SARS-CoV-2 spike protein-induced host inflammatory response signature in human corneal epithelial cells

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Abstract. Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), led to an outbreak of viral pneumonia in December 2019. The present study aimed to investigate the host inflammatory response signature-caused by SARS-CoV-2 in human corneal epithelial cells (HCECs). The expression level of angiotensin-converting enzyme 2 (ACE2) in the human cornea was determined via immunofluorescence. *In vitro* experiments were performed in HCECs stimulated with the SARS-CoV-2 spike protein. Moreover, the expression levels of ACE2, IL-8, TNF-α, IL-6, gasdermin D (GSDMD) and IL-1β in HCECs were detected using reverse transcription-quantitative PCR and/or western blotting. It was identified that ACE2 was expressed in normal human corneal epithelium and HCECs cultured *in vitro*. Furthermore, the expression levels of IL-8, TNF-α and IL-6 in HCECs were decreased following SARS-CoV-2 spike protein stimulation, while the expression levels of GSDMD and IL-1β were increased. In conclusion, the present results demonstrated that the SARS-CoV-2 spike protein suppressed the host inflammatory response and induced pyroptosis in HCECs. Therefore, blocking the ACE2 receptor in HCECs may reduce the infection rate of COVID-19.

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Introduction

Coronavirus disease 2019 (COVID-19) was first identified in December 2019, and it has caused an outbreak of viral pneumonia worldwide (1,2). The severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2), responsible for COVID-19, has potentially severe adverse health effects, such as acute respiratory distress syndrome, difficult-to-correct metabolic acidosis and coagulation dysfunction (3,4). Although several SARS-CoV-2 vaccines have been developed, due to the increasing demand for vaccines, insufficient vaccine production (5), rapid virus mutation (6) and other reasons, SARS-CoV-2 is still endangering human health. The current public health emergency is similar to the SARS outbreak caused by the SARS-CoV in 2002-2003 (7). Moreover, recent research has shown that the SARS-CoV-2 genome shared a sequence homology with the SARS-CoV genome (8).

There are four major structural proteins of SARS-CoV-2, including: Nucleocapsid protein, membrane glycoprotein, small envelope glycoprotein and spike glycoprotein (9) Previous studies have reported that angiotensin-converting enzyme 2 (ACE2), to which the SARS-CoV spike protein binds, mediates SARS-CoV by binding to the S1 domain of the SARS-CoV S protein and promoting viral replication (10,11). As one of the metallopeptidases, ACE2 serves an essential role in mediating the angiotensin II to angiotensin-(1-7) conversion (12). Furthermore, ACE2 receptors can limit some harmful effects of angiotensin II production, such as increased inflammation (13). The enhanced production of angiotensin 1-7 also inhibits the ACE2/angiotensin 1-7/MAS axis and decreasing angiotensin II production (14). Before being confirmed as the functional cellular receptor for SARS-CoV, ACE2 had been extensively studied in heart disease, hypertension and diabetes (11). Moreover, using a computational model, Xu *et al* (8) revealed that the SARS-CoV-2 spike protein has a significant binding affinity to human ACE2, despite replacing four out of five important interface amino acid residues to SARS-CoV. Another study also reported that SARS-CoV-2 used the same cell entry receptor, ACE2, as SARS-CoV in HeLa cells (15).

The ocular surface may be a mode of transmission of SARS-CoV-2, but it remains poorly understood. Previous reports (16-18) have confirmed that SARS-CoV-2 could cause conjunctivitis. Indeed, the initial symptom of several patients infected by SARS-CoV-2 is conjunctivitis (19). A recent study revealed the presence of conjunctivitis is 11.6% in patients with COVID-19 (20). The tissues in contact with air on the ocular surface are primarily the corneal epithelium and conjunctival epithelium. The cornea and conjunctiva are developed from the ectoderm (21). Moreover, the conjunctival epithelial cells migrate to the corneal epithelial cells at the limbus (22,23). Previous studies have confirmed the presence of SARS-CoV-2 in tear and conjunctival swabs from patients with COVID-19 (23,24). In addition, the lack of ocular protection increases the risk of contracting SARS-CoV-2 (25).

The present study focused on the relationship between SARS-CoV-2 and the human corneal epithelium, and aimed to investigate the host inflammatory response signature caused by SARS-CoV-2 in human corneal epithelial cells (HCECs).

Materials and methods

Clinical specimens. Healthy corneas were obtained from organ donors who agreed to donate their corneas after they died. The cornea had been thoroughly tested to ensure its use was safe and it was healthy. The six healthy corneas used in the study were the remaining peripheral corneal tissues after penetrating keratoplasty had been performed. All corneas were healthy without any infection or trauma. All six corneal specimens were collected from The Affiliated Hospital of Qingdao University (Shandong, China) between January 2020 and November 2020. The Ethics Committee of The Affiliated Hospital of Qingdao University approved the use of the corneas at The Affiliated Hospital of Qingdao University. This research adhered to the principles described in the Declaration of Helsinki. Written informed consent was obtained from individuals or their next of kin. The demographic information for postmortem eyes is presented in Table I. Immunofluorescence staining was used to examine ACE2 expression in the corneas.

SARS-CoV-2 spike protein. The SARS-CoV-2 spike protein was purchased from Sino Biological, Inc. (cat. no. MB14JA2203).

In vitro experiments. HCECs were provided by the Ocular Surface Laboratory at the Zhongshan Ophthalmic Center. HCECs were from a primary cell culture. The Ethics Committee of The Affiliated Hospital of Qingdao University approved the use of HCECs at The Affiliated Hospital of Qingdao University. This research adhered to the principles described in the Declaration of Helsinki.

Cells were cultured to 80% confluence in DMEM (HyClone; Cytiva) containing 12% fetal bovine serum (HyClone; Cytiva) and 1% penicillin and streptomycin, and were then stimulated with SARS-CoV-2 spike protein at two different final concentration for 16 h at 37˚C; 10 μg/ml (26) was selected as a lower concentration, and a larger concentration of 50 μg/ml was chosen in order to show the different effects of the concentrations. The negative control group was untreated. After 16 h, reverse transcription-quantitative (RT-q)PCR and western blotting were conducted.

Table I. Demographic information for postmortem eyes.

Case no.	Age, years	Sex	Ethnicity
N1	62	Male	Asian
N2	48	Female	Asian
N3	54	Female	Asian
N4	36	Male	Asian
N5	68	Male	Asian
N6	43	Male	Asian

N, normal.

Immunofluorescence. ACE2 expression in the normal human cornea was evaluated via immunofluorescent staining of frozen sections of corneas after embedding them in an optimum cutting temperature (OCT) compound (Leica Microsystems, Inc.). Corneas were then immediately frozen in liquid nitrogen after embedding in OCT compound (27). Frozen corneal slices (7 μm) were cut using a freezing-microtome (Leica Microsystems GmbH). Slices were blocked with 10% blocking buffer containing rabbit serum (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at 37˚C and were then stained with rabbit anti-human ACE2 antibody (1:100; cat. no. bs-1004R; BIOSS) overnight at 4˚C. Subsequently, slices were incubated with donkey anti-rabbit secondary antibody (1:500; cat. no. ab150061; Abcam) for 1 h and with DAPI solution (Beijing Solarbio Science & Technology Co., Ltd.) for another 10 min at room temperature. The slices were observed and images were captured under a Zeiss Axiovert microscope (Carl Zeiss AG; magnification, x40).

RT-qPCR. Total RNA was extracted from HCECs using the RNAiso Plus kit (Takara Biotechnology Co., Ltd.), and cDNA was obtained by RT of total RNA using the Primescript RT kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. The mRNA expression levels of IL-8, TNF-α, IL-6, gasdermin D (GSDMD) and IL-1β in HCECs were detected as described previously (27). RT-qPCR was performed using an Eppendorf Mastercycler and SYBR-Green (Takara Biotechnology Co., Ltd.). The following thermocycling parameters were used for the amplification: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec, and a final stage of 95˚C for 15 sec, 60˚C for 30 sec and 95˚C for 15 sec. The primer pairs that were used are shown in Table II. All the primers were designed by Takara Biotechnology Co., Ltd. Relative transcription levels were calculated using the relative standard curve method that compares the amount of target normalized to the housekeeping gene β-actin. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (28). Data are presented as the mean ± SD for relative mRNA levels.

Western blot analysis. HCECs stimulated with the SARS-CoV-2 spike protein were analysed using western blotting, as described previously (27). Total protein of HCECs was extracted using the tissue protein lysate (radioimmunoprecipitation assay buffer:phenylmethanesulfonyl:phosphatase inhibitor, 100:1:1; Beijing Solarbio Science

Table II. Nucleotide sequences of human primers used for reverse transcription-quantitative PCR.

Genes	Primer sequence (5'-3')
hIL-8	F: CGG CAA TAG CTC TGT AT
hIL-8	R: CCT TGA AAC TTT GCC TCA
hTNF-α	F: TTC TGT CTA CTG AAC TTC GGG GTG ATC GGT CC
hTNF-α	R: GTA TGA GAT AGC AAA TCG GCT GAC GGT GTG GG
hIL-6	F: CAC AAG TCC GGA GAG GAG AC
hIL-6	R: CAG AAT TGC CAT TGC ACA AC
hGSDMD	F: TGA ATG TGT ACT CGC TGA GTG TGG
hGSDMD	R: CAG CTG CTG CAG GAC TTT GTG
hIL-1β	F: GCT GAT GGC CCT AAA CAG ATG AA
hIL-1β	R: TCC ATG GCC ACA ACA ACT GAC
β-actin	F: GCT CCT CCT GAG CGC AAG
β-actin	R: CAT CTG CTG GAA GGT GGA CA

F, forward; R, reverse; GSDMD, gasdermin D.

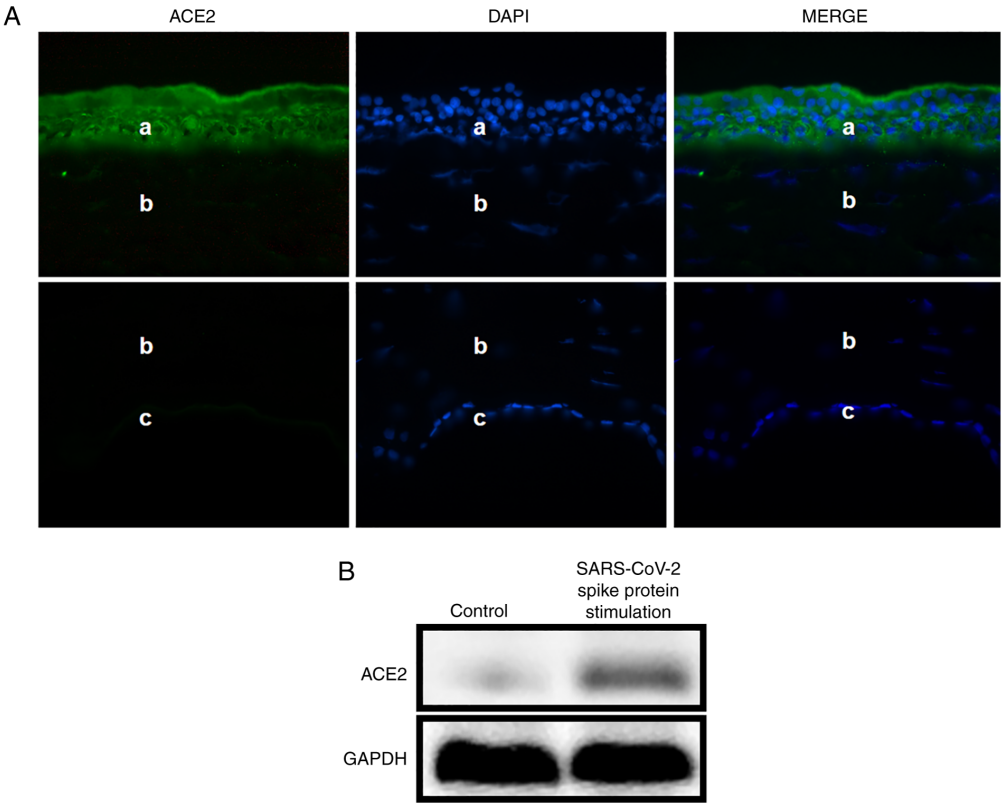

Figure 1. ACE2 is expressed in normal human cornea and HCECs. (A) Immunofluorescence images showing the expression level of ACE2 protein in the normal human corneas (a, epithelium; b, stroma; c, endothelial cells). Blue, nuclear staining (DAPI); Green, ACE2 protein staining. Magnification, x40. HCECs were pretreated with SARS-CoV-2 spike protein for 16 h. (B) Western blotting was used to examine ACE2 expression in HCECs. HCECs, human corneal epithelial cells; ACE2, angiotensin-converting enzyme 2; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

& Technology Co., Ltd.). Protein concentration was measured using a bicinchoninic acid protein assay reagent (Beijing Solarbio Science & Technology Co., Ltd.). Total protein samples (10 μg) were separated by SDS-PAGE on 12% gels and transferred to PVDF membranes (EMD Millipore), which were blocked in 5% bovine serum albumin (Beyotime Institute of Biotechnology) at room temperature for 2 h. Blots were incubated with anti-ACE2 (1:100; cat. no. bs-1004R; BIOSS), anti-GSDMD (1:100; cat. no. sc-393581; Santa Cruz Biotechnology, Inc.), anti-IL-1β (1:1,000; cat. no. AF-401-NA; R&D Systems, Inc.) and anti-GAPDH (1:2,000; cat. no. E-AB-20059; Elabscience, Inc.) at 4˚C overnight, followed by incubation with HRP-linked anti-rabbit (1:500; cat. no. ab150061; Abcam) antibody at room temperature for

2 h. The bands were visualized with Western ECL Blotting Substrates (Bio-Rad Laboratories, Inc.). Digital images were obtained using the Vilber Solo 4S chemiluminescence imaging system (Vilber Lourmat).

Statistical analysis. All data were analyzed with SPSS 25 software (IBM Corp.). The data are presented as the mean ± SD of ≥3 independent experiments. The statistical analysis was performed using a one-way ANOVA followed by LSD-t test, which was used for analysis between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ACE2 is expressed in normal human corneas and HCECs. Immunofluorescence staining was performed to examine the expression level of the ACE2 receptor in normal human corneas. The results demonstrated that the ACE2 protein (green; Fig. 1A) was expressed in the epithelium of normal human corneas (a, epithelium; b, stroma; c, endothelial cells). Western blotting then was used to examine ACE2 expression in HCECs. The protein expression level of ACE2 was higher after the stimulation with SARS-CoV-2 spike protein compared with the control group (Fig. 1B).

SARS-CoV-2 spike protein suppresses the host inflammatory response in HCECs. RT-qPCR was conducted to examine the expression levels of proinflammatory factors in HCECs after stimulation with the SARS-CoV-2 spike protein. Compared with the negative control group, the mRNA expression levels of IL-8 (Fig. 2A), TNF-α (Fig. 2B) and IL-6 (Fig. 2C) were decreased by stimulation with the SARS-CoV-2 spike protein.

SARS-CoV-2 spike protein-induced pyroptosis in HCECs. RT-qPCR and western blotting were used to examine GSDMD and IL-1β expression in HCECs. It was found that the mRNA (Fig. 3A) and protein (Fig. 3B) expression levels of precursor (p)-GSDMD and mature (m)-GSDMD were higher following stimulation with SARS-CoV-2 spike protein at the concentrations of 10 or 50 μg/ml. With regards to IL-1β, the mRNA (Fig. 3C) and protein (Fig. 3D) expression levels of p-IL-1β and m-IL-1β were notably increased after stimulation with 50 μg/ml SARS-CoV-2 spike protein.

Discussion

In total, >8,000 patients were diagnosed with SARS-CoV between 2002-2003. Different from COVID-19, the symptoms on the ocular surface were rarely identified in SARS (29). Since the discovery of COVID-19 in 2019, >100,000,000 people worldwide have been infected with SARS-CoV-2. According to findings by Menachery *et al* (30), the SARS-CoV spike protein-bound to the ACE2 receptor, and replicated efficiently in human airway cells. Moreover, Liu *et al* (31) reported that the SARS-CoV spike protein weakly bound to ACE2 receptors in eyes. These results were consistent with the clinical manifestation of SARS. Although the expression levels of key cytokines, including membrane-associated transmembrane serine protease 2 and ACE2 receptor, have been well studied in respiratory tract cells (32), the relationship between SARS-CoV and ACE2 in ocular cells is not fully understood.

The ocular surface, consisting of conjunctival epithelium and corneal epithelium, is continuously exposed to the environment (33). It has been reported that SARS-CoV-2 can be transmitted via mucous membranes, such as the conjunctiva (34). Previous studies (16,17,35) also have shown that conjunctivitis is an initial clinical manifestation when an individuals is infected by SARS-CoV-2. However, whether and how SARS-CoV-2 can invade the corneal epithelium is yet to be fully elucidated.

Based on the present results, it was suggested that the SARS-CoV-2 spike protein could induce the host inflammatory response by binding to ACE2 in HCECs. The SARS-CoV-2 spike protein binds to the ACE2 receptor and acts as a starting link to mediate the invasion and spread of the virus (10,11). Moreover, the findings of the present study indicated that the SARS-CoV-2 spike protein inhibited the release of proinflammatory factors in the host, which may make it more challenging for the host to eliminate the virus in the early stages of infection. Furthermore, a previous study revealed that IL-6, IL-8 and TNF-α were highly upregulated in patients with SARS (36). In the present study, when the concentration of SARS-CoV-2 spike protein was 10 μg/ml, the mRNA expression levels of IL-8, TNF-α and IL-6 were lower in HCECs compared with those stimulated with 50 μg/ml SARS-CoV-2 spike protein. Thus, the results demonstrated that SARS-CoV-2 could inhibit the release of proinflammatory factors and escaped immune clearance when the viral load was small. However, when the virus content increased, the virus could not escape the immunity of the body, and the expression levels of the proinflammatory factors began to increase.

Pyroptosis is a form of programmed cell death caused by inflammatory bodies (37). It can resist intracellular infection by eliminating damaged cells, thus eliminating pathogens (38). Pyroptosis is dependent on the family of caspases (39) and activation of the pore-forming effector protein GSDMD (40). The precursor-GSDMD protein is 53 kDa in length and is cleaved to produce two major domains: 30 kDa N-terminal fragment of GSDMD (GSDMD-NT) and 20 kDa C-terminal fragment of GSDMD (GSDMD-CT). GSDMD-NT is the main functional domain and is also known as the m-GSDMD (41). The m-GSDMD can cause plasma membrane rupture, resulting in the release of intracellular substances and proinflammatory mediators, such as IL-1β (42). At the same time, GSDMD also serves an important role in IL-1β maturation (41). The present study revealed that when the SARS-CoV-2 spike protein invaded cells, pyroptosis was induced as a cellular defense mechanism in the early stage. However, even when viral load was low (spike protein at a final concentration of 10 μg/ml), cells still began the process of pyroptosis. These findings indicated that although SARS-CoV-2 can inhibit the release of proinflammatory factors in the host to some extent, the body can still eliminate the virus via pyroptosis.

At present, there are numerous research areas for investigations into the SARS-CoV-2 spike protein, ACE2 and pyroptosis. Future studies will aim to determine the complete mechanism of how SARS-CoV-2 invades the human body, and the body's response to the invasion of the virus. However, due to

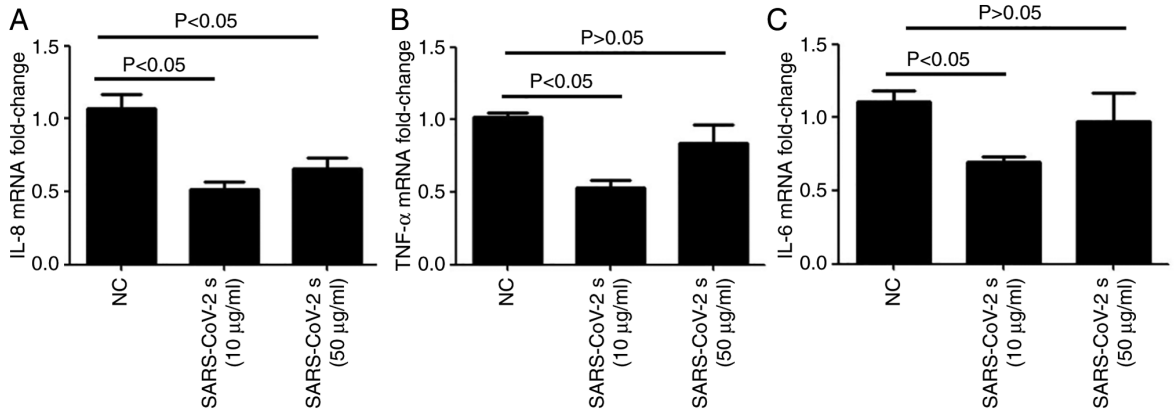

Figure 2. SARS-CoV-2 spike protein suppresses the host inflammatory response in HCECs. HCECs were pretreated with SARS-CoV-2 spike protein for 16 h at a final concentration of 10 or 50 μg/ml, then reverse transcription-quantitative PCR was used to detect the mRNA expression levels of (A) IL-8, (B) TNF-α and (C) IL-6. HCECs, human corneal epithelial cells; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; NC, negative control.

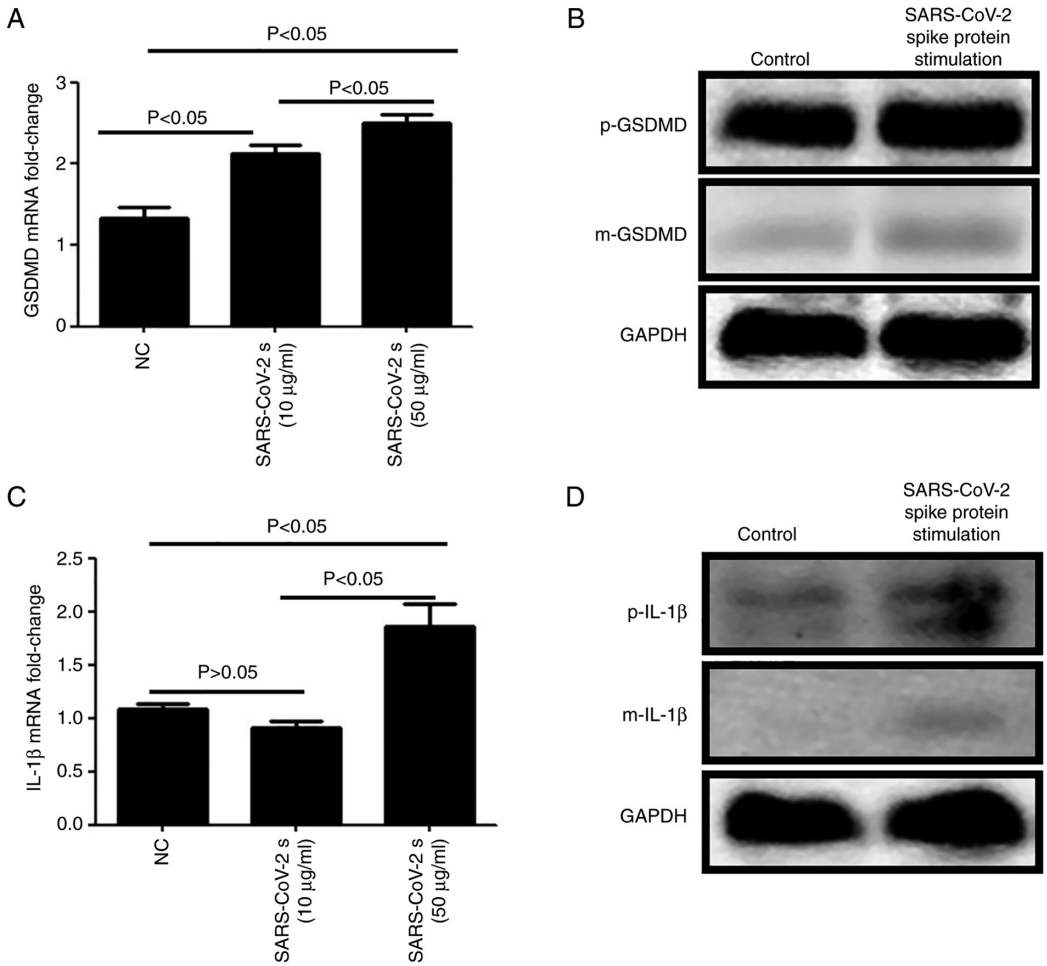

Figure 3. SARS-CoV-2 spike protein-induced pyroptosis in HCECs. HCECs were pretreated with SARS-CoV-2 spike protein for 16 h at a final concentration of 10 or 50 μg/ml, then reverse transcription-quantitative and western blotting were used to examine (A and B) p-GSDMD and m-GSDMD expression, as well as (C and D) p-IL-1β and m-IL-1β expression in HCECs. HCECs, human corneal epithelial cells; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; p-, precursor; m-, mature; GSDMD, gasdermin D; NC, negative control.

the COVID-19 pandemic, experiments can only be conducted to a limited extent. In the future, morphological research and flow cytometry will be conducted to perform multi-dimensional evaluation of pyroptosis, and experiments will be conducted *in vivo*, along with possible long-term follow-up research.

In conclusion, the present study demonstrated that the SARS-CoV-2 spike protein suppressed the host inflammatory response and induced pyroptosis in HCECs. These findings highlight the importance of ocular surface protection in response to the SARS-CoV-2 infection. Moreover, blocking

the ACE2 receptor in HCECs may be an effective method to reduce the infection rate of COVID-19.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GZ, LL and H Yang contributed to data acquisition, analysis and interpretation, and drafted and critically revised the manuscript. GL, SY, CG, LW and H Yan contributed to data acquisition, analysis and interpretation, and critically revised the manuscript. CC and MH contributed to conception, design, data acquisition, analysis and interpretation, and drafted and critically revised the manuscript. CC, GZ, LL and H Yang confirm the authenticity of all the raw data. GZ, LL and H Yang contributed equally to this work. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of The Affiliated Hospital of Qingdao University approved the use of the corneas and approved the use of HCECs. Written informed consent was obtained from individuals or their next to kin.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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