β2-adrenoceptor blocker ICI118551 attenuates Pseudomonas aeruginosa corneal infection in mice

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October 5, 2020

Abstract

Purpose: Our previous studies have confirmed that the catecholamine norepinephrine (NE) promoted the corneal infection and progression of Pseudomonas aeruginosa (P. aeruginosa) keratitis. Here we explored the effects of adrenoceptor (AR) blockers on the severity of P. aeruginosa keratitis in mice. Methods: A total of 630 C57BL/6 mice were used and ocularly inoculated with P. aeruginosa (ATCC 19660). Six AR blockers were topically administrated 6 h before or combined with Tobrex 12 h after bacterial inoculation. Clinical scores, neutrophil infiltration and neutrophil extracellular traps (NETs), proinflammatory factors and bacterial virulence factors expression, bacterial load and biofilm formation were evaluated in vivo. The growth inhibitory and bactericidal activity of ICI118551 was measured in vitro. Results: Among various blockers, the selective β2-AR blocker ICI118551 showed the most significant improvement in disease severity. Prophylactic administration of ICI118551 attenuated the clinical scores, neutrophil infiltration, NETs formation, proinflammatory factors expression of infected corneas, accompanied with the reduction of bacterial load, virulence factors expression, and biofilm formation. When adjunctive treatment with Tobrex, ICI118551 exhibited apparent therapeutic effects with the reduced severity after 12 h of bacterial inoculation. Moreover, ICI118551 inhibited bacterial growth and possessed bactericidal activity in vitro. In addition, ICI118551 had no significant influence on ocular barrier function and intraocular pressure. Conclusions: The selective β2-AR blocker ICI118551 attenuated the severity of P. aeruginosa keratitis in mice, which may represent the potential prophylactic and therapeutic approach to control the P. aeruginosa corneal infection.

Introduction

Microbial keratitis is a kind of vision-threatening corneal disease usually accompanied by ocular injury or contact lens wear (Carnt*et al* ., 2017; Fleiszig *et al* ., 2020; Lakhundi *et al* ., 2017). Once infected, it progresses rapidly despite appropriate antimicrobial therapy (Lakhundi *et al* ., 2017).*Pseudomonas aeruginosa* (*P. aeruginosa*) is identified as a leading pathogen causing microbial keratitis worldwide (Carnt *et al* ., 2017; Lakhundi *et al* ., 2017; Suzuki *et al* ., 2018). Corneal ulcers due to *P. aeruginosa* are reported more destructive than other bacterial corneal ulcers, with significant potential for permanent vision loss (Oka *et al* ., 2015). These symptoms are attributed to the host's inflammatory response, bacterial toxins and exoproducts (Hazlett, 2004).

The pathogenesis of *P. aeruginosa* keratitis involves bacterial virulence factors and host excessive inflammatory response (Hazlett, 2004; Willcox, 2007). *P. aeruginosa* employs a serise of cell-associated and extracellular virulence factors such as pili, flagella, elastase, extoxin A, pyocyanin and biofilm, which can invade or kill corneal cells and induce corneal destruction (Willcox, 2007). Along with these virulence factors, the type-3 secretion system (T3SS) is involved in the pathogenesis of keratitis. The T3SS infuses toxins into host cells, immune evasion, and the Psl exopolysaccharide, which forms tenacious biofilms (Ma *et al* ., 2012; Thanabalasuriar*et al* ., 2019). The formation of biofilm plays an important role in antibiotic resistance and disease progression, which protects*P. aeruginosa* from the phagocytosis of neutrophils, and

induces neutrophils extracellular traps (NETs) formation by neutrophils (Zhao*et al* ., 2019). Although the infiltration of bacteria is prevented to some extent, the biofilm and NETs hinder the rapid killing of bacteria by neutrophils and prolongs the course of disease. The elastase produced by *P. aeruginosa* and neutrophils could cause corneal erosion (Thanabalasuriar *et al* ., 2019).

In addition to bacterial virulence factors, excessive activation of the host defense system is another important factor causing tissue destruction (Willcox, 2007; Yue *et al* ., 2016). The host inflammatory response can eliminate invading pathogens in the cornea (Lee *et al* ., 2015; Okada *et al* ., 2006; Papayannopoulos, 2018; Yang *et al* ., 2016), but also lead to tissue destruction (Willcox, 2007). A reduction of tissue destruction is accompanied by a significant decrease in recruitment of polymorphonuclear neutrophils (PMN) during bacterial infection in corneas of mouse (Pearlman *et al* ., 2008). The conventional drug therapies currently available for*P. aeruginosa* keratitis were antibiotics which would contribute to bacteria elimination (Austin *et al* ., 2017), but the resistance to antibiotics of *P. aeruginosa* is becoming increasingly via intrinsic and acquired mechanisms (Cunrath *et al* ., 2019; Subedi*et al* ., 2018; Vazirani *et al* ., 2015), Therefore, the drug capable of controlling inflammatory response maybe can alleviate inflammation during *P. aeruginosa* corneal infection.

Our previous studies reported that both epithelial injury and extended contact lens wear induced corneal NE secretion. Elevated NE promoted the adhesion and biofilm formation of *P. aeruginosa,* and the infiltration of neutrophils into cornea, which further promoted the*P. aeruginosa* corneal infection and progression in mice (Li*et al* ., 2020; Ma *et al* ., 2020). In this study, we further investigated the effects of various adrenoceptor (AR) blockers on*P. aeruginosa* infection in mouse cornea, and found that the selective β2-AR blocker ICI118551 attenuated the severity of *P. aeruginosa* keratitis in mice, which may represent the potential prophylactic and therapeutic treatment for *P. aeruginosa*infection in cornea.

Methods

Animals, reagents, and antibodies

The adult C57BL/6 mice (age, 8 weeks; weight, 20–25 g; amount, 630) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd, which were maintained in the animal facility of Shandong Eye Institute. All procedures were carried out in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The reagents and antibodies are listed in Supplementary Table 1.

Infection procedure, clinical examination and application of

reagents

Mice were anesthetized and the left corneas were scratched to create three 1-mm incisions using a sterile 25-gauge needle, then inoculated with bacterial suspension containing 1×10^5colony-forming units (CFU) of *P. aeruginosa* (ATCC 19660). The procedure of corneal infection was performed according to the protocol reported previously (Li *et al* ., 2020). Sterile saline was used to dissolve the six AR blockers: the α1/2-AR blocker Phentolamine (50 mg/ml), β1-AR blocker Atenolol (8 mg/ml), β2-AR blocker ICI118551 (5 mg/ml), β2-AR blocker Butoxamine (40 mg/ml), β1/2-AR blocker Timolol (4.4 mg/ml), or β3-AR blocker SR58894A (5 mg/ml), and 5 μL was instilled 30 min before *P. aeruginosa* inoculation, restarted from 6 h after infection, and continued for 3 days (six times a day). Control mice were treated with sterile saline. To determine whether ICI118551 prevents ulceration in infected corneas, tobramycin ophthalmic solution (Tobrex) was used to dissolve ICI118551 (5 mg/mL), and 5 μL was instilled into mouse corneas after *P. aeruginosa* inoculation, starting 12 h after infection and continuing 3 time per day thereafter until 72 h after infection. The corneal disease was graded using a well-established scale[11] and scored at 24, 48, and 72 h post infection.

Determinations of proinflammatory factors and bacterial virulence expression, and Polymorphonuclear Leukocyte (PMN) Infiltration

As previously described (Gao *et al* ., 2015), the corneas were excised, minced, and homogenized in 100 μL of PBS with a TissueLyser (TissueLyser II, Hilden, Germany). The homogenates were divided into 2 parts. The first part was used for quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted using MiniBEST Universal RNA Extraction Kit (Takara, Beijing, China). The cDNA was generated using a PrimeScript RT Reagent Kit, followed by analysis using a real-time PCR kit (Takara) and Rotor-Gene Q systems (Qiagen, Hilden, Germany), based on β-actin as an internal control for the mice and 16s rRNA for *P. aeruginosa* . The primers are presented in Supplementary Table 2. The second part was used for the measurements of myeloperoxidase (MPO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), CXCL2, Exotoxin A, lipopolysaccharide (LPS) expression and elastase activity with corresponding enzyme-linked immunosorbent assay (ELISA) kits.

Immunofluorescence and histochemical staining

As described previously, the corneas were incubated with Ly6G, followed by fluorescein conjugated secondary antibodies, and observed with an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan) (Yoon *et al* ., 2013). For histological staining, the mouse eyes were fixed in 4% formalin, embedded in paraffin, and stained with hematoxylin/eosin.

NET Staining

Sytox green was diluted 1:500 and gently dripped on the surface of the eye, left for 5 minutes, then gently washed off with PBS for 3 times. After that, the cornea was excised, fixed with 4% paraformaldehyde for 15 minutes, and then washed off, as previously described, with a slight modification (Thanabalasuriar *et al* ., 2019). The samples were observed using a confocal laser scanning microscope (Carl Zeiss Meditec, Oberkochen, Germany).

Evaluation ofBiofilm Formation

The corneas were collected, washed, and fixed with 4% paraformaldehyde for 15 minutes. After that samples were washed and stained with 50 μg/mL fluorescein isothiocyanate-conjugated concanavalin A (FITC-ConA) for 20 minutes, as previously described, with a slight modification (Li*et al* ., 2020). The biofilm formation was observed using a confocal laser scanning microscope (Carl Zeiss Meditec, Oberkochen, Germany). FITC-ConA bound to the polysaccharide of the biofilm to emit green fluorescence.

Determination of bacterial load

Each individual cornea was excised and homogenized in sterile saline. Aliquots (100 ml) of serial dilutions were plated onto Luria–Bertani (LB) agar plates in triplicate and cultured at 37 for 24 h. The bacterial colonies were counted and reported as CFU per cornea.

Growth curve measurement

P. aeruginosa isolates were grown on LB agar medium at 37, while planktonic cultures were grown with an initial turbidity of ˜0.02 at 600nm and shaking at 160 rpm. The turbidity of cultures was monitored at 1, 2, 4, 6, 8, 24 h after bacterial incubation.

Viability staining

This experiment was performed using the live/dead baclight bacterial viability kit (Thermo Fisher) (Mah *et al* ., 2003). In brief, concentrate 1ml of the bacterial culture by centrifugation at 5,000 rpm for 5 min. Remove the supernatant and resuspend the pellet in 1ml of 0.9% NaCl for three times. Mix equal volumes of SYTO 9 and propidium iodide (PI) thoroughly and add 3 ul of the mixture for each ml of bacterial suspension. After incubate at room temperature in the dark for 15 minutes, the dye mixture was prepared as microscope slides and observed by confocal fluorescent microscopy (Carl Zeiss Meditec, Oberkochen, Germany).

Negative staining

In brief, concentrate 1ml of *P. aeruginosa* 24 h culture and fix by 3% glutaraldehyde (Tianjin Bodi Chemical Co., Ltd) for 10 min. Then the culture was adsorbed to carbon mesh grids and air dried for 5 min. Next stained with 1% phosphotungstic acid solution 3min and air dried 15 min, then digital images of bacterial were taken with a JEM-1200EX transmission electron microscope (JEOL in Japan).

Flow cytometry

Five eyes were harvested and minced into pieces. Eye tissue was digested in 2mL RPMI-1640 medium containing 0.5 mg/mL collagenase VI (Sigma) for 90 minutes at 37 with shaking (60 rpm). The digested corneal tissue was then passed through a 100 mm filter cell strainer (Corning) to remove the debris. The cells were then pelleted by centrifugation at 400g for 5 minutes and washed once with 1 × PBS. The cells were subjected to were incubated with Ly6G (1:200) staining for flow cytometric analysis. Cell numbers were measured using a flow cytometer (CytoFLEX LX; Beckman Coulter, CA, USA) according to the manufacturers' instruction. Flow cytometric data were analyzed using CytExpert 2.0 software.

Statistical analysis

Data were expressed as mean values ± SD. A commercial statistical/analytical software program (SPSS 20.0; SPSS, Inc., Chicago, IL) was used for statistical analysis. During statistical analysis of bacterial count, bacterial growth curve, MPO assay, flow cytometric analysis, ELISA, and qRT-PCR results, an unpaired two-tailed Student$t$ test was used for comparisons between groups. A nonparametric Mann-Whitney U test was used for clinical scores and viability staining. Differences were considered statistically significant at$P$ values $<.05$.

Results

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To evaluate the effects of various AR blockers on the severity of*P. aeruginosa* keratitis, the mice were administrated topically with Phentolamine, Atenolol, ICI118551, Butoxamine, Timolol, and SR58894A 6 h before bacterial inoculation. As shown in Figure 1A, among the six blockers, only the selective β2-AR blockers ICI118551 and Butoxamine exhibited the most apparent reduced severity of *P. aeruginosa* keratitis after both 24 and 72 h. Interestingly, the nonselective β1/2-AR blocker Timolol caused a mild reduction, while the α1/2-, β1- or β3-AR blockers had no significant reduction of disease severity. Moreover, quantitative analysis of clinical scores (Fig. 1B) and corneal bacterial load (Fig. 1C) after 24 h of infection further confirmed the superior inhibition of β2-AR blockers than other blockers on the severity of *P. aeruginosa* keratitis. In addition, the infiltration of inflammatory cells was also reduced apparently through the administration of β2-AR blockers than other blockers (Fig. 1D), accompanied with the reduced corneal thickness (Supplementary Fig. 1).

ICI118551 attenuates corneal inflammatory response

To evaluate the effect of ICI118551 on inflammatory response, neutrophil infiltration and proinflammatory factor expression were investigated at 48 h after *P. aeruginosa* keratitis infection. Immunofluorescence staining with anti-Ly6G antibody disclosed that neutrophils infiltrated into the anterior stroma in the ICI118551-treatment group, compared with full thickness of the cornea infiltration in the vehicle control corneas (Fig. 2A). The amount and activity of polymorphonuclear neutrophils in the cornea treated with ICI118551 were significantly reduced (Fig. 2B and C, $P < 0.001$). The control group showed a typical NETs structure, while the ICI118551 treatment group only showed a small number of independent neutrophils without obvious network structure (Fig. 2D). Furthermore, the ELISA assay (Figure 2E) and RT-PCR (Supplementary Figure 2) revealed that ICI118551 inhibited the expression levels of TNF-α, IL-1β, and MIP-2 ($P < 0.05$;$P <$ 0.01; $P< 0.01$). These results suggested ICI118551 suppressed the inflammatory response to *P. aeruginosa* keratitis by reducing infiltration of neutrophils and release of inflammatory cytokines.

ICI118551 prophylactic therapy reduced the levels of virulence factors and biofilm formation of *P. aeruginosa* in cornea

We further explored the level of bacterial virulence in the cornea infected by *P. aeruginosa.* Compared with the control group, the expression of virulence factor genes was significantly downregulated in the ICI118551-treated corneas (Figure 3A). Besides, the expression levels of *P. aeruginosa* lipopolysaccharide (LPS), exotoxin A, and elastase were also decreased obviously which were measured by ELISA (Figure 3B; $P < 0.05$). After FITC-ConA staining, the formation of biofilm was observed by confocal microscope. The photographs showed that ICI118551 significantly reduced the density and structure of biofilm (Figure 3C). Above all, it was indicated that the levels of bacterial virulence factors and biofilm formation in the corneas were decreased significantly under the effect of ICI118551.

ICI118551 alleviated damage of the corneal epithelial basement membrane

As the epithelial basement membrane is considered a biological barrier that bacteria must penetrate (Alarcon *et al* ., 2009), changes in the infected corneal basement membrane were tested through collagen type IV staining (Figure 4). In the control group, the cornea was severely edematous at 24 h after infection, and collagen IV staining partially disappeared. Damage to the corneal epithelial basement membrane progressed rapidly, reaching the epithelium basement membrane and deep stroma at 72 h post infection. The epithelial sheet and part of the stroma were lost, and the corresponding collagen IV staining disappeared. In the ICI118551-treated group, the corneal epithelium was slightly edematous, and staining of the epithelial basement membrane showed mild disorder at 24 h post infection, followed by rapid recovery. ICI118551 effectively reduced corneal epithelial basement membrane damage.

Bactericidal activity of ICI118551 against *P. aeruginosain vitro*

To verify the effect of ICI118551 on the growth of *P. aeruginosa* , bacterial growth curve was detected. As shown in Figure 5A, when the bacteria grew up to stable stage, the OD 600 value of the bacterial solution under the influence of ICI118551 decreased significantly compared with that of control. The ICI118551 treatment group showed clearer bacterial liquid at 24 hours post inocubation (Figure 5B). Bacterial survival status of 24 h culture was detected by viability staining, which shown that there were more dead (red, stained with propidium iodide) than live (green, SYTO9-stained) cells in the group treated with ICI118551(Figure 5C, $P < 0.05$). Further negative staining electron microscopy verified that bacterial cells under the influence of ICI118551 were dying and on the verge of rupture (Figure 5D). The data above indicated that ICI118551 has significant bactericidal activity against *P. aeruginosa* .

Optimal drug concentration and initial intervention time of ICI118551

Considering the significant effect of ICI118551 on *P. aeruginosa* keratitis, we further explored the optimal drug concentration and initial intervention time. The effects of three different concentrations of ICI118551 were evaluated on infected corneas, including 1 mg/mL, 2 mg/mL and 5 mg/mL (maximum concentration of solution in sterile saline). Representative photographs and clinical scores quantified 72 hours after infection showed that cornea treated with 5 mg / mL ICI118551 showed higher transparency and lower clinical scores (Figure 6A and B). Moreover, three different administration time of ICI118551 (5 mg/mL) were also optimized. Based on the data of three different administration time (0.5h, 6h, and 12 h after bacterial incubation), it was indicated that the earlier the intervention time, the better the efficacy of ICI118551 against *P. aeruginosa* keratitis of mice with no significant effect on intraocular pressure (Figure 6C and Supplementary Figure 3).

ICI118551 adjunct therapy with Tobrex reduced the severity of *P. aeruginosa*keratitis

The aforementioned results suggested that ICI118551 is effective in reducing *P. aeruginosa* keratitis. Up to date the best treatment for bacterial keratitis remains topical antibiotic (Austin *et al* ., 2017). We then investigated the effect of topically applied ICI118551 combined with antibiotics on the *P. aeruginosa*keratitis. We chose 12 hours after infection as a starting point to treat *P. aeruginosa* keratitis with topical Tobrex in the presence or absence of ICI118551 (5 mg/mL). Figure 7 showed the representative micrographs of infected corneas 12, 24, 48 and 72 h after infection. Before the intervention, the infected eyes in the two groups exhibited some slight opacification and subepithelial haze. While Tobrex-treated eyes exhibited topical dense

opacification and subepithelial haze, the eyes treated with ICI118551 as adjunctive therapy were mostly clear with some areas of slight opacity (Figure 7A). A significant difference was observed 72 h after infection between the clinical scores of corneas cotreated with ICI118551 and Tobrex and those of corneas treated with Tobrex (Figure 7B). Consistent with reduced corneal infection, topical ICI118551 treatment significantly reduced bacterial load, compared with Tobrex alone (Figure 7C; $P< 0.001$).

Discussion

Previous studies reported the use of β2-AR blockers as therapy for pulmonary, cardiovascular, and other diseases (Brodde, 2007; Leineweber & Heusch, 2009; O'Neill *et al* ., 2020; Wenzel *et al* ., 2009). However, the effect of β2-AR blockers on cornea was unclear (Michel, Bond & Summers, 2019). Our study showed that the prophylactic use of the highly selective β2-AR blocker ICI118551 suppressed the severity of *P. aeruginosa* keratitis by reducing neutrophil recruitment, NETs formation, and cytokines release. It also inhibited the formation of biofilm and the production of bacterial virulence factors in cornea. Further bacterial culture *in vitro* confirmed ICI118551 has significantly antibacterial activity against *P. aeruginosa* . Subsequently, the topical ICI118551 treatment combined with Tobrex 12h after infection greatly reduced the severity of *P. aeruginosa* keratitis compared with Tobrex alone, suggesting an adjunct therapy role of ICI118551. To our knowledge, this is the first study to explore the role of β-AR blockers on bacterial keratitis.

Our previous studies have confirmed that extended CL wear could elevate corneal NE content, which promotes the pathogenesis of CL-induced*P. aeruginosa* keratitis in mice. Here in this study, we explored the effects of different AR blockers on the severity of *P. aeruginosa* keratitis in mice. NE and AR blockers have been shown to modulate bacterial infection by interaction with bacteria and immune response (Chavan *et al* ., 2017; Padro & Sanders, 2014; Vaziraniet *al* ., 2015; Xue *et al* ., 2018). Stimulation of the β2-AR pathway was found to be detrimental for host survival to cytomegalovirus infection, while β2-AR deficient mice exhibited a higher resistance to cytomegalovirus infection (Wieduwild *et al* ., 2020). Accordingly, we found that β2-AR blockers have a more obvious effect on reducing the severity of keratitis among six blockers, and ICI118551 has the most significant effect.

Recent evidence suggested ICI118551 significantly inhibited the mobility by binding to β2-AR of neutrophil cell membrane (Reinartz *et al* ., 2016). Moreover, in cornea, β2-AR blockers could inhibit the rate and number of neutrophils trafficking to the injured cornea following corneal abrasion by binding to the β2-AR (Diaz-Salazar *et al* ., 2020; Wieduwild *et al* ., 2020; Xue *et al* ., 2018), thus regulating the release of inflammatory mediators such as IL-6, IL-17A and TNF-α, inactivation nuclear factor-ϰB (NF-ϰB) signaling pathway and reducing host inflammation response. The inflammatory mediators mentioned above would induce inflammation via NF-ϰB signaling pathway (McClellan *et al* ., 2006) and initiate bacterial eradication during the pathogenic process of *P. aeruginosa* infection. Based on our data, the neutrophil infiltration and NETs formation were inhibited from the effect of ICI118551. Indeed, the expression of inflammatory cytokines such as MIP-2, TNF-α and IL-1β were also downregulated along with the suppression of *P. aeruginosa* infection. This constitutes an important indication that ICI118551 reduced the severity of *P. aeruginosa* keratitis via β2-AR pathway and regulation of infiltration of neutrophils.

As reported previously, NE could promote the adhesion and biofilm formation of *P. aeruginosa* . The formation of biofilms by *P. aeruginosa* is thought to increase persistence and antibiotic resistance during infection and like to the release of virulence factors, regulated by complicated signaling systems such as *las* and *rhl*quorum sensing systems (Flemming & Wingender, 2010; Lee & Zhang, 2015; Riquelme *et al* ., 2020). Herein, our data show that the β2-AR blocker ICI118551 reduced the formation of biofilm in the corneas, which is beneficial to the phagocytosis of bacteria by neutrophils. Additionally, recent study reported the non-selective β-blocker, carvedilol, exhibited obvious antibacterial activity by changing the percentage of membrane phospholipids and fatty acids, and influencing the permeability of bacterial cell membranes (Zawadzka *et al* ., 2018). While in this study, we confirmed that β2-AR blocker ICI118551, also has a significant bactericidal effect on *P. aeruginosa* , which can obviously inhibit bacterial growth and the expression levels of virulence factors in cornea. The antibacterial activity of ICI118551 was verified by the staining tests of cell membranes permeability of*P. aeruginosa* incubated with ICI118551 *in vitro* . This is

further indication that ICI118551 not only participated in the regulation of host neutrophil mobility, but also has a non-negligible inhibitory effect on *P. aeruginosa* signal pathways.

In view of the significant inhibition of ICI118551 on *P. aeruginosa* keratitis, we further explored the optimal drug concentration, initial intervention time, and its potential influence on intraocular pressure. From the quantification of the clinical scores, the optimal concentration was 5 mg/ml, the initial intervention time was as earlier as the bacterial inoculation in the cornea. From the dynamic evaluation of intraocular pressure, ICI118551 had no significant effect on this parameter during or after topical administration.

Although invasive *P. aeruginosa* can be readily eliminated by topical antibiotics, persistent inflammation may lead to the progression of corneal ulcer (Gao *et al* ., 2015). Herein, using the established murine model of bacterial keratitis, we examined the therapeutic potential for ICI118551 as an adjunct therapy to Tobrex. We noted that the infected cornea treated with ICI118551+Tobrex exhibited the better improvement in disease severity compared with Tobrex alone. Combined with minimal side effects of topical administration of ICI118551, it merits further development of ICI118551 in the treatment of *P. aeruginosa* keratitis.

Collectively, this study demonstrated that the highly selective β2-AR blocker ICI118551 suppressed*P. aeruginosa* keratitis and alleviate corneal damage of mice. It is suggested that ICI118551 may represent a kind of potential prophylactic and therapeutic approach to control the corneal and other mucosal*P. aeruginosa* infection in the future.

Acknowledgements

We thank Ting Liu, Xia Qi, and Li Ma for their excellent technical assistance.

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Figure legends

Figure 1. Effects of different AR blockers on *P. aeruginosa* keratitis. AR blockers or sterile saline (control) were topically applied respectively to the mice corneas 0.5 h before corneal infection. (A) Representative photographs of corneas at 24 h and 72 h after bacterial inoculation. (B) The corneal clinical scores at 72 h after bacterial inoculation. (C) The bacterial load in each individual cornea 24 h after bacterial inoculation was counted (*, $P< .05$, **, $P < .01$, ***, $P < .001$; n=5). (D) Representative photomicrographs stained with hematoxylin/eosin at 24 h after bacterial inoculation. Abbreviation: E, epithelium.

Figure 2. ICI118551 attenuated neutrophil infiltration, NETs formation and the proinflammatory cytokine levels. The infected corneas were excised 48 h after infection and subjected to immunofluorescence staining with the Ly6G antibody (A), flow cytometric quantification of the cumulative

neutrophil (Ly6G+) percentage among total leukocytes (B), MPO activity assay (C), NETs staining (D), and a proinflammatory cytokine ELISA assay (E) (*, $P < .05$, **, $P < .01$, ***, $P < .001$; n=5).

Figure 3. ICI118551 reduced the levels of virulence factors and biofilm formation of *P. aeruginosa* in corneas. Mice corneas were excised at 24 h after infection and subjected to qRT-PCR (A), ELISA assay (B) and biofilm staining (C). The expression level of flagellin related genes *figD/E/L* , elastase related genes *lasA/B* , and biofilm related gene *pslB* were detected. The content of LPS, exotoxin A, and elastase activity were measured (*, $P < .05$, **$P < .01$; n=5).

Figure 4. ICI118551 reduced corneal epithelial basement membrane damage (E). The infected corneas were subjected to immunofluorescence staining analysis using anti-collagen IV to illuminate the basement membrane (arrowheads) at 24, 48, and 72 h after infection. Abbreviation: E, epithelium.

Figure 5. Bactericidal activity of ICI118551 against *P. aeruginosa* in vitro. The effect of ICI118551 on the growth of *P. aeruginosa* in vitro was detected. (A) Bacterial growth curve. The turbidity of cultures was monitored at 1, 2, 4, 6, 8, 24 h after bacterial incubation (*, $P < .05$, ***, $P < .001$). (B) Comparison of *P. aeruginosa* 24-hour culture. (C) Viablity staining results of *P. aeruginosa* 24-hour culture. Red, stained with propidium iodide; green, stained with SYTO9. (D) Negative staining results of *P. aeruginosa* 24-hour culture. Black bacterial cells represent a complete cell structure, while gray indicates that cells are dissolving (arrow).

Figure 6. Determination of the optimal drug concentration and optimal initial intervention time. The effect of three different concentration (1 mg/ml, 2 mg/ml, and 5 mg/ml), and three different administration time (0.5, 6 and 12 h post infection) of ICI118551 were evalued. Representative images of slit lamp microscopy (A) and clinical scores (B and C) at 24, 48, and 72 h after bacterial inoculation were shown. The bacterial challenge was followed by ICI118551 treatment to contrast the anti-inflammatory effect at different initial intervention times: 0.5, 6 and 12 h represent the time intervals between the two events (*, $P < .05$, **, $P < .01$; n=5).

Figure 7. ICI118551 adjunct therapy combined with Tobrex significantly reduced the severity of *P. aeruginosa* keratitis.ICI118551 combined Tobramycin or Tobramycin was applied topically start from 12 h after infection and 3 times per day. (A) Representative photographs and clinical scores (B) of the tobramycin and ICI118551 combined tobramycin treated corneas at 12 h and 72 h after infection. (C) Bacterial counting of corneas 72 h after infection (*, $P < .05$, **, $P < .01$; n=5).

Supplementary Figure 1. ICI118551 reduced pathological changes in the infected cornea. The thickness of sections stained with hematoxylin/eosin of the infected cornea at 24 h after bacterial inoculation were shown (*, $P < .05$, **, $P < .01$; n=5).

Supplementary Figure 2. ICI118551 negatively regulated the expression of proinflammatory cytokine genes. The expression levels of proinflammatory cytokines, including MIP-2, TNF-α, and IL-1β, were measured by a qRT-PCR (**, $P < .01$, ***, $P < .001$; n=5).

Supplementary Figure 3. The effect of ICI118551 on intraocular pressure. ICI118551 at a concentration of 5 mg/ml or sterile saline (control) was instilled into mice corneas and applied six times/d for 72 h. Intraocular pressure was then detected using a TonoLab tonometer (TioLat, Helsinki, Finland).

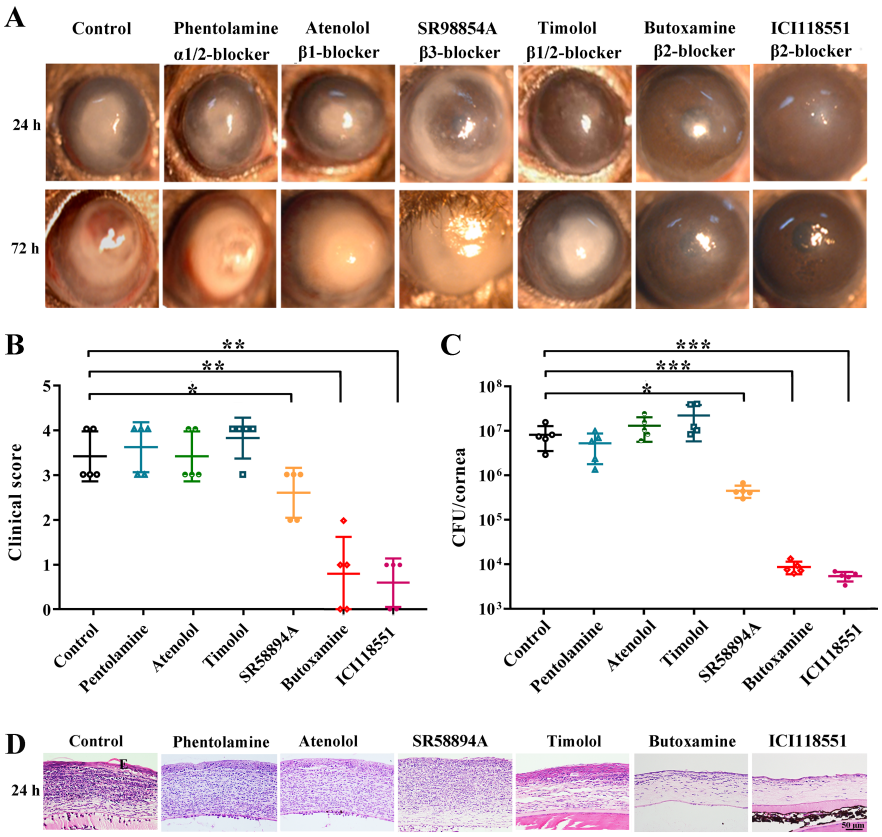


Figure 1

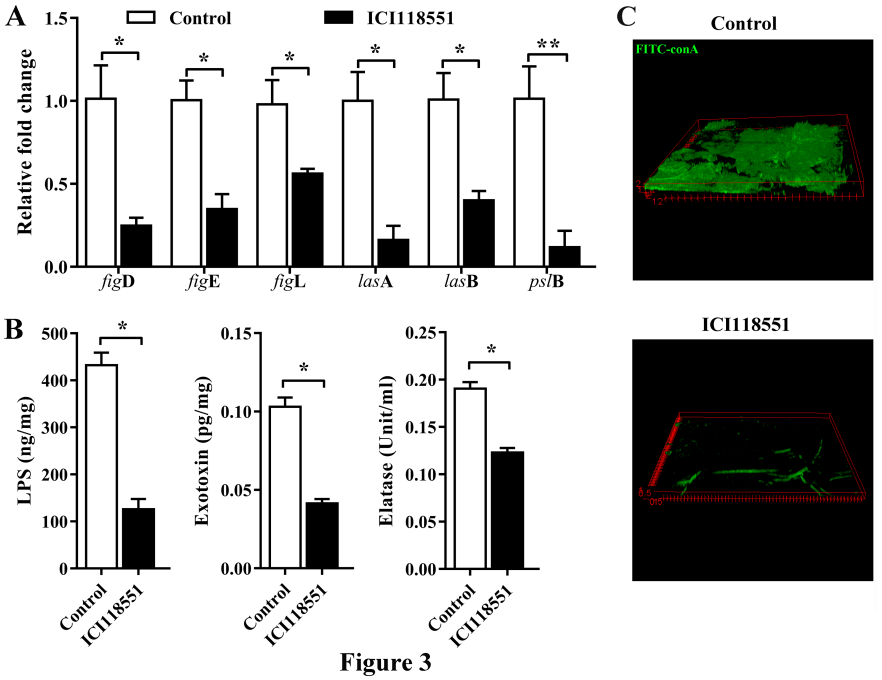


Figure 3

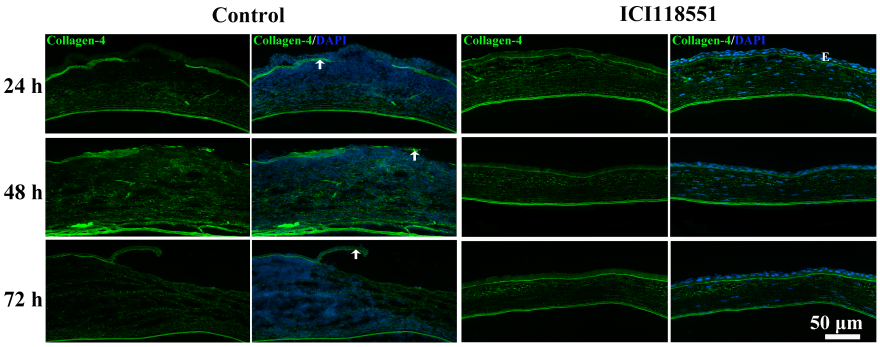
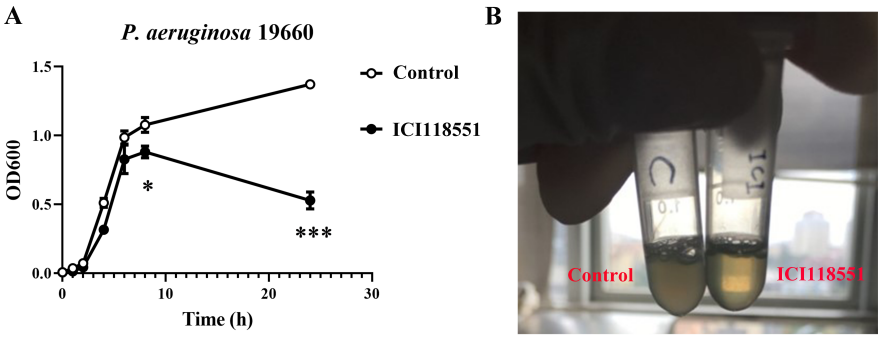


Figure 4



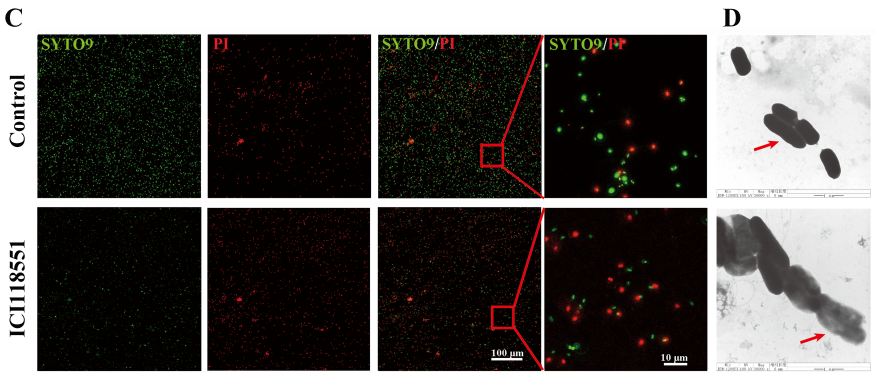


Figure 5

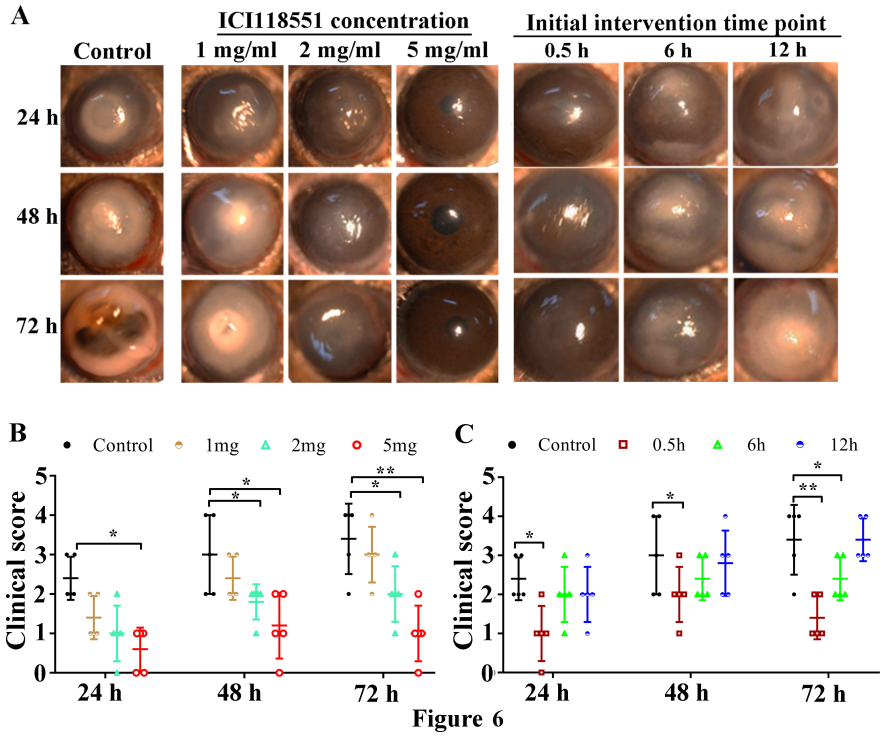


Figure 6

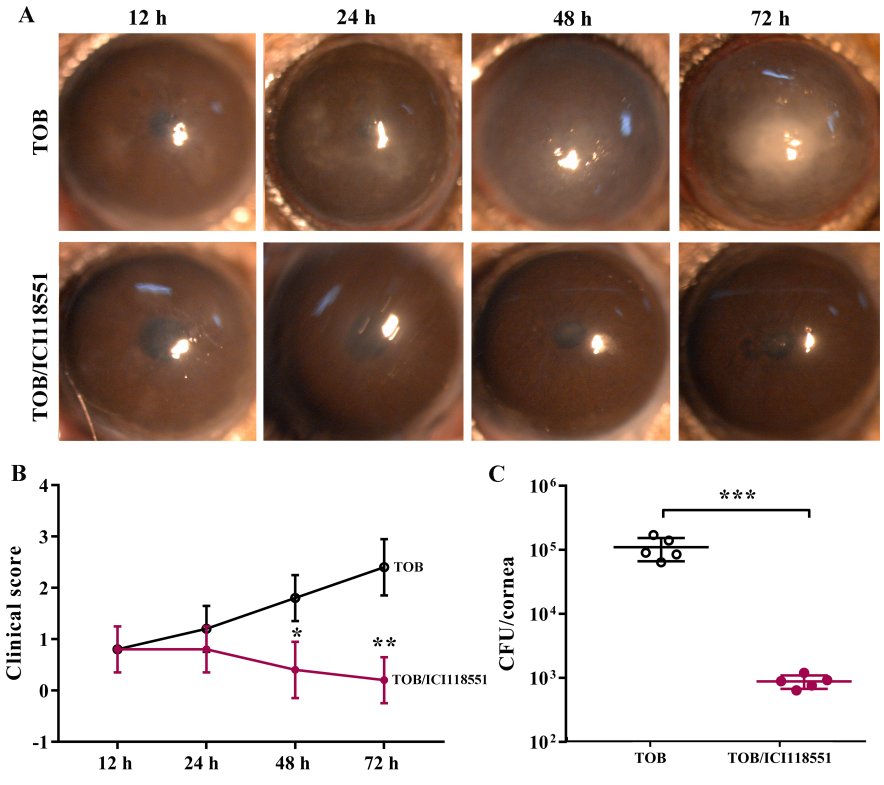
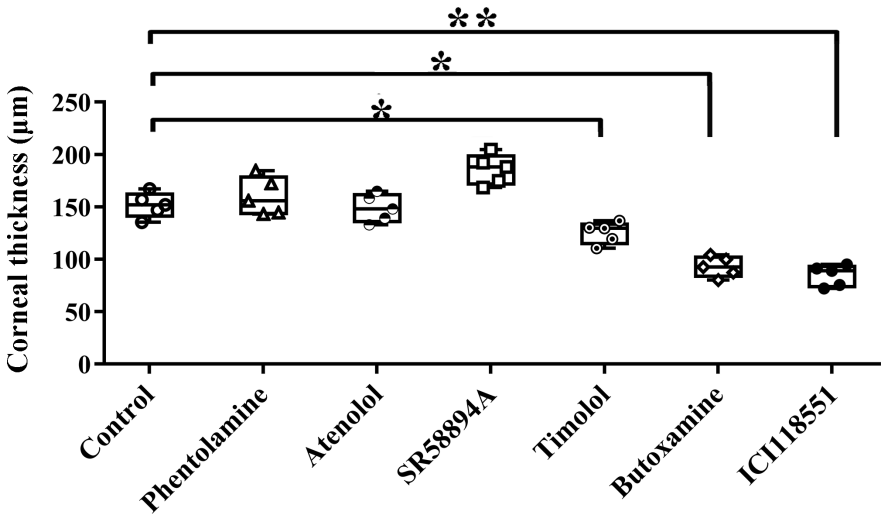
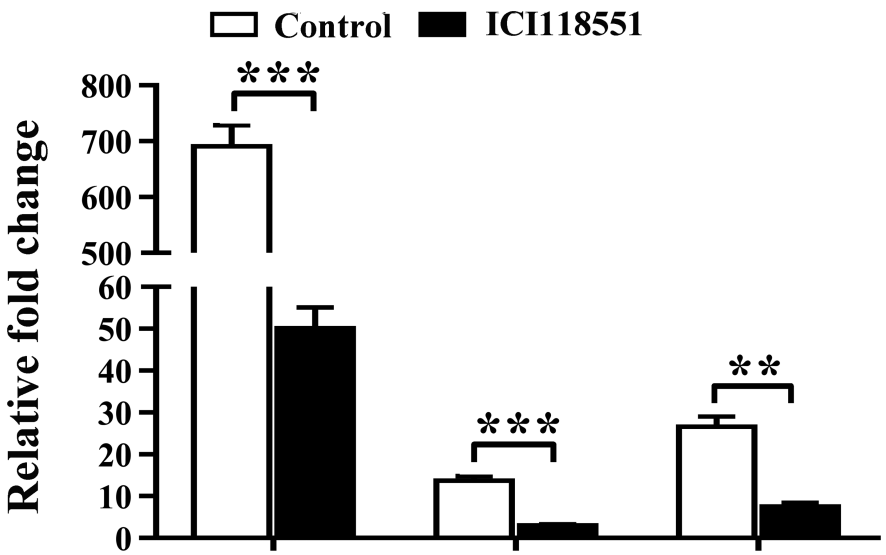


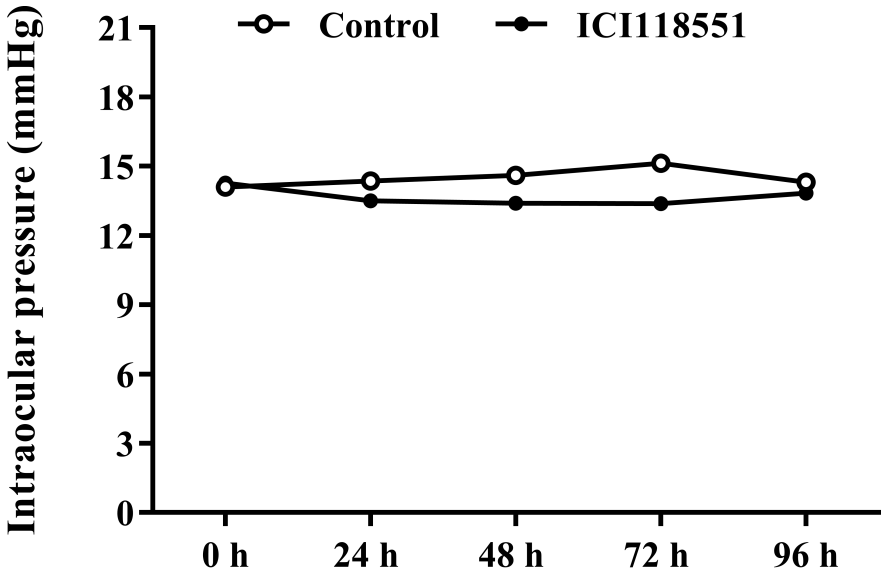
Figure 7



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

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Supplementary Tables-manuscript.pdf available at https://authorea.com/users/364344/articles/484818-%CE%B22-adrenoceptor-blocker-ici118551-attenuates-pseudomonas-aeruginosa-corneal-infection-in-mice

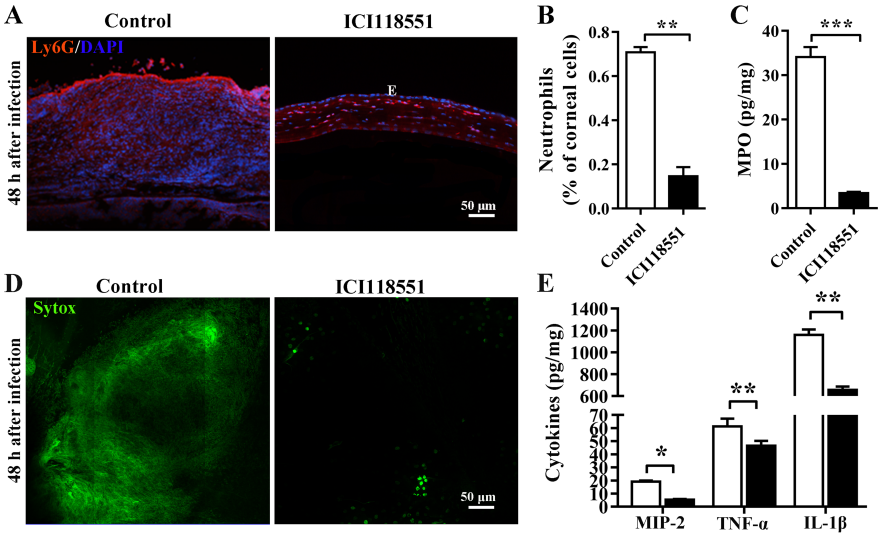

Figure 2