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The ACE2-deficient mouse: A model for a cytokine storm-driven inflammation

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Abstract

Angiotensin converting enzyme 2 (ACE2) plays an important role in inflammation, which is attributable at least, in part, to the conversion of the pro-inflammatory angiotensin (Ang) II peptide into angiotensin 1-7 (Ang 1-7), a peptide which opposes the actions of AngII. ACE2 and AngII are present in many tissues but information on the cornea is lacking. We observed that mice deficient in the *Ace2* gene (Ace2$^{-/-}$), developed a cloudy cornea phenotype as they aged. Haze occupied the central cornea, accompanied by corneal edema and neovascularization. In severe cases with marked chronic inflammation, a cell-fate switch from a transparent corneal epithelium to a keratinized, stratified squamous, psoriasiform-like epidermis was observed. The stroma contained a large number of CD11c, CD68, and CD3 positive cells. Corneal epithelial debridement experiments in young ACE2-deficient mice showed normal appearing corneas, devoid of haze. We hypothesized, however, that these mice are "primed" for a corneal inflammatory response, which once initiated, would persist. In vitro studies reveal that interleukins (IL-1a, IL-1b), chemokines (CCL2, CXCL8), and TNF-α, are all significantly elevated, resulting in a cytokine storm-like phenotype. This phenotype could be partially rescued by treatment with the AngII type 1 receptor (AT1R) antagonist, losartan, suggesting that the observed effect was mediated by AngII acting on its main receptor. Since the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) utilizes human ACE2 as the receptor for entry with subsequent downregulation of ACE2, corneal inflammation in Ace2$^{-/-}$ mice may have a similar mechanism with that in COVID-19 patients. Thus the Ace2$^{-/-}$ cornea, because of easy accessibility, may provide an attractive model to explore the molecular mechanisms, immunological changes, and treatment modalities in patients with COVID-19.

KEYWORDS
cornea, corneal epithelial cells, COVID-19, macrophages, SARS-CoV-2

1 | INTRODUCTION

Angiotensin I converting enzyme 2 (ACE2) is a critical component of the renin-angiotensin system (RAS), due to its ability to hydrolyze angiotensin II (AngII).[1-3] AngII is the major effector peptide of RAS and regulates cell growth, and key events in the inflammatory process.[4] In its pro-inflammatory mode, AngII directly stimulates pro-inflammatory mediators resulting in the infiltration of macrophages and moreover is profibrotic and may foster angiogenesis (4 and references therein).

The expression of ACE2 is most abundant in the kidney and intestine, followed by testis and the heart.[5-7] Moreover, the surface expression of ACE2 was found in lung epithelial cells.[8] Several groups generated ACE2-deficient mice[9-11] with conflicting responses on the contribution of ACE2 to cardiac structure and function, and the control of blood pressure.[12,13] Due to its importance as an entry point for coronaviruses, the effects of ACE2 depletion was tested in lung tissue and shown to be detrimental in the progression of lung injury following experimental perturbations.[14,15] ACE2 depletion also created a "cytokine storm" like inflammation.[16,17] A cytokine storm is a consequence of the secretion of a large number of cytokines and involves recruitment and activation of inflammatory cells such as macrophages.[18,19] Cytokine storms are known to occur in autoimmune diseases[20] and can be triggered by chemical insults such as corneal alkali burns[21] as well as infections, such as COVID-19.[22] In COVID-19 patients, ACE2 is the target of the virus[23] and dramatic increases in plasma cytokines and chemokines such as IL1B, CCL2 (MCP1), CXCL8 (IL8), and TNFα have been observed.[22]

ACE2 is present in the retina[24] and recently, there has been a plethora of information regarding the expression in the cornea and conjunctiva due to the ongoing COVID-19 pandemic.[25-30] During our investigations using an ACE2-deficient mice, we noted that as the ACE2-deficient mice aged, some developed cloudy corneas. In certain mice, cloudy corneas were bilateral, in others they were unilateral, whereas some adult aged mice had clear corneas. Herein, we report that AngII and ACE2 are expressed in limbal and corneal epithelia in humans and mice. Moreover, when challenged with corneal injury, ACE2-deficient mice are "primed" for an increased corneal inflammatory response. Once initiated, inflammation persists, which markedly alters the epithelial and stromal phenotypes. Blockade of the AngII type 1 receptor (AT1R) partially restores the cytokine/chemokine balance due to ACE2 deficiency. Collectively, our findings establish a pivotal role of ACE2 in the cornea and identifies AngII blockade as a potential new target for corneal inflammation. Moreover, the administration of soluble ACE2 protein should have a therapeutic benefit by fostering the degradation of AngII and the formation of Ang 1-7 as it has proposed for other disease entities.[31] Of note, ACE2 is the main receptor for the coronavirus SARS-CoV-2 responsible for the current pandemic.[23] The associated organ injury, particularly acute lung injury, has been shown to involve a decrease in plasma bound ACE2 and administration of such ACE2 have the potential as an ideal treatment, and Ace2$^{-/-}$ mice a unique model to study inflammatory processes akin to those observed in severe COVID-19.

2 | MATERIALS AND METHODS

2.1 | Animal studies

Animal procedures were approved by the Northwestern University Animal Care and Use Committee. We used an ACE2-deficinet mouse (Ace2$^{-/-}$) on a C57Bl/6 background provided to us as a gift by Susan Gurley.[9] To generate debridement wounds in the central corneal epithelium, a rotating diamond burr was gently applied to the surface of the central cornea to remove the corneal epithelium while the peripheral corneal and limbal epithelia remained intact. Gross images of mouse ocular globes were taken using a Leica dissecting scope. Corneal haze was graded on a scale of 0 through 4[32] as follows: 0, no corneal haze; 1, iris detail visible; 2, Pupillary margin visible, iris detail obscured; 3, pupillary margin not visible; 4, cornea totally opaque. Wound closure was examined by topically applying 20 µL of 0.5% fluorescein in PBS and imaging the wound using a Leica dissecting scope under cobalt blue illumination. At the termination of the experiment, mouse whole eyes were fixed in 10% buffered formalin solution and paraffin embedded for histological analysis. H&E staining was conducted as described previously[33] and slides were imaged using an AxioVision Z1 fluorescence microscope system (Carl Zeiss, Oberkochen, Germany).

2.2 | Immunohistochemistry of human and mouse cornea

Normal human corneal tissues were obtained from the Eversight eye banks (Ann Arbor, MI, USA) and embedded in paraffin blocks. Immunohistochemical (IHC) staining of human and Ace2$^{-/-}$ mouse eyes were conducted as described previously.[34] Antigen retrieval of the paraffin embedded sections was performed at 96°C in pH 6.0 citrate buffer for 30min. After blocking in 2.5% normal horse serum, sections were incubated overnight at 4°C with antibodies recognizing: AngiotensinII (AngII) rabbit polyclonal antibody (Penlabs, San Carlos, CA), ACE2 goat polyclonal antibody(R&D systems, Minneapolis, MN), CD68 rabbit polyclonal antibody (Proteintech, Chicago, IL), CD3 rabbit polyclonal antibody (Proteintech, Chicago, IL), and CD11c rabbit polyclonal antibody (Proteintech, Chicago, IL) at

1:100 dilution. After rinsing with PBS containing 0.1% Tween (PBST), the samples were incubated with ImmPRESS-AP (alkaline phosphatase) Polymer Anti-Rabbit or Anti-Goat IgG Reagent (Vector lab, Burlingame, CA) at room temperature for 30 minutes. After rinsing with PBST, chromagen was detected using a Vector Red Alkaline Phosphatase Substrate Kit (Vector lab, Burlingame, CA) for 10-30 minutes. Samples were counterstained with hematoxylin, and dehydrated with graded ethanol and xylene. Images were taken using an AxioVision Z1 fluorescence microscope system (Carl Zeiss, Oberkochen, Germany).

2.3 | Cell culture

Immortalized corneal epithelial cells, hTCEpi, were cultured in keratinocyte serum-free media (Thermo Fisher Scientific, Waltham, MA, USA) as described before.[35] For siRNA knock-down experiments, cells were transfected with 10 nM siRNA SMARTpools against ACE2 and nontarget control (GE Dharmacon, Colorado, USA) as previously described.[33] Two days after transfection, cells were treated with losartan (100μM) for 24 hours, and processed for total RNA isolation. Human monocytic THP-1-Dual cells (InvivoGen, San Diego, CA, USA) were cultured with RMPI 1640 with 10% heat inactivated FBS. About 10nM 12-O-tetradecanoylphorbol-l3-acetate (PMA) for 24 hours is used to induce THP-1-Dual into macrophages. Murine macrophagic RAW-Dual (InvivoGen, San Diego, CA, USA) cells were cultured with DMEM with 10% heat activated FBS.

2.4 | Modified Boyden chamber assay

For the chemotaxis assay, the hTCEpi cells (5×10^4 cells/500 μl) transfected with siControl or siACE2 were plated in the lower chamber of 24-well culture plates for 24 hours. The RAW-Dual cells (4×10^4 cells/200 μl) were resuspended in DMEM with 10% heated-FBS media, and placed in Transwell chambers (Corning Inc, Corning, NY, USA) in 24 well culture plates. DMEM with 10% heated-FBS was added to both the transwell and lower chamber. After 48 hours, transwell chambers were rinsed with PBS, and the cells on top were removed with a cotton tip applicator. The RAW-Dual cells that migrated to the bottom of the transwell were stained with crystal violet (Sigma-Aldrich, St. Louis, MO, USA), and counted under an inverted Zeiss microscope.

2.5 | Real time quantitative PCR (qPCR)

Total RNAs were isolated from hTCEpi cells and purified with a miRNeasy kit (Qiagen, Hilden, Germany). Real-time

qPCR (RT-qPCR) was performed with a Roche LightCycler 96 System using the Roche FastStart Essential DNA Green Master (Roche, Branchburg, NJ, USA) according to the manufacturer's instructions. The primer pairs for RT-qPCR were designed using the IDT PrimerQuest Primer Design Tool (IDT, Coralville, IA). Primers (Table S1) were used to recognize: human IL1A, IL1B, IL6, CCL2, INOS, CXCL8, TNFA. Relative gene expression was calculated using the 2ΔΔCt.

2.6 | Statistical analysis

Unpaired t test were performed to determine statistical significance. The data are shown as means ± standard deviation (*SD*). The differences were considered significant for P values of <.05. All experiments were replicated at least three times.

3 | RESULTS

3.1 | AngII and Ace2 are expressed in limbus and cornea

Immunohistochemical analysis revealed that the expression of AngII was strong throughout the human limbal and corneal epithelia as well as the stromal vessels (Figure 1A,B). In contrast, ACE2 expression was prominent in the limbal basal cells and vasculature (Figure 1C). AngII and ACE2 were detected throughout the wild-type (WT) mouse corneal epithelium (Figure 2A,C) but AngII appeared to be more patchy in its distribution (Figure 2A). In the ACE2-deficient mice (Ace2$^{-/-}$), no ACE2 was detected in the corneal epithelium (Figure 2D), whereas AngII expression was markedly increased (Figure 2B) compared with the WT (Figure 2A).

3.2 | Loss of ACE2 results in marked corneal changes

Aged Ace2$^{-/-}$ mice developed striking changes in corneal epithelial morphology as well as significant stromal angiogenesis and an inflammatory infiltrate (Figure 3). Interestingly these changes appeared in approximately 70% of aged adult (60-80 weeks old) mice. In many instances, these changes only occurred in one eye. The contralateral eye appeared to have a normal corneal epithelium and a stroma containing highly organized collagen bundles with numerous keratocytes but devoid of inflammatory cells (Figure S1). Those Ace2$^{-/-}$ mice that developed inflammation were readily recognized clinically by a cloudy, hazy cornea (Figure 3B-D). A wide spectrum of corneal epithelial alterations were detected, ranging from a thin, disorganized epithelium (Figure 3F) to a more stratified,
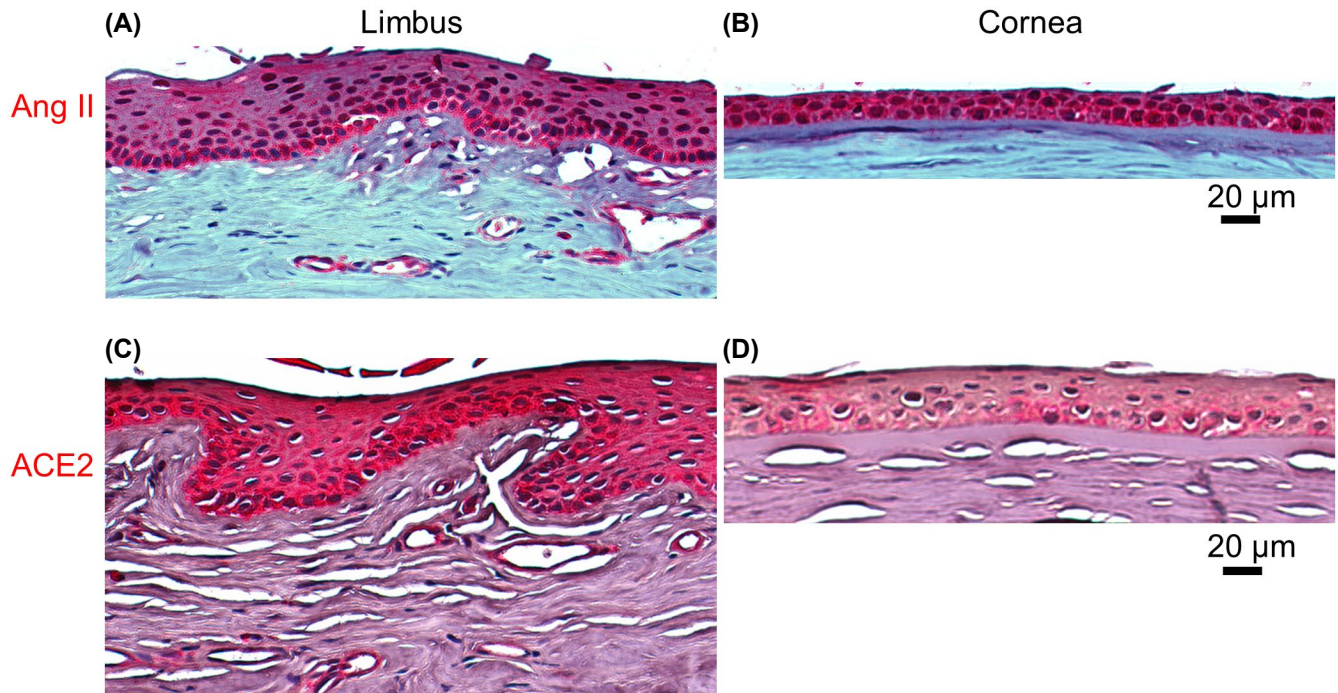
(A) Limbus   **(B)** Cornea

Ang II

ACE2

20 μm

FIGURE 1 Human corneal and limbal epithelial cells express AngII and ACE2. Immunohistochemical staining detects AngII (A, B) and ACE2 (C, D) in human limbal (A, C) and corneal (B, D) epithlia. N = 3
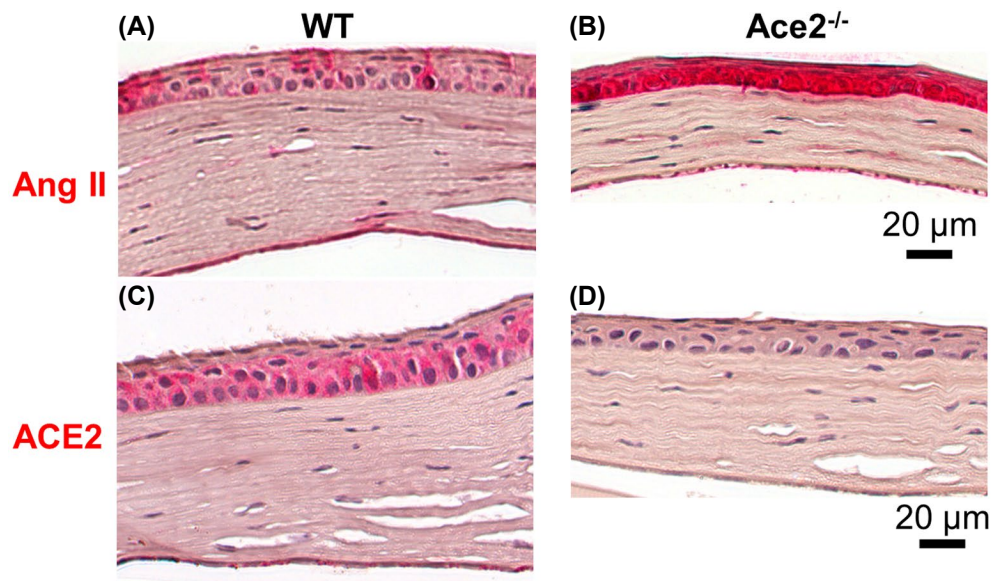
(A) WT   **(B)** Ace2$^{-/-}$

Ang II

20 μm

ACE2

20 μm

FIGURE 2 ACE2-deficient corneal epithelium of young adult mice with normal appearing corneas shows increased AngII expression. Immunostaining detects AngII (A, B) and ACE2 (C, D) in WT (A, C) and Ace2$^{-/-}$ (B, D) epithelia. N = 3

thickened epidermoid tissue (Figure 3H). In all eyes exhibiting haze or cloudiness the stroma consisted of a densely packed admixture of polymorphonuclear, mononuclear, and lymphocytic infiltrates along with numerous vascular profiles (Figure 3F-H). On higher magnification, the infiltrates were identified as neutrophils by H&E, and as macrophages, T cells, and dendritic cells identified by immunohistochemistry staining for CD68, CD3, and CD11c cells, respectively (Figure 4).

3.3 | Ace2$^{-/-}$ mice are "primed" to develop marked inflammation in response to a minor stress

Since in 30% (6/19 mice) of Ace2$^{-/-}$ mice, the cloudy corneas were not bilateral, this would suggest that the lesions are a direct sequelae of external perturbation rather than arising from a systemic deficiency. To test this
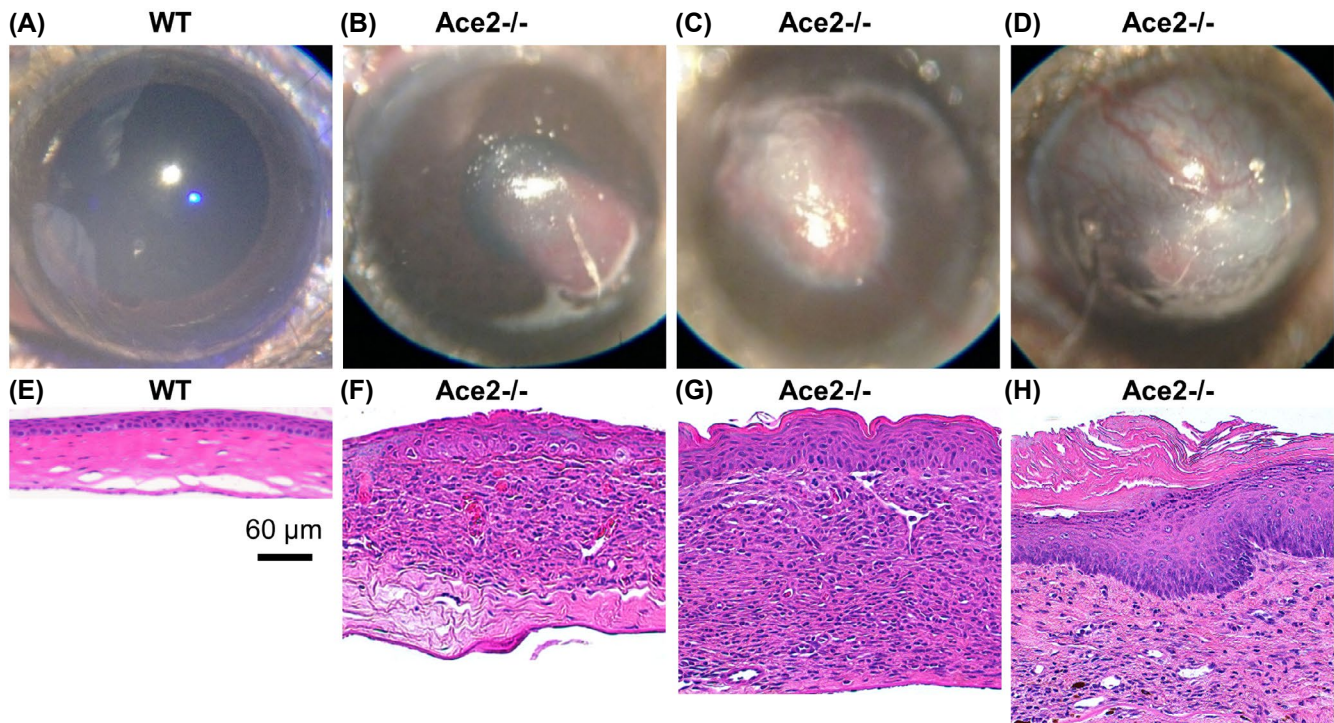
FIGURE 3 Aged ACE2-deficient mice show corneal inflammation and epithelial defects. (A-D) Representative gross images of WT (A) and Ace2$^{-/-}$ mouse eyes (60-80 weeks old) (B-D) were taken using a dissecting scope. Clear and transparent corneas were observed in aged WT mice eyes (A), while increased central corneal haze with corneal neovascularization were noted in aged Ace2$^{-/-}$ mice eyes (B-D). (E-H) Representative H&E stained histological images of WT mouse cornea with intact corneal epithelium and regular stroma (E) and Ace2$^{-/-}$ mouse corneas (F-H) with markedly thickened cornea and a large number of polymorphonuclear, mononuclear, and lymphocytic infiltrates along with numerous vascular profiles. Twenty-six aged Ace2$^{-/-}$ mice (60-80 weeks old) were analyzed. About 19 mice developed corneal haze. Among the 19 mice with corneal haze, 13 were bilateral and 6 were unilateral. Total 32 eyes with cloudy cornea were observed

idea, we made small 1mm circular debridement wounds in young Ace2$^{-/-}$ mice with clear corneas and compared the response to aged-matched WT littermates. In this well adopted procedure, the type of wound removes the corneal epithelium but does not result in a significant inflammatory infiltrate.[36] Immediately following wounding, all mice received topical application of a 0.5% fluorescein stain, and the rate of epithelial healing was visualized. As expected, WT mice sealed wounds within 24 hours (Figure 5A), whereas the Ace2$^{-/-}$ mice still had detectable fluorescein staining at this time (Figure 5A). Three days post wounding, WT mice had clear corneas devoid of fluorescein staining. Similarly, the Ace2$^{-/-}$ mice had minimal fluorescein staining 3days post wound; however, there was development of haze prompting further clinical evaluation of corneal clarity[32] (Figure 5B). Day 7 post wounding, WT mice appeared normal, whereas the Ace2$^{-/-}$ mice still had cloudy corneas and patches of fluorescein staining detected on the surface, suggestive of a defective epithelial barrier.

One day post wounding, the WT mice had completely re-epithelialized the surface as evidenced by a thin, 1-2 cell layered, well-organized epithelium (Figure 6A,B). This was contrasted by the Ace2$^{-/-}$ mice, which were characterized by a single, thin layer of disorganized epithelial cells, lacking evidence of stratification (Figure 6C,D). At day 1 post wounding, the stroma of the WT mice appeared normal consisting of a plywood-like organization of collagen bundles, interspersed with numerous keratocytes. Few if any inflammatory cells were noted (Figure 6A,B). The stroma of the Ace2$^{-/-}$ mice was filled with inflammatory cells (primarily polymorphonuclear cells) and numerous vascular profiles, characteristic of a brisk inflammation (Figure 6C,D). Seven days post wounding, WT mice had a multi-layered, stratified epithelium characteristic of an unwounded mouse (Figure 6E,F) overlying a normal appearing stroma devoid of inflammatory cells. The corneal epithelium from Ace2$^{-/-}$ mice, 7 days post wounding was variable in appearance. For example, some mice had a markedly thickened multi-layered epithelium (Figure 6G), whereas some mice had a thin, single-layered epithelium (Figure 6H). In all cases the Ace2$^{-/-}$ mice, 7 days post wounding, had a stroma with variable amounts of inflammatory cells and vascular remnants (Figure 6G,H).
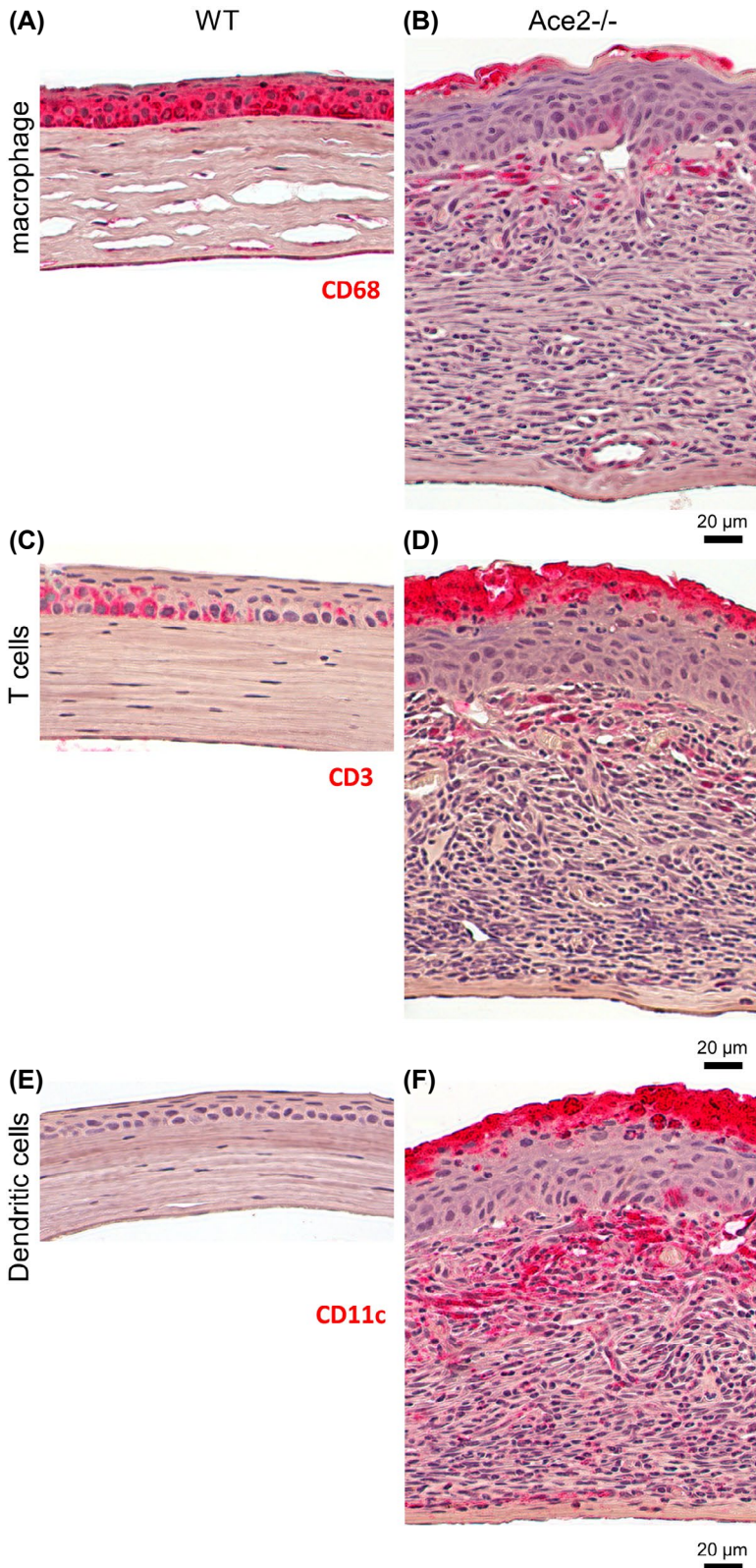
(A) WT

(B) Ace2-/-

macrophage

CD68

20 μm

(C)

(D)

T cells

CD3

20 μm

(E)

(F)

Dendritic cells

CD11c

20 μm

FIGURE 4 Aged ACE2-deficient mice show infiltration of immune cells. WT (A, C, E) and Ace2$^{-/-}$ (B, D, F) mouse corneas were stained for CD68 (marker for macrophage), CD3 (marker for T cells), and CD11c (marker for dendritic cells). N = 3

3.4 | Induction of cytokine expression in epithelial cells lacking Ace2 contribute to the primed inflammatory response in the Ace2$^{-/-}$ mice

Following a perturbation such as wounding, epithelial cells produce pro-inflammatory cytokines, which are known to initiate the inflammatory response.[37] To investigate the mechanism of this initial phase of inflammation in the context of ACE2, we knocked-down ACE2 in a corneal epithelial cell line (hTCEpi) and evaluated the expression of several cytokines. Knockdown of ACE2 in hTCEpi cells with an siRNA smartpool increased the expression of IL1A, IL1B,
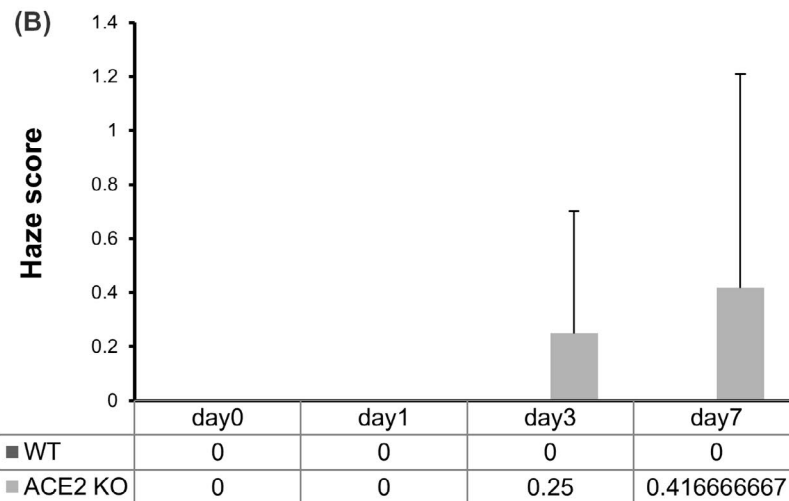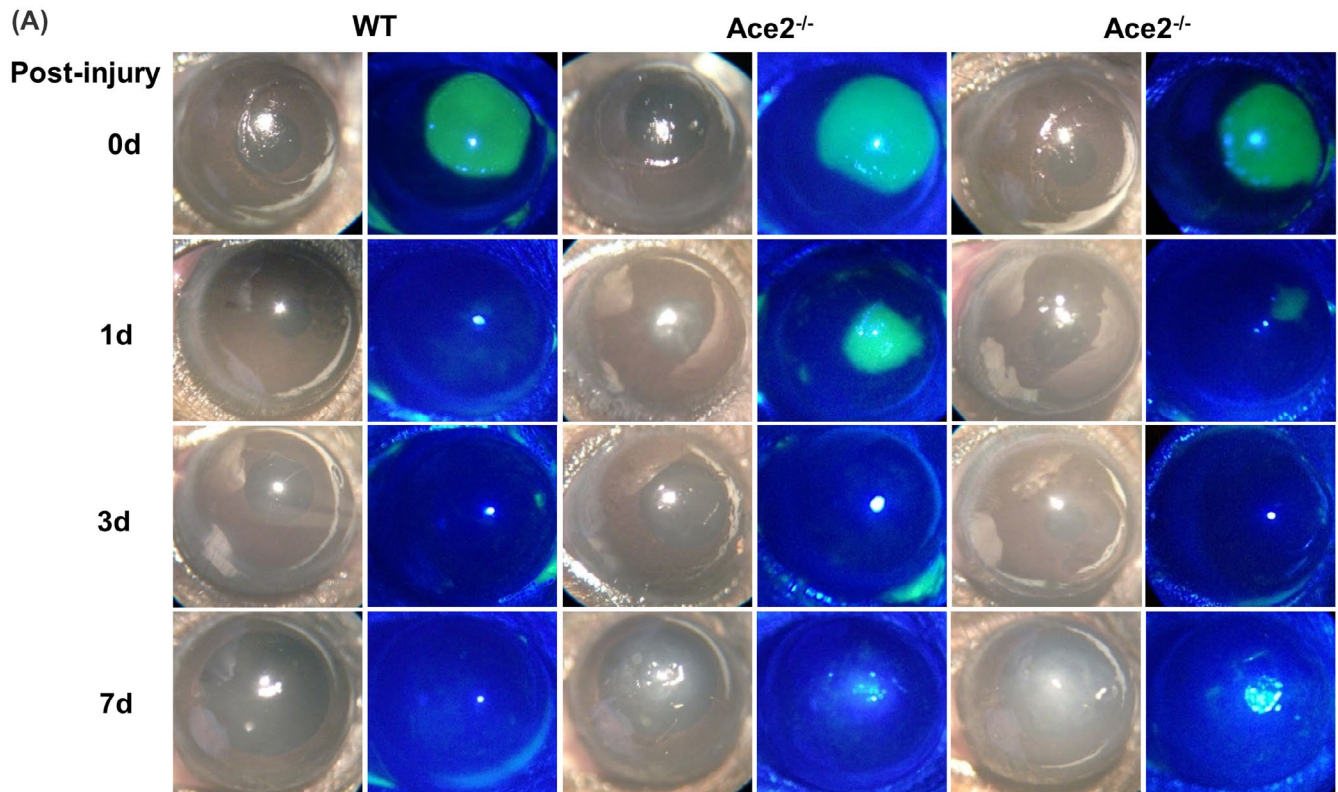
FIGURE 5 Young adult ACE2-deficient mice show corneal haze after central corneal debridement wounding. Clinical and fluorescein images were taken at 1, 3, and 7 days post injury. Green fluorescence represents areas devoid of epithelium (ie, corneal wounds). Corneal haze was observed in $ACE2^{-/-}$ mouse corneas at day 7 post injury. N = 6

TNFA, CCL2, and CXCL8, compared to the siRNA control (Figure 7).

To explore whether such an increase in cytokine expression in corneal epithelial cells enhanced recruitment of immune cells, we conducted trans-well chemotaxis assays using a modified Boyden chamber co-cultured with the RAW-Dual macrophage cell line against hTCEpi cells on the bottom chamber. Increased migration of macrophages was observed when ACE2 was knocked-down in hTCEpi cells (Figure S2).This suggests that ACE2 deficiency was at least in part responsible for the recruitment of macrophages into the inflammatory milieu. The increases observed in CCL2 and TNFA are associated with the recruitment and activation of macrophages.[38-41] Therefore, we explored the possibility that ACE2-deficient epithelial cells could activate macrophages. We exposed a PMA-stimulated THP-1-Dual human monocytic cell line to conditioned media from hTCEpi cells lacking ACE2. Such treatment significantly increased the expression of IL1A, IL1B, IL6, TNFA, CCL2, and CXCL8 compared with control hTCEpi conditioned media (Figure S3).
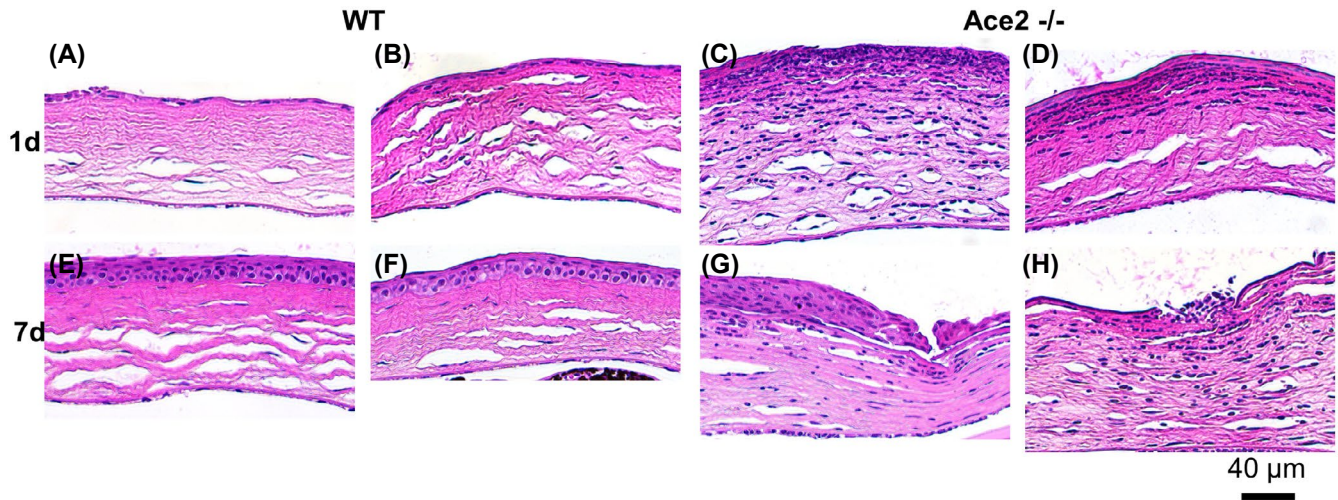
FIGURE 6 Young adult ACE2-deficient mice show corneal inflammation and epithelial defect after central corneal debridement wounding. WT (A, B, E, F) and Ace2$^{-/-}$ (C, D, G, H) mouse corneas were stained with H&E. Representative histological images of WT (A, B, E, F) and Ace2$^{-/-}$ (C, D, G, H) mouse corneas show a dramatic infiltration of immune cells in Ace2$^{-/-}$ but not in WT mouse corneas at day 1 post injury (A-D) and at day 7 post injury (E-H). Each image is representative of an individual mouse. N = 8
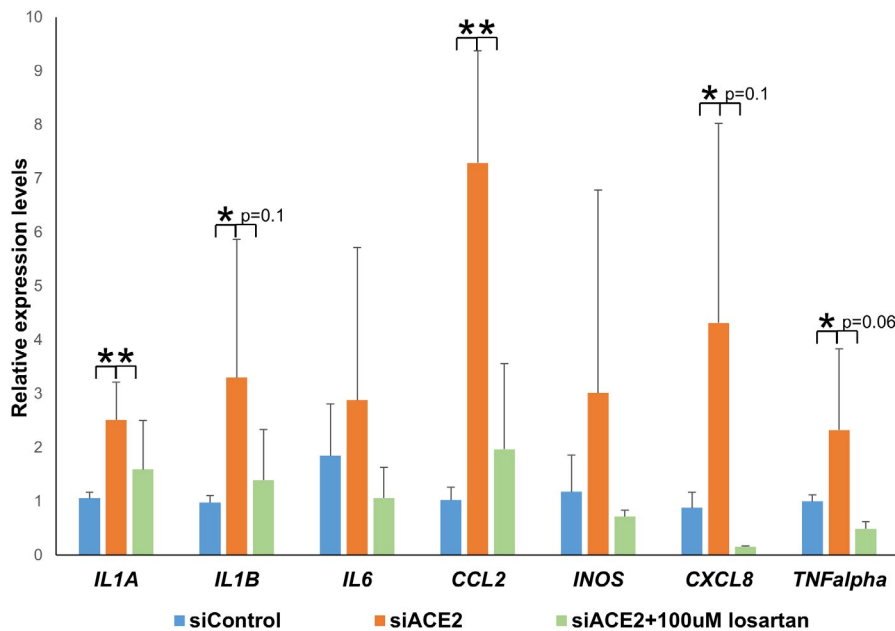

FIGURE 7 Losartan treatment can partially reverse pro-inflammatory activity in ACE2 depleted corneal epithelial cells. hTCEpi cells transfected with siControl or siACE2 were treated with losartan for 24 hours. Total RNAs were isolated from these cells for RT-qPCR for inflammation-related genes. N = 8. *$P < .05$

These findings indicate that loss of ACE2 in hTCEpi can activate macrophages.

To examine if ACE2 deficiency-induced cytokine production is due to an increase in AngII activity, we investigated whether treatment of corneal epithelial cells with the AT1R antagonist, losartan could rescue the phenotype. hTCEpi deficient in ACE2 were treated with 100 μm losartan for 24 hours and the cytokine response was evaluated. Losartan treatment decreased the expression of IL1A, TNFA, and CCL2 (Figure 7). This suggests that ACE2 deficiency is responsible for the excessive expression of cytokines in the inflammatory milieu via the activation of AngII.

4 | DISCUSSION

RAS components are found in ocular tissues of various species, as well as humans. The major components including renin, angiotensinogen, and ACE2 have been identified in the retina, ciliary body, vitreous fluid, iris, choroid, aqueous fluid, sclera, and conjunctiva[42] and references therein. Until recently, little if any attention has been focused on defining RAS in the limbus/cornea.[27-29] We now demonstrate that ACE2 is present in both human and mouse limbal and corneal tissues and that the genetic deficiency of ACE2 results in a marked inflammatory response in corneal epithelial and

stromal tissues. Moreover, the deficiency of ACE2 resulted in marked upregulation of AngII, the main peptide that is degraded normally by ACE2.

As ACE2-deficient mice aged, more than 70% developed cloudy corneas in either one or both eyes. This led us to postulate that external stress (eg, scratching or wounding) to the corneal surface resulted in an inflammatory event that could not be resolved due to the inability to regulate AngII. To test this concept, we induced gentle debridement of the corneal epithelium, which in WT mice does not result in a significant inflammatory response.[36] In the Ace2$^{-/-}$, this procedure caused a significant inflammatory response in young mice with otherwise normal appearing corneas (Figures 5, 6). This strongly supports the idea that Ace2$^{-/-}$ mice are "primed" for an inflammatory response and results in unresolvable inflammation after a mild perturbation. Similarly, Ace2$^{-/-}$ mice lungs following an insult resulted in worsened inflammatory cell infiltration and lung damage compared with WT mice; while there were no differences in unperturbed lungs between Ace2$^{-/-}$ and WT mice.[14,15,43] In contrast, ACE2 deficiency led to increases in inflammation in mouse kidney and aorta even without perturbation.[44,45] Although the lung and cornea are disparate tissues, the similarity in inflammatory responses raises the interesting question of what are the underlying mechanisms driving inflammation.

As noted earlier in ACE2-deficient mice, cloudy corneas were frequently observed in older (>60 weeks) mice and sometimes only in one eye. This most likely reflects an earlier external trauma. However, it is also possible that cloudy corneas seen in older mice are the result of an enhanced accumulation of reactive oxygen species due to alterations in RAS, as it has been shown that ACE2-deficient mice exhibit increased oxidative stress.[46] It is well established that with age, the well-developed corneal antioxidant defense systems diminish, leading to ROS accumulation and oxidative stress (47 and references therein). This combination of ROS and oxidative stress can lead to many age-related ocular diseases of the anterior segment (for reviews see 47,48). In other tissues, such as the brain, AngII has been shown to induce NADPH oxidase-dependent ROS production in isolated cerebral microvessels, resulting in cerebrovascular dysregulation.[49]

In the ACE2-deficient mice, once an inflammatory response is initiated, inflammation persists becoming permanent, which remodels the stromal microenvironment. This microenvironmental change results in a wide range of epithelial phenotypes. The most dramatic is a cell fate switch from the transparent corneal epithelium to a keratinized, stratified squamous, psoriasiform-like epidermis. A similar cell-fate switch was reported in mice deficient in leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1).[50] In these mice, loss of LRIG1 resulted in a pro-inflammatory state in the cornea through upregulation of Stat3. The persistence of the inflammation eventually resulted in the generation of an epithelium that resembled human psoriasis.

When ACE2 is silenced in a corneal epithelial cell line, interleukins (IL1A, IL1B, IL6), chemokines (CCL2, CXCL8), and TNFA, are all significantly elevated, resulting in a cytokine storm-like phenotype (51 and references therein). We observed that losartan treatment significantly blocked the induction of IL1A, TNFA, and CCL2 by ACE2 depletion in cell cultures but only slightly altered the expression of IL1B and CXCL8, indicating a partial rescue. Losartan is one of the AngII receptor blockers (ARBs). Unlike other ARBs, the effect of losartan can be overcome by high concentrations of AngII, which means that losartan is surmountable.[52] In addition, losartan needs to be converted in the liver to form its active metabolite EXP3174, which is 10-40 times more potent than losartan.[53] These two features of losartan may explain why we only see partial rescue in vitro. Nonetheless, these findings clearly establish that the recruitment and activation of the macrophage component of the inflammatory response is related to AngII acting via the AT1 receptor.

In the aggregate, our findings suggest that provision of ACE2 may provide a new strategy for treating corneal inflammatory conditions, as well as other indications such as kidney disease. Of potential clinical significance is the possibility that the Ace2$^{-/-}$ mouse cornea might be an excellent model for investigations into the etiology and treatment of a coronavirus-induced inflammation.[54] The severe acute respiratory system coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and the novel human coronavirus (SARS-CoV-2) have all been demonstrated to utilize human ACE2 as the receptor for entry.[23,55,56] In such a scenario, the coronavirus binds to and reduces the expression of ACE2; such a reduction increases AngII, leading to unregulated inflammation. Recently, it was demonstrated that patients infected with SARS-CoV-2 had interstitial mononuclear inflammatory infiltrates, dominated by lymphocytes in both lungs.[57] As the condition worsened, a dramatic cytokine-storm-driven inflammation was noted.[57] This phenotype bears striking similarities to what we report in the Ace2$^{-/-}$ mice, suggestive that corneal inflammation in Ace2$^{-/-}$ mice may have a similar mechanism with that in SARS-CoV-2 infected patients. Thus, the eye becomes extremely attractive as a model to explore the molecular mechanisms, immunological changes, and treatment modalities in patients with a SARS-CoV-2 infection for the following reasons: (i) mice cannot be infected with the SARS-CoV-2 making it difficult to find a direct experimental model; (ii) not having to use the live virus makes the Ace2$^{-/-}$ safer; and (iii) the relative ease of inducing and clinically detecting a corneal ACE2-deficient inflammatory response (corneal haze) is extremely convenient for observation.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

Drs. Batlle and Wysocki are co-inventors of a patent: "Active Low Molecular Weight Variants of Angiotensin Converting Enzyme 2"; and have applied for a patent for their use for coronavirus infection. Dr Batlle is the Founder of Angiotensin Therapeutics Inc.

AUTHOR CONTRIBUTIONS

R. M. Lavker contributed to experimental design, data interpretation, and manuscript writing; H. Peng contributed to experimental design and conduct, data interpretation, and manuscript writing; N. Kaplan contributed to experimental design and conduct, data interpretation, and manuscript writing; W. Yang contributed to experimental conduct; J. Wang contributed to experimental conduct, data interpretation, and manuscript writing; J. Wysocki made the corneal observations in the Ace2$^{-/-}$; K. Lu contributed to experimental design, data interpretation, and manuscript writing; D. Batlle contributed to experiment design and manuscript writing.

REFERENCES

1. Guy JL, Jackson RM, Acharya KR, Sturrock ED, Hooper NM, Turner AJ. Angiotensin-converting enzyme-2 (ACE2): comparative modeling of the active site, specificity requirements, and chloride dependence. *Biochemistry*. 2003;42:13185-13192.

2. Serfozo P, Wysocki J, Gulua G, et al. Ang II (angiotensin II) conversion to angiotensin-(1–7) in the circulation is POP (prolyloligopeptidase)-dependent and ace2 (angiotensin-converting enzyme 2)-independent. *Hypertension*. 2020;75:173-182.

3. Wysocki J, Ye M, Rodriguez E, et al. Targeting the degradation of angiotensin II with recombinant angiotensin-converting enzyme 2: prevention of angiotensin II-dependent hypertension. *Hypertension*. 2010;55:90-98.

4. Suzuki Y, Ruiz-Ortega M, Egido J. Angiotensin II: a double-edged sword in inflammation. *J Nephrol*. 2000;13:S101-S110.

5. Donoghue M, Hsieh F, Baronas E, et al. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1–9. *Circ Res*. 2000;87:1–9.

6. Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem*. 2000;275:33238-33243.

7. Li MY, Li L, Zhang Y, Wang XS. Expression of the SARS-CoV-2 cell receptor gene ACE2 in a wide variety of human tissues. *Infect Dis Poverty*. 2020;9:45.

8. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol*. 2004;203:631-637.

9. Gurley SB, Allred A, Le TH, et al. Altered blood pressure responses and normal cardiac phenotype in ACE2-null mice. *J Clin Invest*. 2006;116:2218-2225.

10. Yamamoto K, Ohishi M, Katsuya T, et al. Deletion of angiotensin-converting enzyme 2 accelerates pressure overload-induced cardiac dysfunction by increasing local angiotensin II. *Hypertension*. 2006;47:718-726.

11. Crackower MA, Sarao R, Oudit GY, et al. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature*. 2002;417:822-828.

12. Oudit GY, Crackower MA, Backx PH, Penninger JM. The role of ACE2 in cardiovascular physiology. *Trends Cardiovasc Med*. 2003;13:93-101.

13. Gurley SB, Coffman TM. Angiotensin-converting enzyme 2 gene targeting studies in mice: mixed messages. *Exp Physiol*. 2008;93:538-542.

14. Kuba K, Imai Y, Rao S, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med*. 2005;11:875-879.

15. Imai Y, Kuba K, Rao S, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature*. 2005;436:112-116.

16. Rodrigues Prestes TR, Rocha NP, Miranda AS, Teixeira AL, Simoes ESAC. The anti-inflammatory potential of ACE2/angiotensin-(1–7)/mas receptor axis: evidence from basic and clinical research. *Curr Drug Targets*. 2017;18:1301-1313.

17. Tyburski JG, Dente C, Wilson RF, et al. Differences in arterial and mixed venous IL-6 levels: the lungs as a source of cytokine storm in sepsis. *Surgery*. 2001;130:748-752.

18. Ferrara JL, Abhyankar S, Gilliland DG. Cytokine storm of graft-versus-host disease: a critical effector role for interleukin-1. *Transplant Proc*. 1993;25:1216-1217.

19. Lee DW, Gardner R, Porter DL, et al. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood*. 2014;124:188-195.

20. Behrens EM, Koretzky GA. Review: cytokine storm syndrome: looking toward the precision medicine era. *Arthritis Rheumatol*. 2017;69:1135-1143.

21. Bian F, Pelegrino FS, Pflugfelder SC, Volpe EA, Li DQ, de Paiva CS. Desiccating stress-induced MMP production and activity worsens wound healing in alkali-burned corneas. *Invest Ophthalmol Vis Sci*. 2015;56:4908-4918.

22. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. 2020;395:497-506.

23. Zhou P, Yang XL, Wang XG, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. 2020;579:270-273.

24. Tikellis C, Johnston CI, Forbes JM, et al. Identification of angiotensin converting enzyme 2 in the rodent retina. *Curr Eye Res*. 2004;29:419-427.

25. Napoli PE, Nioi M, d'Aloja E, Fossarello M. The ocular surface and the coronavirus disease 2019: does a dual "ocular route" exist? *J Clin Med*. 2020;9:1269.

26. Xie LX, Zhou QJ, Gao H, Shi WY. Strengthening basic and clinical research on ocular infection caused by coronavirus. *Zhonghua Yan Ke Za Zhi*. 2020;56:250-252.

27. Desautels JD, Moshirfar M, Martheswaran T, Shmunes KM, Ronquillo YC. Risks posed to corneal transplant recipients by COVID-19-affected donors. *Ophthalmol Ther*. 2020;6:1-9.

28. Hui KPY, Cheung MC, Perera R, et al. Tropism, replication competence, and innate immune responses of the coronavirus SARS-CoV-2 in human respiratory tract and conjunctiva: an analysis in ex-vivo and in-vitro cultures. *Lancet Respir Med*. 2020.https://doi.org/10.1016/S2213-2600(20)30193-4

29. Zhang BN, Wang Q, Liu T, et al. Expression analysis of 2019-nCoV related ACE2 and TMPRSS2 in eye tissues. *Zhonghua Yan Ke Za Zhi*. 2020;56:E011.

30. Aiello F, Gallo Afflitto G, Mancino R, et al. Coronavirus disease 2019 (SARS-CoV-2) and colonization of ocular tissues and secretions: a systematic review. *Eye*. 2020; 1-6. https://doi.org/10.1038/s41433-020-0926-9

31. Wysocki J, Schulze A, Batlle D. Novel variants of angiotensin converting enzyme-2 of shorter molecular size to target the kidney renin angiotensin system. *Biomolecules*. 2019;9(12):886.

32. Gupta N, Kalaivani M, Tandon R. Comparison of prognostic value of Roper Hall and Dua classification systems in acute ocular burns. *Br J Ophthalmol*. 2011;95:194-198.

33. Kaplan N, Dong Y, Wang S, et al. FIH-1 engages novel binding partners to positively influence epithelial proliferation via p63. *FASEB J*. 2020;34:525-539.

34. Park JK, Peng H, Katsnelson J, et al. MicroRNAs-103/107 coordinately regulate macropinocytosis and autophagy. *J Cell Biol*. 2016;215:667-685.

35. Peng H, Kaplan N, Hamanaka RB, et al. microRNA-31/factor-inhibiting hypoxia-inducible factor 1 nexus regulates keratinocyte differentiation. *Proc Natl Acad Sci USA*. 2012;109:14030-14034.

36. Stepp MA, Zieske JD, Trinkaus-Randall V, et al. Wounding the cornea to learn how it heals. *Exp Eye Res*. 2014;121:178-193.

37. Wilson SE, Mohan RR, Mohan RR, Ambrosio R Jr, Hong J, Lee J. The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. *Prog Retin Eye Res*. 2001;20:625-637.

38. Kusnadi A, Park SH, Yuan R, et al. The cytokine TNF promotes transcription factor SREBP activity and binding to inflammatory genes to activate macrophages and limit tissue repair. *Immunity*. 2019;51(241–257):e249.

39. Park SH, Kang K, Giannopoulou E, et al. Type I interferons and the cytokine TNF cooperatively reprogram the macrophage epigenome to promote inflammatory activation. *Nat Immunol*. 2017;18:1104-1116.

40. Zou K, Wang Y, Hu Y, Zheng L, Xu W, Li G. Specific tumor-derived CCL2 mediated by pyruvate kinase M2 in colorectal cancer cells contributes to macrophage recruitment in tumor microenvironment. *Tumour Biol*. 2017;39:1010428317695962.

41. Chen C, He W, Huang J, et al. LNMAT1 promotes lymphatic metastasis of bladder cancer via CCL2 dependent macrophage recruitment. *Nat Commun*. 2018;9:3826.

42. Lgic R. Four decdes of ocular renin-angiotensin and kallikrein-kinin systems (1977–2017). *Exp Eye Res*. 2018;166:74-83.

43. Lin CI, Tsai CH, Sun YL, et al. Instillation of particulate matter 2.5 induced acute lung injury and attenuated the injury recovery in ACE2 knockout mice. *Int J Biol Sci*. 2018;14:253-265.

44. Jin HY, Chen LJ, Zhang ZZ, et al. Deletion of angiotensin-converting enzyme 2 exacerbates renal inflammation and injury in apolipoprotein E-deficient mice through modulation of the nephrin and TNF-alpha-TNFRSF1A signaling. *J Transl Med*. 2015;13:255.

45. Thomas MC, Pickering RJ, Tsorotes D, et al. Genetic Ace2 deficiency accentuates vascular inflammation and atherosclerosis in the ApoE knockout mouse. *Circ Res*. 2010;107:888-897.

46. Wysocki J, Ortiz-Melo DI, Mattocks NK, et al. ACE2 deficiency increases NADPH-mediated oxidative stress in the kidney. *Physiol Rep*. 2014;2:e00264.

47. Nita M, Grzybowski A. The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults. *Oxid Med Cell Longev*. 2016;2016:3164734.

48. Vallabh NA, Romano V, Willoughby CE. Mitochondrial dysfunction and oxidative stress in corneal disease. *Mitochondrion*. 2017;36:103-113.

49. Kazama K, Anrather J, Zhou P, et al. Angiotensin II impairs neurovascular coupling in neocortex through NADPH oxidase-derived radicals. *Circ Res*. 2004;95:1019-1026.

50. Nakamura T, Hamuro J, Takaishi M, et al. LRIG1 inhibits STAT3-dependent inflammation to maintain corneal homeostasis. *J Clin Invest*. 2014;124:385-397.

51. Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. Into the eye of the cytokine storm. *Microbiol Mol Biol Rev*. 2012;76:16-32.

52. Burnier M. Angiotensin II type 1 receptor blockers. *Circulation*. 2001;103:904-912.

53. Israili ZH. Clinical pharmacokinetics of angiotensin II (AT1) receptor blockers in hypertension. *J Hum Hypertens*. 2000;14(Suppl 1):S73-S86.

54. Hanff TC, Harhay MO, Brown TS, Cohen JB, Mohareb AM. Is there an association between COVID-19 mortality and the renin-angiotensin system-a call for epidemiologic investigations. *Clin Infect Dis*. 2020.

55. Gralinski LE, Baric RS. Molecular pathology of emerging coronavirus infections. *J Pathol*. 2015;235:185-195.

56. Batlle D, Wysocki J, Satchell K. Soluble angiotensin-converting enzyme 2: a potential approach for coronavirus infection therapy? *Clin Sci (Lond)*. 2020;134:543-545.

57. Xu Z, Shi L, Wang Y, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med*; 2020;8(4):420–422.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.