

# ACE2 Expression Is Upregulated in Inflammatory Corneal Epithelial Cells and Attenuated by Resveratrol

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**PURPOSE.** The ocular surface is considered an important route for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) transmission. The expression level of the SARS-CoV-2 receptor angiotensin-converting enzyme 2 (ACE2) is vital for viral infection. However, the regulation of ACE2 expression on the ocular surface is still unknown. We aimed to determine the change in ACE2 expression in inflamed corneal epithelium and explore potential drugs to reduce the expression of ACE2 on the ocular surface.

**METHODS.** The expression of the SARS-CoV-2 receptors ACE2 and TMPRSS2 in human corneal epithelial cells (HCECs) was examined by qPCR and Western blotting. The altered expression of ACE2 in inflammatory corneal epithelium was evaluated in TNF $\alpha$ - and IL-1 $\beta$ -stimulated HCECs and inflamed mouse corneal epithelium, and the effect of resveratrol on ACE2 expression in HCECs was detected by immunofluorescence and Western blot analysis.

**RESULTS.** ACE2 and TMPRSS2 are expressed on the human corneal epithelial cells. ACE2 expression is upregulated in HCECs by stimulation with TNF $\alpha$  and IL-1 $\beta$  and inflamed mouse corneas, including dry eye and alkali-burned corneas. In addition, resveratrol attenuates the increased expression of ACE2 induced by TNF $\alpha$  in HCECs.

**CONCLUSIONS.** This study demonstrates that ACE2 is highly expressed in HCECs and can be upregulated by stimulation with inflammatory cytokines and inflamed mouse corneal epithelium. Resveratrol may be able to reduce the increased expression of ACE2 on the inflammatory ocular surface. Our work suggests that patients with an inflammatory ocular surface may display higher ACE2 expression, which increases the risk of SARS-CoV-2 infection.

**Keywords:** ACE2, SARS-CoV-2, human corneal epithelial cells, Inflammatory corneas, resveratrol

Coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, has become a global public health emergency. According to the World Health Organization,<sup>1</sup> more than 47 million confirmed cases of COVID-19 have been reported worldwide, and the death toll from COVID-19 has exceeded 1.2 million people as of November 3, 2020. Current studies have demonstrated that older people and those with hypertension, diabetes mellitus, respiratory disease, and cancer are more susceptible to SARS-CoV-2 infection, leading to significantly increased mortality and severity of COVID-19.<sup>2-6</sup> Thus it is urgent to identify potential risk factors and seek effective prevention to control the spread of the disease.

Coronaviruses bind to their host receptor ACE2 with high affinity via S1 unit of its spike (S) protein, which facilitates viral attachment to the surface of target cells. Subsequently the host serine protease TMPRSS2 primes the S protein for cell entry and allows fusion of viral and cellu-

lar membranes.<sup>7,8</sup> ACE2 is a key receptor of SARS-CoV-2 and is highly expressed in multiple organs, such as the lung, small intestine, and kidney, and the cardiovascular system in humans. Recent studies have reported that ACE2 is expressed in the epithelium of the human cornea and conjunctiva.<sup>9</sup> SARS-CoV-2 can directly infect ocular surface tissue, leading to keratoconjunctivitis,<sup>10-12</sup> and viral keratoconjunctivitis has been found to be the initial clinical presentation of COVID-19.<sup>13</sup> In addition, in a previous study, positive SARS-CoV-2 RNA was detected in conjunctival swab samples collected from COVID-19 patients with conjunctivitis, whereas patients without conjunctivitis were negative for SARS-CoV-2 RNA.<sup>14</sup> These results suggest that the ocular surface tissue is an important route of SARS-CoV-2 transmission and that the eye may be one of the most infected organs in COVID-19 patients.

Previous studies have indicated that ACE2 protein level is associated with the occurrence and severity of COVID-19.

Smith et al.<sup>3</sup> reported that cigarette smoking exposure increases ACE2 expression and smokers are particularly susceptible to severe SARS-CoV-2 infections. Pinto et al.<sup>15</sup> showed patients with comorbidities with higher ACE2 expression have much more chances of developing severe COVID-19. These studies suggest the critical role of ACE2 for SARS-CoV-2 infection and elevated ACE2 expression may be a high risk for COVID-19 transmission. The ocular surface exposes to the air and contact to the virus directly. However, the regulation of ACE2 expression on the ocular surface is still unknown. Chen et al.<sup>16</sup> recently performed a cross-sectional study of 534 patients with COVID-19 in Wuhan and reported that in the 534 patients with COVID-19, dry eye (112, 20.97%), blurred vision (68, 12.73%) and foreign body sensation (63, 11.80%) ranked as the three most common COVID-19-related ocular symptoms, which prompted us to investigate whether the expression of ACE2 on the ocular surface is affected under inflammatory conditions.

Here, we demonstrate that ACE2 is highly expressed in human corneal epithelial cells and can be upregulated by stimulation with TNF $\alpha$  and IL-1 $\beta$ . ACE2 expression is also significantly increased in inflamed mouse corneal epithelium, including dry eye and alkali-burned corneas. In addition, resveratrol attenuates the increased ACE2 expression in inflammatory HCECs, which indicates that resveratrol may be able to help to reduce the transmission of SARS-CoV-2. To the best of our knowledge, this work for the first time demonstrates that the expression of the SARS-CoV-2 receptor ACE2 is upregulated under inflammatory conditions in human corneas, and resveratrol may be able to reduce the increased expression of ACE2 on the inflammatory ocular surface.

## METHODS

### Patients and Specimens

Human corneal epithelial tissue was collected from four healthy adult myopic patients undergoing laser-assisted subepithelial keratomileusis (EK) for the treatment of mild to moderate myopia. Topical proparacaine and 20% alcohol were applied to the ocular surface to loosen the corneal epithelium. Approximately 9 mm of the central epithelium was removed with sterile cellulose sponges and immediately stored at  $-80^{\circ}\text{C}$ . Primary pterygium tissue was collected from pterygium excisions of four patients. The mRNA was extracted from the corneal epithelium and pterygium tissue according to the manufacturer's instructions. This study was approved by the Research and Ethics Committees of Tianjin Eye Hospital and was conducted according to the Declaration of Helsinki.

### Cell Culture and Determination of Cell Viability

Immortalized HCECs obtained from ATCC were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Human corneal keratocytes were obtained from ScienCell Research Laboratories (San Diego, CA, USA) and cultured in a defined proprietary culture medium (FM, Cat. no. 2301). For the TNF $\alpha$  treatment, HCECs were grown in six-well plates and treated with 5 ng/mL or 10 ng/mL recombinant human TNF $\alpha$  in RPMI 1640 medium for 12 or 24 hours after starvation in RPMI 1640 medium. For the IL-1 $\beta$  stimulation, HCECs were treated with 20 ng/mL recombinant human

IL-1 $\beta$  in RPMI 1640 medium for 24 hours after starvation in RPMI 1640 medium.

Cell viability was evaluated by the MTT assay. In brief, HCECs were seeded in 96-well plates and grown to 90% confluence. Cells were then maintained in serum-free 1640 medium for 24 hours and were subsequently incubated with different concentrations of resveratrol (0, 2.5, 5, 10, 20, 50, and 100  $\mu\text{M}$ ) in serum-free 1640 medium for 24 hours at  $37^{\circ}\text{C}$ . Then, 20  $\mu\text{L}$  of MTT solution was added to each well, and cells were incubated for an additional four hours. Next, the medium was discarded, and the obtained formazan crystals were solubilized by adding 150  $\mu\text{L}$  of dimethyl sulfoxide. Absorbance measurements were read at 570 nm using a microplate reader (BioTek, Winooski, VT, USA).

### Animal Model of Corneal Injury and Treatment

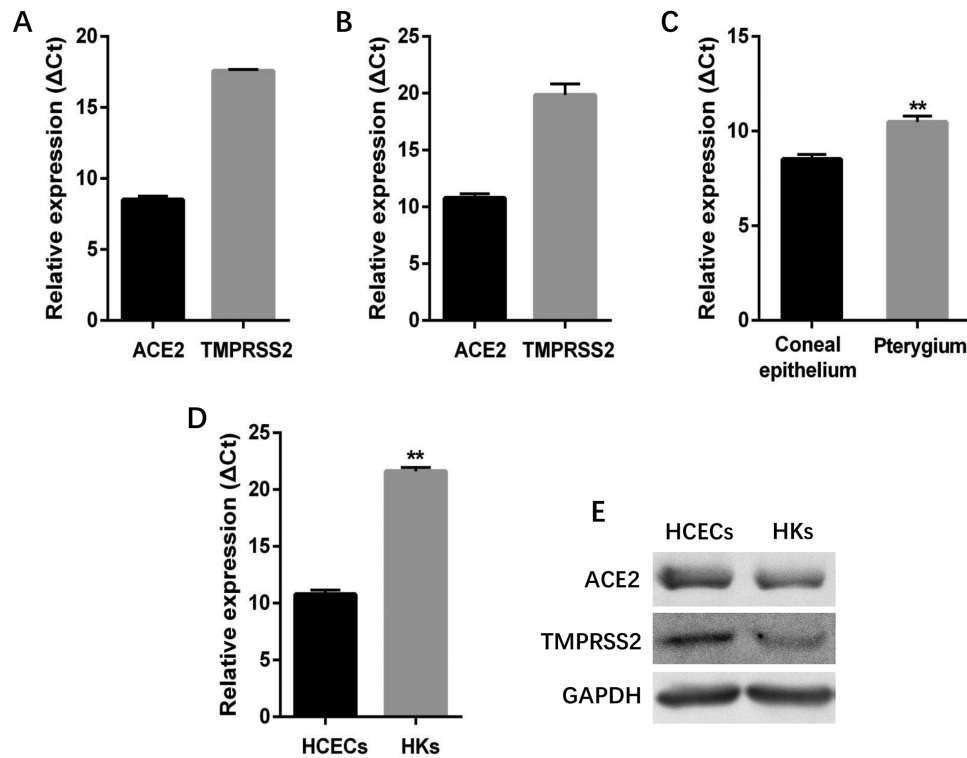
Six- to eight-week-old male C57BL/6 mice and 10-week-old male BALB/c mice were used in this study. This study was approved by the Institutional Animal Care and Use Committee of Tianjin Medical University. Mice were anesthetized with an intraperitoneal injection of 40 mg/kg pentobarbital and received a topical drop of tetracaine. The corneal alkali-burn model was induced by placing a filter paper disc (2 mm in diameter) soaked with 1 N NaOH on the center of the corneal surface of C57BL/6 mice for 10 seconds. After the removal of the disc, the ocular surface was rinsed with 10 mL saline solution immediately.<sup>17</sup> The dry eye model was induced by benzalkonium chloride (BAC) as previously described<sup>18</sup> in BALB/c mice. Briefly, a 5  $\mu\text{L}$  droplet of 0.2% BAC was administered to the right eye via a micropipette twice daily over two weeks. The ocular conditions were observed under a slit-lamp microscope.

### Quantitative PCR

Total RNA was extracted from cells and the human corneal epithelium using a Qiagen RNeasy Mini Kit according to the manufacturer's instructions and was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega). Real-time PCR was performed using a Bio-Rad iCycler with SYBR Primix Ex Taq (Takara) as described previously.<sup>19</sup> The primer sequences were as follows: ACE2 (forward, 5'-CGAGTGGCTAATTTGAAACCAAGAA-3', reverse, 5'-ATTGATACGGCTCCGGGACA-3')<sup>20</sup>; TMPRSS2 (forward, 5'-ACTCTGGAAGTTCATGGGCAG-3', reverse, 5'-TGAAGTTTGGTCCGTAGAGGC-3')<sup>21</sup>; GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) (forward, 5'-TGCCCTCAACGACCACTTTG-3', reverse, 5'-CTGGTGGTCCAGGGGTCTTA-3'). Gene expression was normalized to the GAPDH mRNA level.

### Western Blot Analysis

Corneal epithelial cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing a protease inhibitor cocktail. The protein lysate was mixed with loading buffer, electrophoresed via SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked and then incubated with primary antibodies (anti-ACE2 [15348; Abcam, Cambridge, MA, USA], anti-TMPRSS2 [Abcam, 92323] and an anti-GAPDH antibody [CB1001; Merck Millipore, Burlington, MA, USA]). GAPDH was used as an internal control. The



**FIGURE 1.** Analysis of the gene expression of receptors of SARS-CoV-2 in the human corneal epithelium, pterygium, human corneal epithelial cells and keratocytes. RT-qPCR of the expression levels of *Ace2* and *Tmprss2* relative to that of *GAPDH* in corneal epithelial cells obtained from patients (A) and HCECs (B). (C) *Ace2* expression was determined and compared between the corneal epithelium and pterygium tissue from patients by RT-qPCR. (D) *Ace2* expression was compared between HCECs and HKs by RT-qPCR. (E) Western blotting was used to determine ACE2 and TMPRSS2 expression in HCECs and HKs. \*\* $P < 0.01$ .

intensity of the immunoreactive bands was analyzed using ImageJ.

### Histological and Immunofluorescence Staining

HCECs and mouse eyes were fixed with 4% formaldehyde at 4°C. The mouse samples were dehydrated and embedded with O.C.T. (Optimal Cutting Temperature compound), and cryosections were generated at 10 μm. The tissue sections were stained with hematoxylin and eosin (H&E) for histomorphologic analysis. The cell slides and mouse eye sections were immunostained using a primary antibody against ACE2 (15348; Abcam) or NFκB p65 (ab231481) at 4°C overnight. Subsequently, the cell slides and mouse eye sections were incubated with the secondary Alexa Fluor 488-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific, Waltham, MA, USA) for one hour at room temperature. The sections were mounted with antifade mounting medium after incubation with DAPI for three minutes. Confocal images were acquired under a Leica DMi8 microscope (Leica, Wetzlar, Germany).

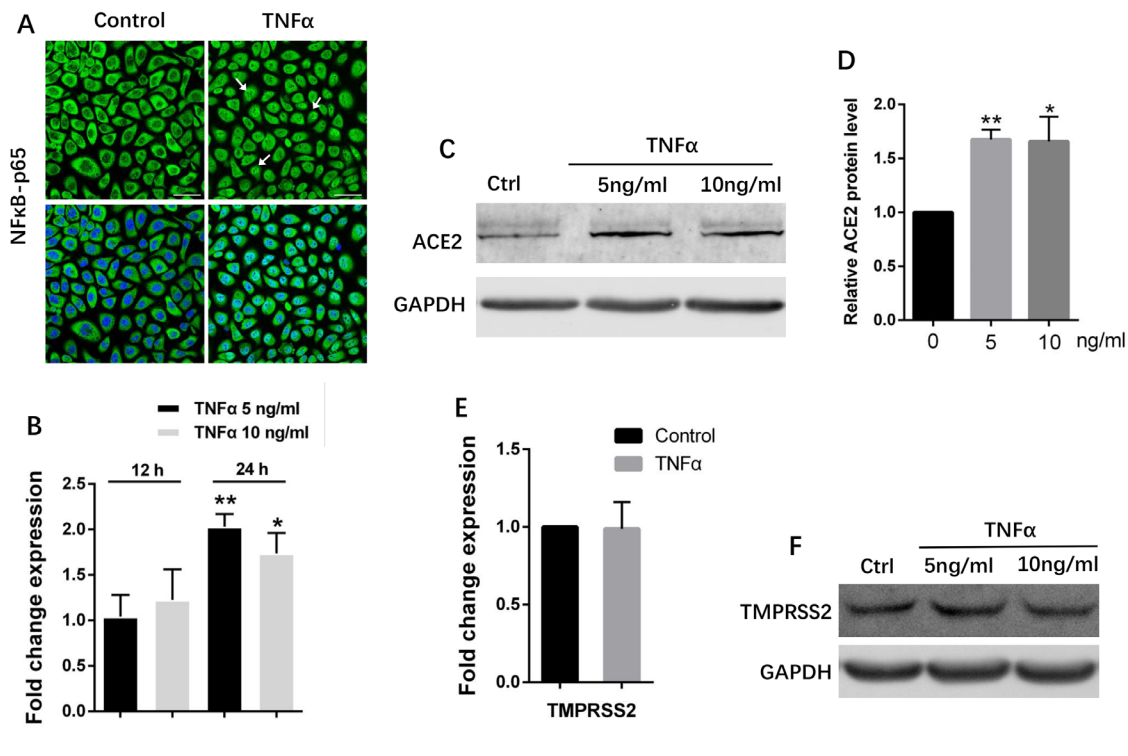
### Statistical Analysis

All data are presented as the mean ± SEM. Two-tailed unpaired Student's *t* test conducted with GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA) was used to perform statistical analyses. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

### ACE2 and TMPRSS2 are Expressed on the Human Ocular Surface

A recent study exploring a single-cell RNA sequencing (scRNA-Seq) dataset showed coexpression of ACE2 and TMPRSS2 in the corneal and conjunctival epithelium, implicating these tissues as target entry tissues for SARS-CoV-2 on the ocular surface. We first verified the expression levels of ACE2 and TMPRSS2 in the human corneal epithelium obtained from four patients during EK and found much higher ACE2 mRNA levels, with a ΔCt of  $8.513 \pm 0.25$ , in the human corneal epithelium than those of TMPRSS2 (ΔCt of  $17.57 \pm 0.10$ ) (Fig. 1A). Consistently, immortalized human corneal epithelial cells (HCECs) exhibited similar expression patterns of ACE2 (ΔCt:  $10.78 \pm 0.38$ ) and TMPRSS2 (ΔCt:  $19.84 \pm 0.97$ ) (Fig. 1B) as those found in the corneal epithelium from patients. In addition, because of the limited available normal human conjunctival tissues, ACE2 expression in pterygium tissue was examined and was found to be significantly lower (ΔCt:  $10.48 \pm 0.32$ ) than that in the human corneal epithelium ( $P = 0.0029$ ) (Fig. 1C). In addition, ACE2 expression in HCECs was also much higher than that in human corneal keratocytes (ΔCt:  $21.48 \pm 0.22$ ) ( $P < 0.0001$ ) (Fig. 1D). Consistently, HCECs displayed much higher protein levels of ACE2 and TMPRSS2 than HKs (Fig. 1E). Because of the high abundance of ACE2 expression, we used HCECs to investigate changes in the



**FIGURE 2.** Expression changes of ACE2 and TMPRSS2 in human corneal epithelial cells stimulated by TNF $\alpha$ . (A) NF $\kappa$ B nuclear translocation was assessed by immunofluorescence staining of the NF $\kappa$ B p65 (green) subunit in HCECs with or without TNF $\alpha$  treatment for one hour. The arrows indicate the nuclear localization of p65. (B) RT-qPCR was performed to determine the Ace2 expression level in HCECs treated with or without TNF $\alpha$  at the indicated concentration for 12 and 24 hours. The level of gene expression in control samples is designated as 1. (C) Western blotting was performed to determine the ACE2 protein levels in HCECs exposed to TNF $\alpha$  at the indicated concentration for 24 hours, and the quantified data are shown in D. (E) RT-qPCR was performed to determine the TmpRSS2 expression level in HCECs treated with or without TNF $\alpha$  for 24 hours. (F) Western blotting was performed to determine the TMPRSS2 protein levels in HCECs exposed to TNF $\alpha$  at the indicated concentration for 24 hours. Data are presented as the mean  $\pm$  SEM from at least three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ . Scale bars: 50  $\mu$ m.

receptor of SARS-CoV-2 under inflammatory conditions in subsequent experiments.

### ACE2 Expression is Stimulated by Proinflammatory Cytokines in Human Corneal Epithelial Cells

To determine the altered expression of ACE2 in corneal epithelial cells under inflammatory conditions, we treated HCECs with TNF $\alpha$ , a proinflammatory cytokine, to trigger inflammation. We first performed immunofluorescence staining to test the response of HCECs to TNF $\alpha$  treatment by analyzing the activation of NF $\kappa$ B signaling. As shown in Figure 2A, a significant increase in the nuclear translocation of p65, a subunit of NF $\kappa$ B, was observed in TNF $\alpha$ -treated HCECs, suggesting that TNF $\alpha$  promotes an inflammatory response in HCECs. We subsequently examined the expression of ACE2 in HCECs stimulated with TNF $\alpha$  at both the mRNA and protein levels. TNF $\alpha$  treatment did not alter the mRNA level of ACE2 after 12 hours in HCECs (Fig. 2B). However, after 24 hours of treatment with 5 ng/mL and 10 ng/mL TNF $\alpha$ , ACE2 mRNA in HCECs was increased by almost twofold ( $P = 0.0018$  and  $P = 0.03$ , respectively) (Fig. 2B). Consistent with the RT-qPCR analysis results, compared with that of the control group, the protein level of ACE2 was also remarkably elevated after 24 hours of stim-

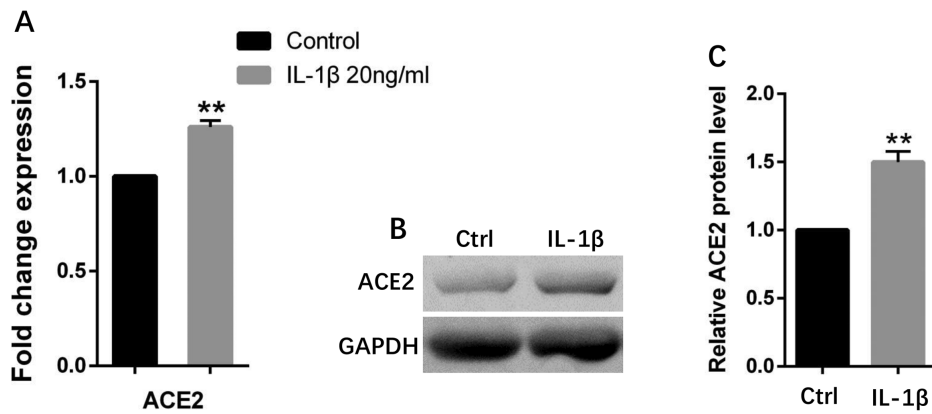
ulation with TNF $\alpha$  in HCECs ( $P = 0.0016$  at 5 ng/mL TNF $\alpha$  and  $P = 0.045$  at 10 ng/mL TNF $\alpha$ ) (Figs. 2C, 2D). However, no significant change in TMPRSS2 expression was observed at either the mRNA or protein level after 24 hours of stimulation with TNF $\alpha$  in HCECs (Figs. 2E, 2F).

To verify the change in ACE2 expression in response to inflammatory cytokines in HCECs, IL-1 $\beta$  was used to stimulate HCECs, and we observed that both the mRNA and protein levels of ACE2 in HCECs were increased significantly by 20 ng/mL IL-1 $\beta$  (Figs. 3A–3C). These results indicate that the expression of ACE2 in human corneal epithelial cells is upregulated by inflammatory cytokine stimulation.

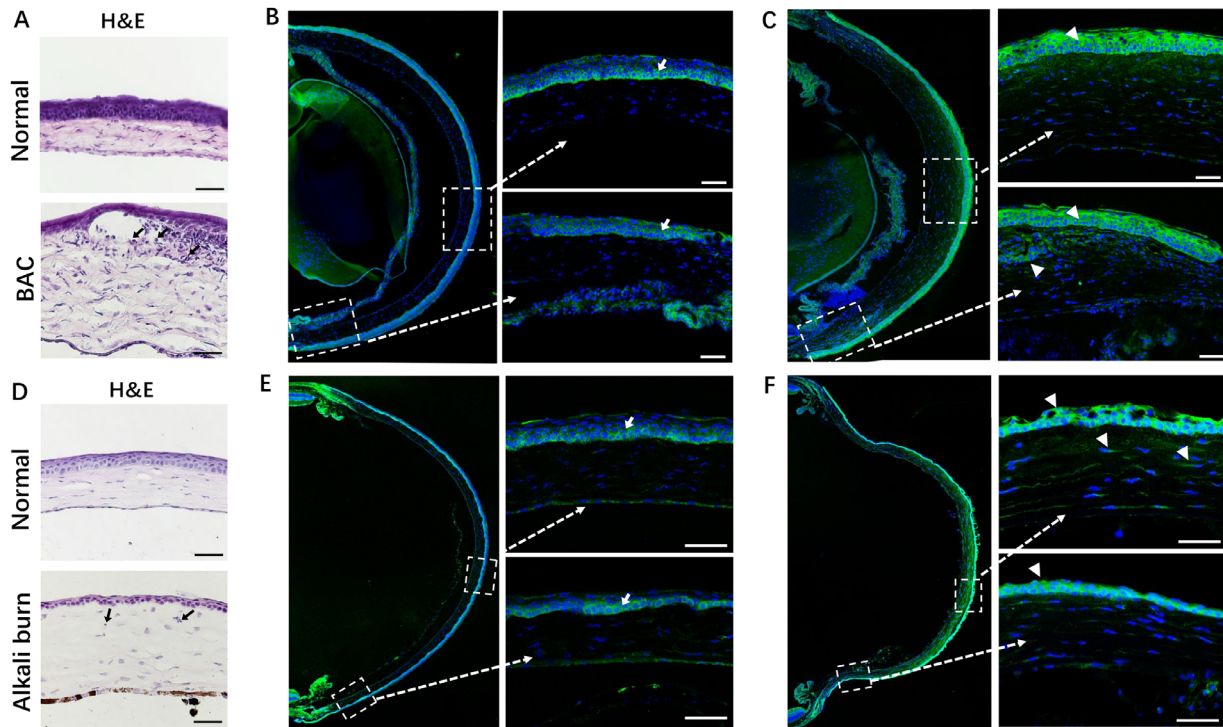
### ACE2 Expression is Elevated in Inflammatory Mouse Corneal Epithelium

To investigate ACE2 expression in inflammatory corneal epithelium, we used the corneal alkali-burn and BAC-induced dry eye mouse models, which are widely used to study corneal inflammation. Histological analysis showed that corneas that received 0.2% BAC twice daily developed severe epithelial damage, with many inflammatory cells infiltrating under the epithelium (Fig. 4A, arrows). After three weeks of alkali-burn injury, the re-epithelialized corneas displayed fewer irregular epithelial cell layers (2–3 layers) and mild inflammation with a small number of





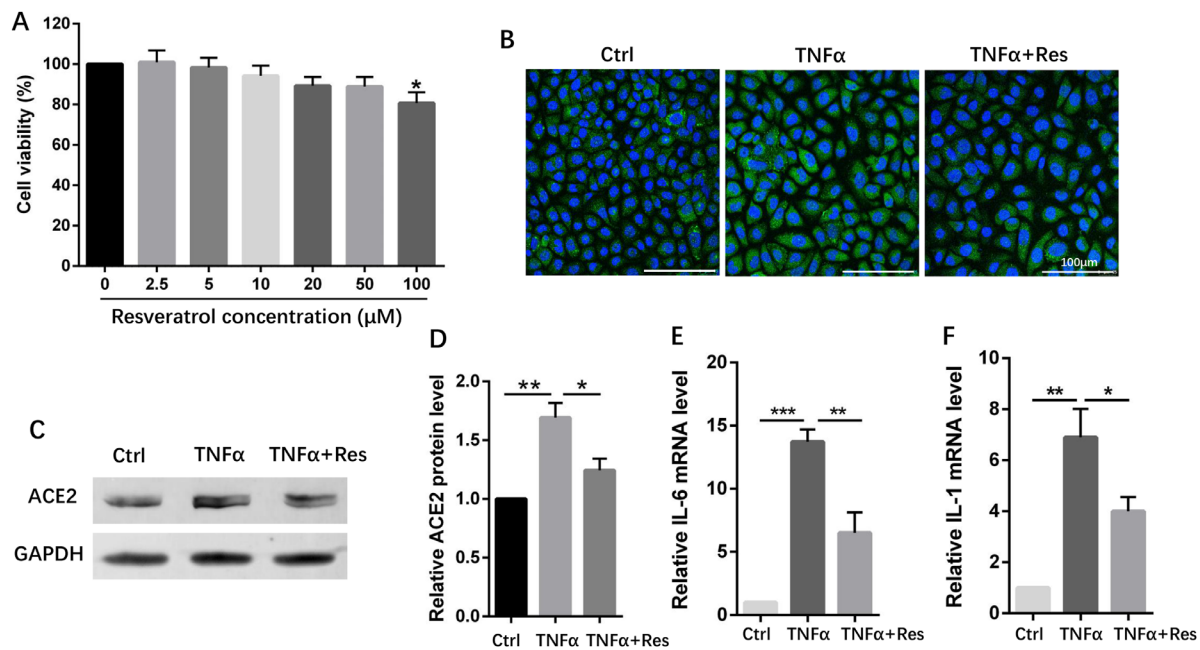
**FIGURE 3.** Expression changes of ACE2 in human corneal epithelial cells stimulated by IL-1 $\beta$ . **(A)** RT-qPCR was performed to determine the Ace2 expression level in HCECs treated with or without IL-1 $\beta$  at the indicated concentration for 24 hours. The level of gene expression in control samples is designated as 1. **(B)** Western blotting was performed to determine the ACE2 protein levels in HCECs exposed to 20 ng/mL IL-1 $\beta$  for 24 hours, and the quantified data are shown in C. \* $P < 0.05$ , \*\* $P < 0.01$ .



**FIGURE 4.** Elevated expression of ACE2 in the epithelium of injured mouse corneas. **(A)** Representative images of H&E staining **(A)** and immunofluorescence staining of ACE2 (green) with sections of corneas from normal BALB/c **(B)** and BALB/c mice after 14 consecutive days of twice daily exposure to BAC **(C)**. Representative images of H&E staining **(D)** and immunofluorescence staining of ACE2 in sections of corneas from normal C57BL/6 **(E)** and alkali-burned C57BL/6 mice after three weeks **(F)**. Low magnification of the entire cornea is displayed on the left panel and higher magnifications from the boxed areas on the left panel are shown on the right in **B, C, E, and F**. The black arrows indicate inflammatory cells. Note that higher ACE2 expression is shown in basal cells compared with that in wing and superficial cells in normal mouse corneas (white arrows), whereas strong expression of ACE2 is observed in the whole epithelial layers and stroma in injured corneas (white arrowheads). Scale bars: 50  $\mu$ m.

inflammatory cells in the stroma (Fig. 4D, arrows), whereas the normal corneal epithelium had four or five cell layers. A previous study reported strong ACE2 expression in mouse corneas comparable to that in lung tissue by RT-PCR analysis.<sup>22</sup> Consistently, we observed that in corneas from both normal C57BL/6 and BALB/c mice, ACE2 was expressed in epithelial cells, especially in basal epithelial cells in both the central cornea and limbus (Figs. 4B, 4C, 4E, 4F;

arrows), and in corneal endothelial cells, but a weak signal was detected in the stroma by immunofluorescence (Figs. 4B, 4C, 4E, 4F). Compared with normal BALB/c mouse corneas, corneas treated with BAC for 14 consecutive days displayed a stronger signal of ACE2 expression in the whole epithelial layer, including basal, wing and superficial cells, and in the corneal stroma (Figs. 4B and 4C, arrowheads). Similarly, alkali-burned corneas with mild inflammation



**FIGURE 5.** Resveratrol attenuates the elevated expression of ACE2 induced by TNF $\alpha$  in HCECs. (A) Cell viability (determined by the MTT assay) of HCECs treated with different concentrations of resveratrol for 24 hours. The results were normalized to the vehicle group. (B) Immunofluorescence staining of ACE2 (green) in HCECs in the presence or absence of TNF $\alpha$  or resveratrol for 24 hours, and the quantified data are shown in D. (C) Western blot analysis of ACE2 expression in HCECs in the presence or absence of TNF $\alpha$  or resveratrol for 24 hours, and the quantified data are shown in D. RT-qPCR of the expression levels of IL-6 (E) and IL-1 (F) relative to that of GAPDH in HCECs in the presence or absence of TNF $\alpha$  or resveratrol for 24 hours. Res, resveratrol. \* $P < 0.05$ , \*\* $P < 0.01$ . Scale bars: 50  $\mu$ m.

exhibited high ACE2 expression throughout the whole repaired epithelial layer in both the central cornea and limbus (Figs. 4E, 4F; arrowheads), suggesting that ACE2 expression is upregulated in inflammatory mouse corneas.

### Resveratrol Attenuates the Elevated Expression of ACE2 Induced by TNF $\alpha$ in Corneal Epithelial Cells

To explore potential agents to protect the ocular surface from SARS-CoV-2 infection, we investigated the effects of resveratrol on the regulation of ACE2 expression in inflammatory HCECs. We first tested the cytotoxicity of HCECs exposed to multiple concentrations of resveratrol for 24 hours, and only 100  $\mu$ M resveratrol provoked a significant reduction in HCEC viability ( $P = 0.023$ ) (Fig. 5A). Therefore, we used a relatively low concentration (5  $\mu$ M or 10  $\mu$ M) of resveratrol to pretreat HCECs for two hours, then administered 5 ng/mL TNF $\alpha$  and, finally, incubated them for 24 hours. Immunofluorescence staining showed that TNF $\alpha$  treatment stimulated ACE2 expression significantly compared with that of the normal control. ACE2 expression was much lower in the resveratrol with TNF $\alpha$  treatment group than in the TNF $\alpha$  treatment group (Fig. 5B). Consistently, Western blot analysis showed that TNF $\alpha$  significantly upregulated ACE2 expression ( $P = 0.005$ ), and resveratrol treatment significantly attenuated the increase of ACE2 in HCECs ( $P = 0.048$ ) (Figs. 5C, 5D). Furthermore, we found that resveratrol exhibited potent anti-inflammatory activity in HCECs. As shown in Figures 5E and 5F, resveratrol significantly reduced the production of IL-1 and IL-6 stimulated by TNF $\alpha$  in HCECs, which may contribute to attenuating the increase in ACE2 triggered by TNF $\alpha$  in HCECs.

### DISCUSSION

Our data demonstrated that the corneal epithelium exhibited higher expression of ACE2, a functional receptor of SARS-CoV-2, than pterygium tissue and corneal keratocytes in humans. ACE2 expression was stimulated by inflammatory cytokines in human corneal epithelial cells and in inflamed mouse corneas induced by BAC administration and alkali burn. In addition, we also found that resveratrol reduced the increased expression of ACE2 triggered by TNF $\alpha$  in HCECs, suggesting that it might be able to decrease susceptibility to SARS-CoV-2 at the ocular surface. Although detailed clinical studies with human populations are needed before making a conclusive claim, our study indicates that patients with inflammatory corneal epithelium may display higher ACE2 expression, which may increase the risk of SARS-CoV-2 infection and requires extra caution during the COVID-19 pandemic.

Several studies have recently reported high expression of Ace2 and Tmprss2 on the ocular surface,<sup>9,22</sup> and Collin et al.<sup>23</sup> found coexpression of ACE2 with TMPRSS2 throughout the limbal and peripheral corneal epithelium and superficial conjunctiva in normal humans using a sc RNA seq dataset, suggesting that the ocular surface may be a site of SARS-CoV-2 entry. We first validated the expression of Ace2 and Tmprss2 in the human corneal epithelium and immortalized HCECs, and both of these genes were expressed in the human corneal epithelium and HCECs. Due to ethical concerns, obtaining adult healthy human conjunctival tissues is difficult; thus, we compared Ace2 expression in the corneal epithelium, conjunctiva from pterygium tissue and corneal keratocytes. The human corneal epithelium showed much higher ACE2 expression than

that in pterygium tissue and keratocytes. These results suggest that corneal epithelial cells can be used as a model to study changes in ACE2 expression because of the high abundance of ACE2 expression on the ocular surface.

Inflammation is ubiquitous in ocular surface diseases,<sup>24</sup> such as dry eye, infection, and chemical damage, as well as in a high percentage of COVID-19 patients with dry eye and conjunctivitis,<sup>14</sup> which prompted us to investigate the altered expression of ACE2 in the corneal epithelium under inflammatory conditions. Inflammatory cytokines disrupt the barrier function of human corneal epithelial cells<sup>25</sup> and mediate ocular inflammation by activating the NF $\kappa$ B pathway and prompting the secretion of inflammatory factors.<sup>26–28</sup> We therefore tested the inflammatory response of HCECs by evaluating activation of NF $\kappa$ B signaling induced by TNF $\alpha$ . Subsequently, we observed that both the mRNA and protein levels of ACE2 were upregulated in HCECs treated with TNF $\alpha$  and IL-1 $\beta$ , suggesting that ACE2 expression can be upregulated by inflammatory stimuli in HCECs, whereas no significant change in TMPRSS2 expression was observed. Consistent with this result, elevated ACE2 expression triggered by various viruses and inflammatory cytokines has also been reported in HEK293 cells and lung tissue.<sup>3,29</sup>

The corneal alkali-burn and dry eye models are representative models used to study corneal inflammation. Multiple inflammatory factors, such as TNF $\alpha$ , IL-1, and IL-6, have been found to be increased in both animal models and patients with dry eye or chemical burns.<sup>28,30</sup> Thus we used these two corneal injury models to investigate the altered expression of ACE2. BAC is frequently used to induce dry eye in BALB/c mice, leading to elevated levels of IL-1 and TNF and disruption of epithelial organization.<sup>18,31</sup> Consistent with the results of a previous study,<sup>18</sup> corneas that received 0.2% BAC twice daily developed epithelial defects with a number of infiltrating inflammatory cells. In normal mouse corneas, ACE2 expression was mainly restricted to epithelial basal cells and no obvious ACE2 expression was detected in superficial epithelial cells or the stroma. However, the BAC-treated epithelium displayed high expression of ACE2 in the whole epithelial layer, and it was interesting that elevated ACE2 expression was also observed in inflammatory corneal stroma. Alkali-burned mouse corneas showed complete loss of corneal epithelial cells after injury, followed by the regeneration of epithelial cells at later stages, which is consistent with the results of previous studies.<sup>32</sup> Inflammation in alkali-burned mouse corneas is usually present for at least 28 days.<sup>33,34</sup> After observing several time points after injury, including three days, one week, two weeks, and three weeks, we detected strong ACE2 expression in re-epithelialized corneas with mild inflammation, which generated multiple epithelial layers three weeks after induction of alkali-burn injury. Taken together, our results demonstrate that ACE2 expression in corneas is upregulated under inflammatory conditions. Similar to our results, a recent study reported a significant increase in ACE2 mRNA and protein levels in conjunctival tissues of inflamed conjunctiva, conjunctival nevi, conjunctival cysts, conjunctival papilloma, and conjunctival polyps compared to those in normal conjunctival tissues.<sup>35</sup> Taken together, our results may explain why patients with dry eye and keratoconjunctivitis are more likely be positive for SARS-CoV-2 RNA,<sup>14,16</sup> which may be attributed to the increased ACE2 expression on the ocular surface.

To identify potential drugs that reduce ACE2 expression on the ocular surface, we tested several candidates that have been reported to prompt the repair of corneal damage, and we found that resveratrol was effective in attenuating the increased expression of ACE2 induced by TNF $\alpha$  in HCECs. Resveratrol is a well-known activator of SIRT1, a mammalian form of the sirtuin family of proteins.<sup>36</sup> The pathway regulated by SIRT1 affects metabolism, cell survival, cellular senescence, inflammation-immune function, cell cycle, and stress resistance.<sup>37</sup> On the ocular surface, resveratrol has been reported to decrease inflammation, oxidative damage, and angiogenesis in the cornea.<sup>38,39</sup> In our study, resveratrol exhibited a strong anti-inflammatory effect in HCECs, and it significantly reduced the production of multiple inflammatory cytokines, such as IL-1 and IL-6, in HCECs. IL-6 and IL-1 $\beta$  have been reported to induce ACE2 transcription in multiple cell types and tissues.<sup>40,41</sup> Therefore the reduction of inflammatory cytokine secretion in TNF $\alpha$ -stimulated HCECs after treatment with resveratrol may help to elucidate the mechanisms by which resveratrol regulates ACE2 expression.

In conclusion, our work highlights that ACE2 is highly expressed in the human corneal epithelium and is upregulated in inflammatory corneas. Resveratrol attenuates the increased expression of ACE2 in inflammatory HCECs. The main purpose of this work is to shed light on patients with inflammatory ocular surfaces, who may have higher expression of ACE2, and extra caution should be taken for ophthalmologist and ocular surface patients to take necessary eye protection and treat ocular surface disorders actively during the COVID-19 pandemic.

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