-Rel-specific siRNA can efficiently prevent and treat autoimmune diseases including psoriasis, EAE, and RA. However, non-viral vectors have sometimes failed to show the expected therapeutic efficacy because of poor delivery efficiency to certain types of cells, especially T cells. In recent years, AAV-mediated gene transfer has been proving to be an effective treatment for immune-mediated inflammatory diseases of the eye.<sup>37</sup> AAV gene transfer has the advantages of efficient gene delivery and long-term transgene production after a single dose. Various AAV serotypes exhibit preferred tropism due to the use of different cellular surface receptors. For example, AAV8 and AAV9 are the most efficient AAV vectors to infect corneal cells,<sup>37</sup> but AAV6 is a vector with high infection efficiency for immune cells,<sup>38</sup> including T cells.<sup>39</sup> Gene transfer using AAV vectors has several advantages when treating ocular disease. The relatively small tissue area, accessibility of the eye for direct treatment, and overall ocular compartmentalization allow low doses of AAV vectors to produce therapeutic effects. In addition, low vector doses limit systemic exposure and thereby reduce the possibility of immune responses to the viral capsid or transgene.<sup>40</sup> In fact, two AAV therapeutics have been approved by the US Food and Drug Administration, including voretigene neparvovec (Luxturna) and nusinersen (Spinraza). In addition, 64 ocular clinical trials involving AAV gene therapy are currently listed in clinicaltrials.gov. In the current study, we demonstrated that





**FIGURE 7.** AAV6 expressing *c-Rel*-specific shRNA and PTXF showed no side effects on tear production or corneal sensitivity. (A, B) BABL/c mice without corneal transplantation were treated with AAV-shNC (NC) or AAV-shRel (shRel) as described in the Materials and Methods section. Mice engrafted with allogeneic corneas were used as control. Tear production (A) and cornea sensitivity (B) were examined at the indicated time points after treatment. (C, D) BABL/c mice without corneal transplantation received eye drops of PBS or PTXF as described in the Materials and Methods section. Tear production (C) and cornea sensitivity (D) were examined at the indicated time points after treatment. Results shown are representative of two independent experiments. PKP, penetrating keratoplasty. Data are presented as mean  $\pm$  SD.



**FIGURE 8.** Schematic illustration of how *c-Rel* inhibitors reduce corneal transplant rejection. NF- $\kappa$ B *c-Rel* is preferentially expressed in activated lymphocytes and monocytes and promotes the expression of various pro-inflammatory cytokines, including TNF- $\alpha$ , IL-17A, IL-2, IFN- $\gamma$ , IL-12, IL-1 $\beta$ , and IL-23. Targeting *c-Rel* from the mRNA level using AAV6-shRel or from the protein level using PTXF downregulated the expression of *c-Rel* inflammatory targets in T-cells, dendritic cells, and macrophages, thereby reducing corneal transplant rejection.

AAV6 expressing *c-Rel*-specific shRNA can efficiently reduce corneal transplant rejection in mice. It is worth noting that AAV6 can successfully transduce human but not mouse T cells. Although corneal transplant rejection is mainly mediated by T cells, inflammatory cytokines produced by macrophages and dendritic cells are also involved in the rejection of corneal graft either directly or through regulating T cell function. We suggest that AAV6-mediated targeting of *c-Rel* may reduce transplant rejection in mice by modulating the function of macrophage and dendritic cells.

PTXF is a nonspecific phosphodiesterase inhibitor with favorable anti-inflammatory effects and immunoregulatory properties.<sup>41</sup> It has been approved by the FDA for the treatment of a wide variety of clinical conditions, including peripheral vascular disease, where blood flow may be impaired in the microvasculature. PTXF acts through multiple targets in vivo, including as a potent inhibitor of *c-Rel* protein expression. However, PTXF does not affect the expression of RelA, which is another member of the NF- $\kappa$ B family.<sup>33</sup> Although our study demonstrated that treatment with PTXF can significantly reduce *c-Rel* protein expression and corneal transplant rejection, it remains to be determined whether PTXF affects additional signaling pathways that favor corneal graft survival. On the other hand, it has been reported that PTXF impairs the immunosuppressive function of activated Tregs<sup>35</sup> but enhances the function of inflammatory Tregs.<sup>32</sup> Therefore, it remains to be determined whether long-term *c-Rel* blocking will have an effect on Treg function.

In summary, we have shown that increased expression of *c-Rel* is a risk factor for corneal transplant rejection and that targeting *c-Rel* can significantly reduce acute corneal transplant rejection.

## Acknowledgments

Supported by grants from the National Natural Science Foundation of China (82271129, 82201152, 82271052); Natural Science Foundation of Shandong Province (ZR2021QH013); Postdoctoral Innovation Project of Shandong Province (202103021); Young Taishan Scholars (tsqn201909188); and Qingdao Municipal Science and Technology Bureau (21-1-4-rjkk-11-nsh).

Disclosure: **Q. Zheng**, None; **R. Liu**, None; **B. Jiang**, None; **J. Sun**, None; **T. Wang**, None; **Q. Ruan**, None

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