IL-36α Exerts Proinflammatory Effects in *Aspergillus fumigatus* Keratitis of Mice Through the Pathway of IL-36α/IL-36R/NF-κB

Yawen Niu, Changjie Ren, Xudong Peng, Cui Li, Qiang Xu, Liting Hu, Ziyue Zhang, Guiqiu Zhao, and Jing Lin

Department of Ophthalmology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, China

Correspondence: Guiqiu Zhao, Department of Ophthalmology, The Affiliated Hospital of Qingdao University, No. 16 Jiangsu Road, Qingdao, Shandong Province 266000, China; zhaoguiqiu_good@126.com.
Jing Lin, Department of Ophthalmology, The Affiliated Hospital of Qingdao University, No. 16 Jiangsu Road, Qingdao, Shandong Province 266000, China; linjing_yk@126.com; yankelinjing@126.com.

Received: September 7, 2020
Accepted: January 17, 2021
Published: April 14, 2021

Citation: Niu Y, Ren C, Peng X, et al. IL-36α exerts proinflammatory effects in *Aspergillus fumigatus* keratitis of mice through the pathway of IL-36α/IL-36R/NF-κB. *Invest Ophthalmol Vis Sci.* 2021;62(4):16. https://doi.org/10.1167/iovs.62.4.16

Purpose. To explore the role of IL-36α in corneas infected by *Aspergillus fumigatus*.

Methods. The experimental group was comprised of 15 corneas with fungal keratitis, and 15 healthy donor corneas were included in the control group. IL-36α was detected in normal and infected corneas of humans and C57BL/6 mice. Mice corneas were infected with *A. fumigatus* with or without pretreatment of recombinant mouse (rm) IL-36α and IL-36α neutralizing antibody (Ab). Primary macrophages were stimulated with 75% ethanol-killed *A. fumigatus* with or without pretreatment of rmIL-36α. The severity of the disease was documented by clinical score and photographs with a slit lamp. PCR, western blot, and immunostaining were used to determine the expression of IL-36α, IL-1β, IL-6, and TNF-α. Polymorphonuclear neutrophilic leukocyte infiltration was assessed by myeloperoxidase (MPO) assay and flow cytometry. Macrophage infiltration was tested by immunofluorescent staining and flow cytometry.

Results. IL-36α mRNA and protein were significantly elevated in human and mice corneas after infection. The rmIL-36α treatment of C57BL/6 mice increased clinical score, MPO levels, macrophage infiltration, and expression of the proinflammatory cytokines IL-1β, IL-6, and TNF-α compared with the infected controls, which showed a decrease due to IL-36α Ab treatment. In primary macrophages, IL-36α expression was also significantly increased by *A. fumigatus*. The rmIL-36α treatment upregulated IL-1β, IL-6, and phosphorylated nuclear factor (NF)-κB expression, which was significantly inhibited by rmIL-36Ra.

Conclusions. IL-36α act as a proinflammatory cytokine in *A. fumigatus* keratitis by promoting the infiltration of neutrophils and macrophages and increasing the secretion of IL-1β, IL-6, and TNF-α, in addition to regulating expression of phosphorylated NF-κB.

Keywords: IL-36α, *Aspergillus fumigatus*, keratitis, mice

Fungal keratitis is a type of infectious keratopathy with a high rate of blindness.[1,2] The incidence of fungal keratitis is increasing yearly due to increased ocular trauma, especially agricultural trauma and the excessive use of antibiotics or corticosteroids. The primary pathogens include *Fusarium*, *Curvularia*, *Aspergillus*, and *Mycotoruloides*. *Aspergillus fumigatus* features an armory of virulence determinants that elicit a strong host immune response.[3] *Aspergillus* is one of the most common pathogens associated with fungal keratitis. When *A. fumigatus* invades, the host recognizes and attempts to eradicate the pathogenic microorganism via the host's immune system.

IL-36α, a newly discovered member of the IL-1 family, has been regarded as a proinflammatory cytokine in various infectious and immune diseases. It contributes to the local inflammatory response by promoting the secretion of various cytokines, such as chemokines, recruiting macrophages, and neutrophils; activating dendritic cells; and promoting the polarization of Th cells and other mechanisms.[4–6] IL-36α is now considered to be critical for the host defense against bacteria, virus, and fungi. Previous studies have shown that the increased expression of IL-36α in psoriatic lesions can promote the secretion of proinflammatory factors, activate immune cells, and exacerbate skin inflammation.[7] The enhanced expression of IL-36α in lung tissues infected with bacteria or viruses upregulates IL-6 and CXCL8 expression and promotes neutrophil infiltration.[6,8] It is worth noting that recent studies have shown that IL-36α is significantly increased in Pseudomonas *aeruginosa* keratitis[9]; however, it remains untested as to whether *A. fumigates* can elevate IL-36α and then trigger inflammatory responses.

Thus, this current study investigated the expression and function of IL-36α with regard to innate immunity to *A. fumigatus* keratitis in mice. Our data provide evidence that IL-36α mRNA and protein levels are upregulated in cornea after infection. Exogenous recombinant mouse (rm) IL-36α resulted in worsened disease due to its effect on the infiltration of neutrophils and macrophages,

secretion of proinflammatory cytokines, and expression of IL-36R.

Materials and Methods

Clinical Specimens

In total, 15 healthy donor corneas were used for corneal transplantation, and the remainder of the peripheral corneal tissues were collected. Fifteen patients (15 eyes) with *A. fumigatus* keratitis underwent penetrating keratoplasty, and corneas with lesions were collected. The corneal epithelium was scraped. Scrapings were collected in 500 µL of RNAiso reagent (Takara Bio, Shiga, Japan) and used to test IL-36α mRNA levels. Corneal specimens from patients with fungal keratitis and from healthy donors were collected and prepared according to experimental requirements. Hematoxylin and eosin (H&E) staining was used to observe the pathological changes in corneas. Immunohistochemistry was performed to detect the protein expression of IL-36α in human corneas. Research adhered to the tenets of the Declaration of Helsinki. The human corneal tissue providers all signed informed consent forms for specimen processing. The experiment had the approval of the hospital's ethics committee.

Animals and Corneal Infection

Female 8-week-old C57BL/6 mice were purchased from Changzhou Cavens Laboratory Animal Co., Ltd. (Jinan, Shandong, China). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized using 8% chloral hydrate and placed beneath a stereoscopic microscope. The central corneal epithelium (2-mm diameter range) of the left eye was removed. A 5-µL aliquot (1×10^8 CFU/mL) of *A. fumigatus*, strain 3.0772 (China General Microbiological Culture Collection Center, Beijing, China), was topically applied to the ocular surface. The ocular surface was then covered with a soft contact lens, and the eyelids were sutured. Mice corneas were collected for real-time RT-PCR at 1, 3, and 5 days post-infection (PI), and for western blot at 1, 3, and 5 days PI. Eyeballs were removed at 3 days PI for immunofluorescence.

Corneal Response to Infection

A clinical score was recorded for each mouse at 1, 3, and 5 days PI for statistical comparison of disease severity. Photography with a slit lamp was used to illustrate disease.

Mouse Peritoneal Primary Macrophages Isolation

C57BL/6 mice were injected intraperitoneally (IP) with 1 mL of 3% thioglycolate medium. Seven days after injection, the mice were sacrificed. Then, 5 mL of RPMI 1640 medium was injected into the abdominal cavity. The abdomen was gently massaged to fully wash the abdominal cavity with the medium. The liquid was sucked out and collected. Peritoneal lavage was repeated once more.

Primary Macrophage Culture and *A. fumigatus* Stimulation

Primary macrophages were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA) at 37°C and 5% CO_2. Cells were incubated with *A. fumigatus* hyphae (to the final concentration of 5×10^6 CFU/mL) for 0, 4, 8, 12, 16, 18, and 24 hours.

Recombinant Mouse IL-36α Treatment

The left eyes of C57BL/6 mice ($n = 5$ per group and time) were injected subconjunctivally with rmIL-36α protein (1 µg/5 µL; R&D Systems, Minneapolis, MN, USA) or control (1 µg/5 µL IgG) 1 day before infection. At 1 and 3 days PI, each mouse was injected intraperitoneally with 1 µg of rmIL-36α or IgG control diluted in 100 µL PBS. Primary macrophages were pretreated with rmIL-36α protein for 2 hours and then incubated with *A. fumigatus*. The cells were then analyzed by RT-PCR and western blot.

IL-36α Neutralizing Antibody Treatment

IL-36α neutralizing antibody (1 µg/5 µL; R&D Systems) or IgG control (1 µg/5 µL) was given subconjunctivally into the left eyes of C57BL/6 mice ($n = 5$ per group and time) 1 day before infection. At 1 and 3 days PI, each mouse was injected IP with 1 µg of IL-36α Ab or IgG control diluted in 100 µL PBS.

Real-Time RT-PCR

C57BL/6 mice corneas were collected at the indicated times after treatment. Total corneal RNA was isolated by using Takara Bio RNAiso Plus reagent and quantified by spectrophotometry. RNA (2 µg) was used for first-strand cDNA synthesis according to the protocol for a reverse-transcription system. cDNA products were diluted 1:25 with diethylpyrocarbonate-treated water, and a 2-µL cDNA aliquot was used for real-time RT-PCR (20 µL total reaction volume) according to the manufacturer's instructions. Takara Real-Time PCR Master Mix was used for the PCR reaction with primer concentrations of 5 µM. All reactions were performed with the following cycling parameters: 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds, and a final stage of 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. Relative transcription levels were calculated by using the relative standard curve method, which compares the amount of target normalized to the housekeeping gene β-actin. Data are shown as the mean ± SEM for relative mRNA levels.

Western Blot Analysis

Corneas and cells were ground and lysed in radioimmunoprecipitation assay (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) lysis buffer containing 1-mM phenylmethanesulfonyl fluoride (Beijing Solarbio) for 2 hours. They were then centrifuged at 4°C, 12,000 rpm, for 10 minutes. The supernatant was collected and tested for protein concentration. After SDS sample buffer was added, followed by boiling, total protein was separated on 12% or 15% acrylamide SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Beijing Solarbio). The membranes were blocked with Western Blocking

Investigative Ophthalmology & Visual Science

Buffer (Beyotime Biotechnology, Shanghai, China) at room temperature for 2 hours and were then incubated with primary antibody to IL-36α (1:1000; R&;D Systems), IL-1β (1:1000; Abcam, Cambridge, UK), IL-6 (1:1000; Abcam), TNF-α (1:1000; Abcam), phosphorylated (p) NF-κB (1:1000; CST, Wuhan, China), total NF-κB (1:1000; CST), glyceraldehyde 3-phosphate dehydrogenase (1:2000; Elabscience, Wuhan, China), β-actin (1:2000; Elabscience), or β-tublin (1:2000; Elabscience) at 4°C overnight. After washing in PBS containing 0.05% TWEEN 20 (Bio-Rad, Hercules, CA, USA) for three times, the membranes were incubated with corresponding peroxidase-conjugated secondary antibodies (1:5000; Elabscience) at room temperature for 1 hour. The blots were then developed by using chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitation of Corneal Polymorphonuclear Neutrophilic Leukocytes

A myeloperoxidase (MPO) assay was used to quantitate the number of polymorphonuclear neutrophilic leukocytes. Previous studies have reported that mice *A. fumigatus* keratitis response is most obvious at 3 days PI[10]; therefore, corneas ($n = 6$ per group and time) were removed at 3 days PI and processed according to the manufacturer's instructions for the MPO test kit (Njjcbio, Nanjing, China).

Immunochemistry

After fixation and removal of endogenous peroxidase with 3% hydrogen peroxide, cells were incubated with goat-blocking antibody at 37°C for 20 minutes. After that, the cells were reacted with IL-36α antibody (Abcam) for 2 hours, then with a biotin-conjugated anti-rabbit secondary antibody at 37°C for 40 minutes, followed by peroxidase-conjugated streptavidin for 15 minutes. Slices were developed with diaminobenzidine. The immunostaining data were quantified by Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Immunofluorescent Staining

Eyeballs were removed ($n = 3$ per group and time) at 3 days PI from C57BL/6 mice, immersed in 0.01-M PBS, embedded in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA, USA), and frozen in liquid nitrogen. Ten-micrometer sections were cut, mounted to poly-L-lysine-coated glass slides, and stored at 37°C overnight. After a 5-minute fixation in acetone, slides were blocked with 0.01-M PBS containing 10% blocking serum for 30 minutes at room temperature. The sections were incubated at a 1:100 dilution of the rat anti-F4/80 antibodies (Abcam) at 4°C overnight. This was followed by FITC-conjugated goat anti-rat secondary antibody (1:500, 1 hour at room temperature without light; Abcam). Isotype IgG was used as the negative control. Finally, sections were visualized and digital images captured with a Zeiss Axio Vert microscope (Carl Zeiss Microscopy, Jena, Germany) at 40× magnification.

Flow Cytometry

Samples were analyzed by flow cytometry with a Beckman flow cytometer (Beckman Coulter, Brea, CA, USA) and FlowJo software (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). The primary antibodies used in this experiment were CD11b-FITC, Ly6G-PE, CD45-FITC, and F4/80-PC7 (BioLegend, San Diego, CA, USA). All the experiments were repeated three times.

Enzyme-Linked Immunosorbent Assay

The cell supernatant was collected and centrifuged at 1000 g for 20 minutes, and 100 µL of each sample was then assayed in duplicate for IL-1β and IL-6 protein according to the manufacturer's instructions (BioLegend).

Statistical Analysis

The difference in clinical scores between the two groups at each time was tested by the Mann–Whitney U test. An unpaired, two-tailed Student's t-test was used to determine the statistical significance of the real-time RT-PCR, western blot, ELISA, MPO, and cell-sorting data. Data were considered significant at $P < 0.05$. All experiments were repeated once to ensure reproducibility, and data from a representative experiment are shown as mean ± SEM.

Results

IL-36α Expression in Human Corneal Epithelium

H&E staining showed stroma infiltration in *Aspergillus*-infected cornea (Figs. 1A, 1B). The mRNA and protein expression of IL-36α in normal uninfected and *Aspergillus*-infected human corneas were tested by real-time RT-PCR and immunostaining. Results indicated that relative IL-36α mRNA levels were significantly higher in the epithelium of *Aspergillus*-infected cornea than in normal corneal epithelium ($P < 0.001$) (Fig. 1C). To confirm these data, the presence of IL-36α protein was determined by immunostaining. IL-36α protein levels were elevated in infected corneas ($P < 0.01$) (Figs. 1D–1F). IL-36α was mainly expressed in the epithelium of fungal keratitis and especially in the cytoplasm.

Expression of IL-36α in C57BL/6 Mouse Corneas

In order to observe the disease response of control and infected corneas, we used a slit-lamp microscope to take photographs at 1, 3, and 5 days PI (Figs. 2A–2D). Compared with the control group, the clinical score gradually increased and reached a peak at 3 days PI in the infected group ($P < 0.01$) (Fig. 2E). To further explore whether IL-36α was involved in *A. fumigatus* keratitis, we tested the mRNA and protein expression of IL-36α in control and *Aspergillus*-infected C57BL/6 mouse corneas using real-time RT-PCR, western blot, and immunofluorescence. Data showed that, in infected mice corneas at 1, 3, and 5 days PI, the levels of IL-36α mRNA ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) (Fig. 2F) and protein ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) (Figs. 2G, 2H) were upregulated compared to control mice corneas. The photographs showed that IL-36α protein expression (green) was significantly elevated in infected mice corneas compared to control corneas (Fig. 2I). IL-36α was mainly expressed in the epithelium of infected corneas and especially in the cytoplasm.

Investigative Ophthalmology & Visual Science

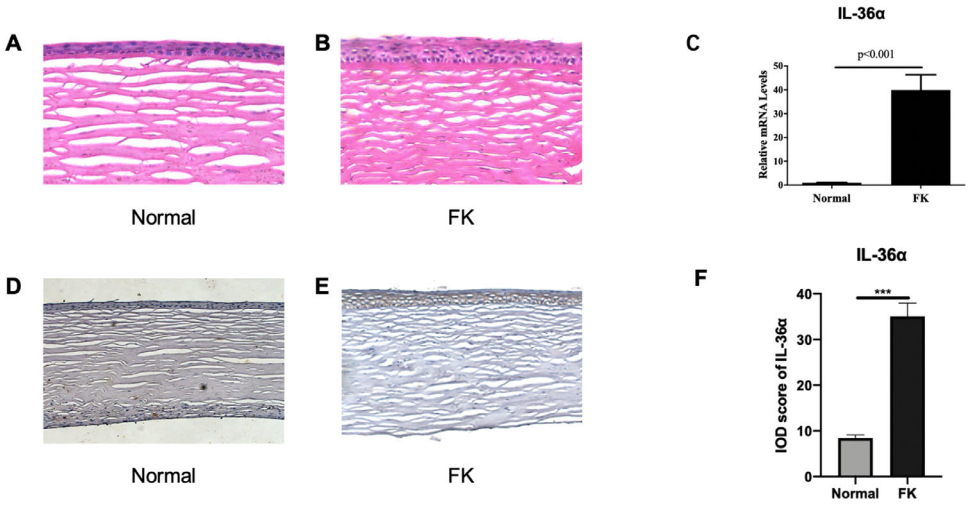

Figure 1. IL-36α expression in human corneal epithelium. The H&E staining showed normal (**A**) and *Aspergillus*-infected (**B**) corneas. Relative IL-36α mRNA levels (**C**) were higher in the epithelium of *Aspergillus*-infected corneas than in normal corneal epithelium. Immunostaining and the quantitative estimation (**F**) demonstrated that IL-36α protein expression markedly increased in the infected corneal epithelium (**E**) versus controls (**D**). All data are mean ± SEM; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Magnification, 200× (**A**, **B**, **D**, **E**). FK, fungal keratitis.

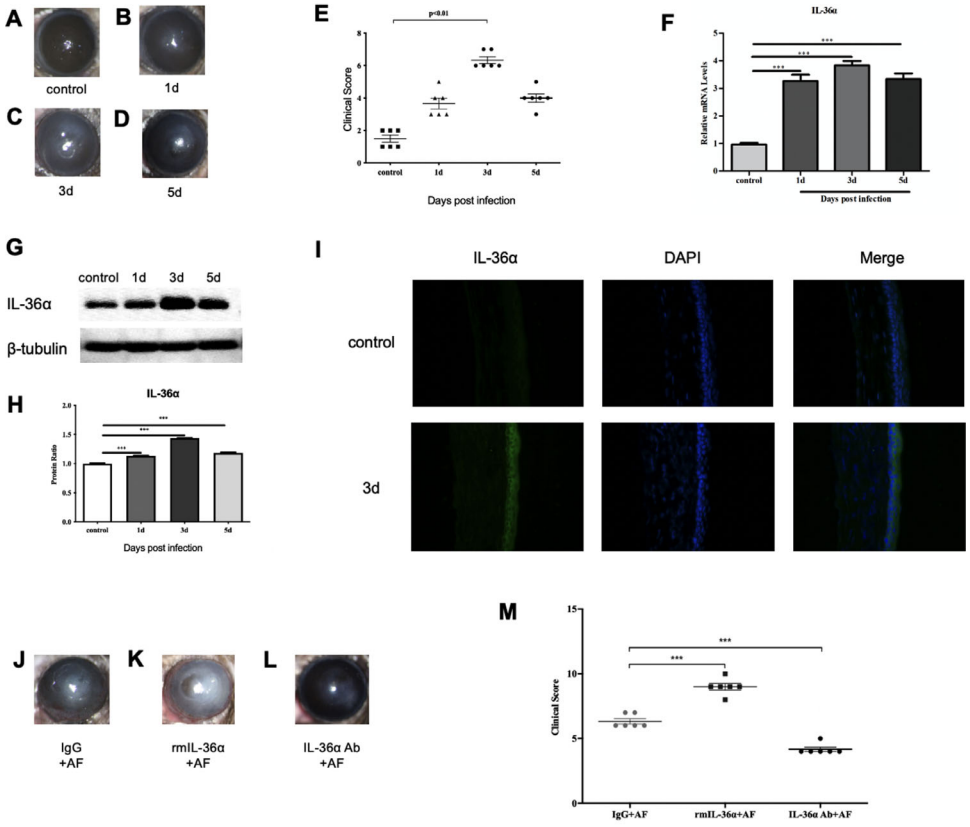

Figure 2. Expression of IL-36α in C57BL/6 mouse corneas. (**A–D**) Photographs taken with a slit lamp at 1, 3, and 5 days PI show the changes of disease severity. (**E**) Clinical scores seen at 1, 3, and 5 days PI. (**F**) IL-36α mRNA and (**G**, **H**) protein levels were upregulated in infected mice corneas at 1, 3, and 5 days PI compared to control mice corneas. (**I**) Immunofluorescent staining demonstrated that IL-36α protein expression (*green*) was significantly elevated in infected mice corneas compared to control corneas. (**J–L**) Disease severity and (**M**) clinical scores were increased in the rmIL-36α-treated group compared with the IgG-treated group and decreased in the IL-36α Ab-treated group. All data are mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Magnification, 25× (**A–D**), 400× (**I**), and 25× (**J–L**). AF, *A. fumigatus*.

Effect of IL-36α on Neutrophils and Macrophages Infiltration

To investigate the role of IL-36α in vivo, we next treated mice with rmIL-36α and IL-36α Ab. First, we observed the disease response of corneas treated with rmIL-36α or IL-36α Ab and the control corneas treated with IgG. Results showed that treatment with rmIL-36α significantly aggravated the inflammatory response (Figs. 2J, 2K) and increased clinical scores ($P < 0.001$) (Fig. 2M) compared with the IgG-treated controls. IL-36α Ab treatment reduced the inflammatory response (Figs. 2J, 2L) and decreased clinical scores ($P < 0.001$) (Fig. 2M).

Next, we tested MPO levels and used flow cytometry to assess the number of neutrophils in corneas. Data showed that rmIL-36α treatment significantly upregulated MPO levels and the number of neutrophils in corneas compared with the IgG-treated controls ($P < 0.001$ and $P < 0.001$, respectively) (Figs. 3A–3C). IL-36α Ab pretreatment led to the downregulation of MPO levels and the number of neutrophils ($P < 0.001$ and $P < 0.001$, respectively) (Figs. 3A–3C).

We also used immunofluorescence and flow cytometry to determine the number of macrophages in corneas. Immunostaining results showed that the number of macrophages (green) was increased after rmIL-36α treatment when compare to the IgG-infected controls (Fig. 3D). IL-36α Ab treatment decreased the number of macrophages (green) in corneas (Fig. 3D). Flow cytometry results showed similar results ($P < 0.001$) (Figs. 3E, 3F).

Effect of IL-36α on Cytokines (IL-1β, IL-6, and TNF-α) in Corneas Infected by *A. fumigatus*

We used real-time RT-PCR and western blot to test IL-1β, IL-6, and TNF-α expression. Results showed that rmIL-36α significantly increased the mRNA and protein levels of IL-1β ($P < 0.001$ (mRNA levels of IL-1β, Fig. 4A), $P < 0.001$ (protein levels of IL-1β P31, Fig. 4E), and $P < 0.01$ (protein levels of IL-1β P17, Fig. 4F), respectively) (Figs. 4A, 4D–4F); IL-6 ($P < 0.01$ (mRNA levels of IL-6, Fig. 4B) and $P < 0.001$ (protein levels of IL-6, Fig. 4G), respectively) (Figs. 4B, 4D, 4G); and TNF-α ($P < 0.001$ (mRNA levels of TNF-α, Fig. 4C) and $P < 0.01$ (protein levels of TNF-α, Fig. 4H), respectively) (Figs. 4C, 4D, 4H) compared with the IgG infected group. The expression of IL-1β ($P < 0.001$ (mRNA levels of IL-1β, Fig. 4I), $P < 0.001$ protein levels of IL-1β P31, Fig. 4M), and $P < 0.001$ (protein levels of IL-1β P17, Fig. 4N), respectively) (Figs. 4I, 4L–4N); IL-6 ($P < 0.001$ (mRNA levels of IL-6, Fig. 4J) and $P < 0.001$ (protein levels of IL-6, Fig. 4O), respectively) (Figs. 4J, 4L, 4O); and TNF-α ($P < 0.001$ (mRNA levels of TNF-α, Fig. 4K) and $P < 0.001$ (protein levels of TNF-α, Fig. 4P), respectively) (Figs. 4K, 4L, 4P) was downregulated in IL-36α Ab-treated corneas compared to IgG-treated corneas.

Role of IL-36α in Primary Macrophages

IL-36α mRNA levels were upregulated in the stimulated primary macrophages at 4, 8, 12, and 16 hours ($P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) compared with the untreated cells, and they peaked at 12 hours (Fig. 5A). IL-36α protein levels also increased after stimulation by *A. fumigatus* hyphae for 6, 12, and 18 hours ($P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively) and peaked at 18 hours compared with untreated cells (Figs. 5B, 5C).

Next, we sought to explore whether IL-36α regulates the expression of cytokines in macrophages. Data showed that, after treatment with rmIL-36α for 4, 8, and 12 hours, the mRNA levels of IL-1β ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) (Fig. 5D) and IL-6 ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) (Fig. 5E) were significantly increased compared with IgG control cells. After rmIL-36α treatment for 6, 12, and 18 hours, the protein levels of IL-1β ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) (Fig. 5F) and IL-6 ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) (Fig. 5G) were also significantly increased. *A. fumigatus* hyphae stimulation upregulated IL-1β and IL-6 mRNA and protein levels. The rmIL-36α treatment further increased the mRNA and protein levels of IL-1β ($P < 0.001$ and $P < 0.001$, respectively) (Figs. 5H, 5J) and IL-6 ($P < 0.00$ and $P < 0.01$, respectively) (Figs. 5I, 5K) compared with IgG-stimulated control cells.

IL-36α Regulates Cytokines by IL-36R/NF-κB in Primary Macrophages

Primary macrophages were treated with rmIL-36Ra to test whether IL-36α increased the expression of proinflammatory cytokines by IL-36R. Macrophages were stimulated with *A. fumigatus* hyphae or rmIL-36α with or without rmIL-36Ra. Data showed that rmIL-36Ra treatment decreased the mRNA levels of IL-1β ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 6A) and IL-6 ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 6B) when compared to control infected cells or rmIL-36α-treated cells. After rmIL-36Ra treatment, the protein levels of IL-1β ($P < 0.001$ and $P < 0.001$) (Fig. 6C), IL-6 ($P < 0.001$ and $P < 0.001$) (Fig. 6D), and p-NF-κB ($P < 0.001$) (Figs. 6E, 6F) were also downregulated.

Discussion

IL-36α is emerging as a key player in immune responses.[11] IL-36α is a proinflammatory cytokine that recruits and activates immune cells such as neutrophils and macrophages through increased secretion of adhesion molecules, chemokines, and cytokines.[11,12] Results presented in this study indicate that IL-36α mRNA expression was low in the normal human corneal epithelium, and that its expression was significantly upregulated in the corneal epithelium of patients with fungal keratitis. Immunohistochemical staining results showed that, compared with the normal control group, IL-36α protein staining was significantly enhanced in the corneal tissue of patients with fungal keratitis, and it was mainly localized in the corneal epithelium. Corneal epithelial tissue is the first line of defense against fungal invasion.[13] After being invaded by fungi, the corneal epithelium recognizes fungus and toxins, initiates immune responses, and secretes cytokines to mediate inflammatory cells to the site of the infection. A large number of immune cells, cytokines, and chemokines are involved in the removal of fungus and repairing the cornea.[14,15]

As a type of proinflammatory factor, IL-36α can activate immune cells and promote the secretion of cytokines.[4] The high expression of IL-36α in fungal infected corneal tissues suggests that it may be involved in the innate immune response process of fungal keratitis. Such findings

Investigative Ophthalmology & Visual Science

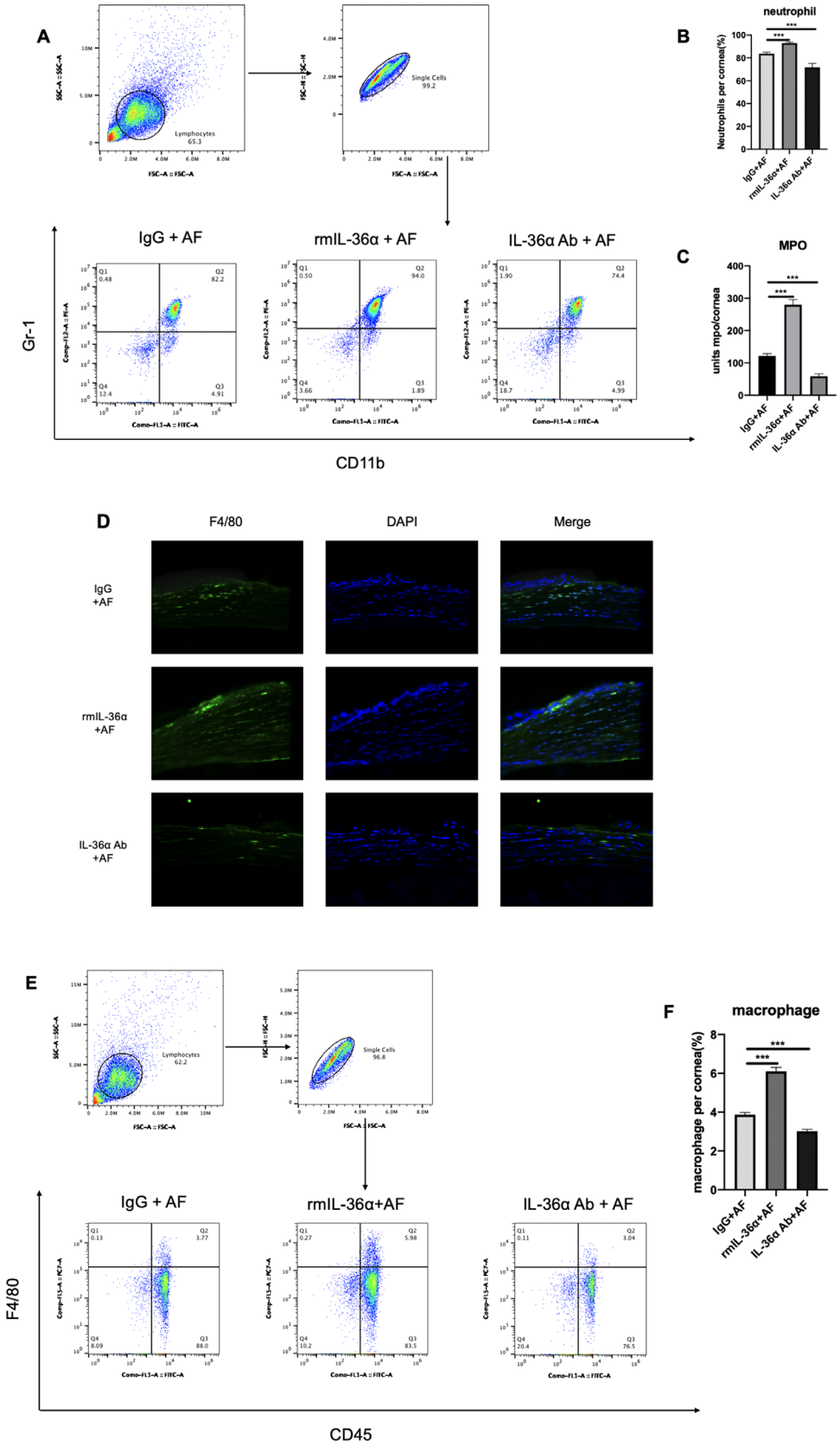

Figure 3. Effect of IL-36α on neutrophils and macrophages infiltration. (**A**, **B**) The number of neutrophils in corneas and (**C**) MPO levels were upregulated in the rmIL-36α-treated group compared with the IgG-treated control and were downregulated in the IL-36α Ab-treated group. (**D**) Immunostaining (macrophages, *green*) and (**E**, **F**) flow cytometry results showed that the number of macrophages was increased after rmIL-36α treatment when compared to the IgG-infected controls and was decreased after IL-36α neutralization. All data are mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Magnification, 400× (**D**).

Investigative Ophthalmology & Visual Science

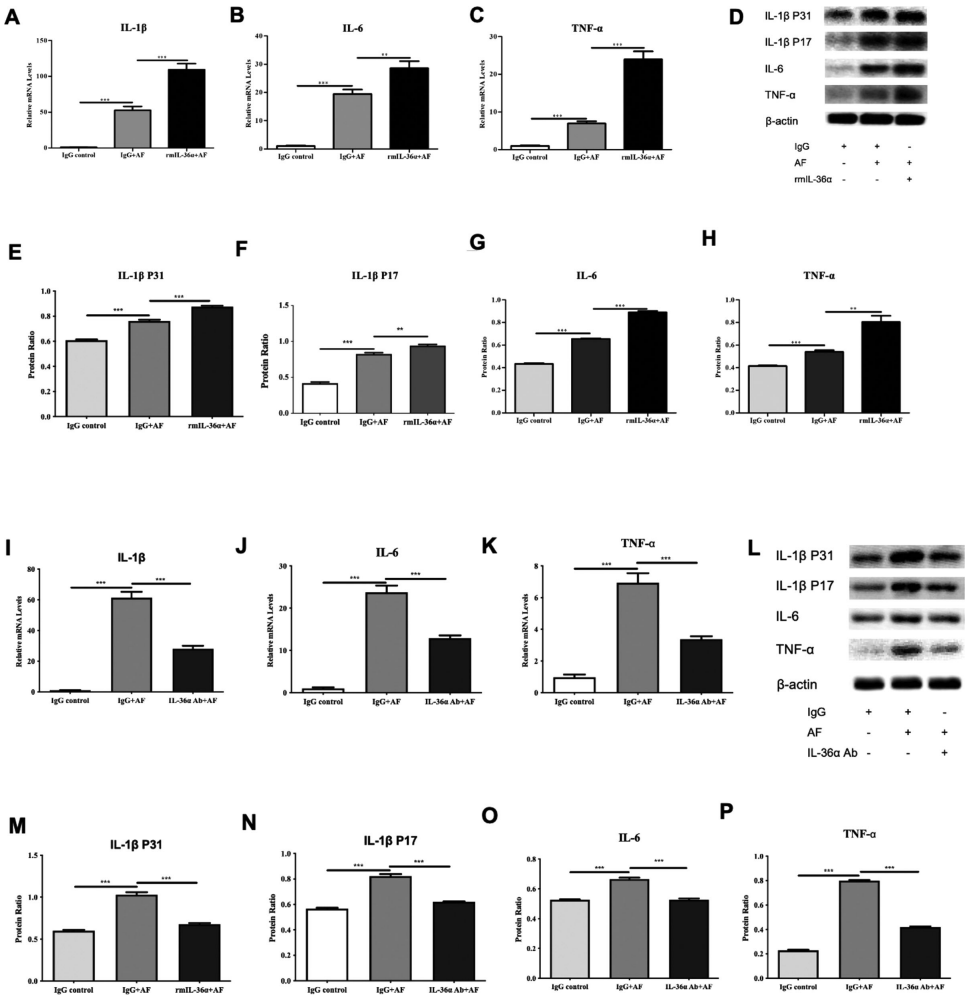

Figure 4. Effect of IL-36α on cytokines (IL-1β, IL-6, and TNF-α) in corneas infected by *A. fumigatus*. IL-1β (**A**, **D**–**F**), IL-6 (**B**, **D**, **G**), and TNF-α (**C**, **D**, **H**) mRNA and protein levels were significantly increased in the rmIL-36α-treated group compared with the IgG-treated control group. IL-36α neutralization decreased the expression of IL-1β (**I**, **L**–**N**), IL-6 (**J**, **L**, **O**), and TNF-α (**K**, **L**, **P**). All data are mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

are consistent with results from a previous study by another laboratory showing that IL-36α was significantly upregulated in skin tissues infected with *Staphylococcus aureus*, and it was then involved in tissue inflammation.[16] To further verify this report, animal models of *A. fumigatus* keratitis were established. Results showed that *A. fumigatus* upregulated IL-36α expression in the mice cornea and that the increased levels of IL-36α were consistent with the severity of corneal inflammation. These data, suggesting that IL-36α plays a potential role in the development of *A. fumigatus* keratitis, are consistent with results in a previous study from another laboratory indicating that IL-36α was induced after infection and correlated with the development of *Legionella pneumophila* pneumonia.[17] This was further confirmed by an in vitro study that reported that the expression of IL-36α was significantly increased in the primary macrophages stimulated with *A. fumigatus* hyphae.

Macrophages, as immunomodulators, play an important role in the phagocytosis of fungal spores, hyphae, and antigen presentation.[18,19] The upregulation of IL-36α induced by *A. fumigatus* suggests that IL-36α may act through macrophages and then participate in the immune regulation process of fungal keratitis. These findings are also consistent with those of a previous study, which reported that IL-36α was produced by pulmonary macrophages upon exposure to *Pseudomonas aeruginosa*.[20] Our in vivo and in vitro data suggest that IL-36α may play an important role in the early stage of fungal keratitis.

In order to fully determine the role of IL-36α in corneal *A. fumigatus* infection, rmIL-36α protein and IL-36α neutralizing antibody were given to C57BL/6 mice. Clinical score and slit-lamp data indicate that the injection of C57BL/6 mice with rmIL-36α protein significantly aggravated disease outcome. The rmIL-36α protein significantly increased the corneal ulcer area, degree of corneal edema, and turbidity; there was much corneal neovascularization in the limbus, and the clinical inflammation score was significantly increased. IL-36α neutralizing antibody reduced disease response and clinical score. These findings suggest that IL-36α acts as a proinflammatory factor in the immune response of fungal keratitis to aggravate the corneal inflammation.

IL-1 is a powerful innate immunity inducer and a proinflammatory mediator in acute and chronic inflammatory responses.[21] IL-36α, as a member of the IL-1 family, plays an important proinflammatory role in the inflammatory

Investigative Ophthalmology & Visual Science

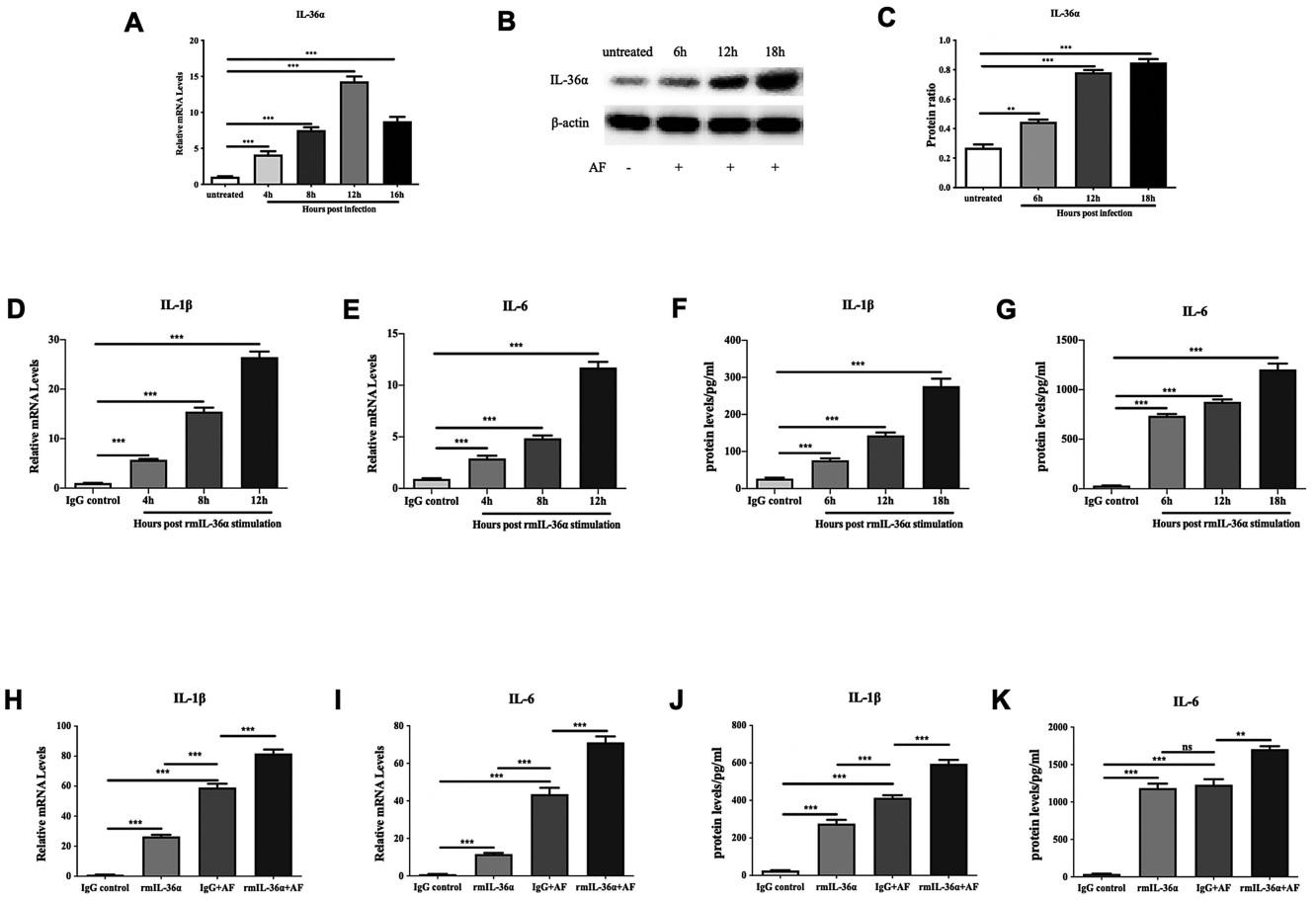

Figure 5. Role of IL-36α in primary macrophages. (**A**) IL-36α mRNA levels were upregulated in the stimulated primary macrophages at 4, 8, 12, and 16 hours compared with untreated cells and peaked at 12 hours. (**B**, **C**) IL-36α protein levels also increased after stimulation by *A. fumigatus* hyphae for 6, 12, and 18 hours, and they also peaked at 18 hours compared with untreated cells. (**D**, **F**) IL-1β and (**E**, **G**) IL-6 mRNA and protein levels were significantly increased after rmIL-36α treatment compared with IgG control cells. rmIL-36α treatment further increased the mRNA and protein levels of IL-1β (**H**, **J**) and IL-6 (**I**, **K**) compared with stimulated control cells. All data are mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

response. IL-36α can regulate the activity of immune cells such as dendritic cells, Th1 cells, and CD4+ T lymphocytes, and it is widely involved in innate and adaptive immune processes.[22] We found that rmIL-36α treatment significantly upregulated MPO levels and the number of neutrophils in corneas compared with the IgG control. Immunostaining and flow cytometry results showed that the number of macrophages was significantly increased after rmIL-36α treatment. IL-36α neutralizing antibody treatment decreased the number of neutrophils and macrophages in corneas. Neutrophils and macrophages are important innate immune cells that can phagocytose pathogens and kill pathogenic microorganisms, present antigens, trigger specific immune responses, and promote the secretion of inflammatory factors.[18,19,23] They are essential in the inflammatory response to corneal *A. fumigatus* infection; however, excessive immune cell infiltration can lead to severe damage to corneal tissue. These findings are consistent with those of a study reporting that intratracheal instillation of recombinant mouse IL-36α induced neutrophil influx in the lungs of wild-type C57BL/6 mice in vivo.[8] Our findings are also consistent with those of another study on inflammatory bowel disease that observed that IL-36α can increase the infiltration of neutrophils and macrophages in the small intestine tissue and then aggravate intestinal damage.[24] Our results provide evidence that IL-36α plays an essential role in the pathogenesis of corneal *A. fumigatus* infection in C57BL/6 mice by inducing infiltration of neutrophils and macrophages.

Further detection of proinflammatory factors in mice cornea infected with *A. fumigatus* after rmIL-36α pretreatment showed that rmIL-36α significantly upregulated IL-1β, IL-6, and TNF-α expression. Previous studies showed that IL-36α regulated the immune response of *A. fumigatus* keratitis in mice by promoting the recruitment of macrophages; however, *A. fumigatus* significantly increased IL-36α expression in primary macrophages. Therefore, we studied the mechanism of IL-36α in *A. fumigatus* infection in primary macrophages and found that rmIL-36α further increased IL-1β and IL-6 expression induced by *A. fumigatus* in macrophages. As is well known, IL-1β and IL-6 are important proinflammatory factors that can activate and recruit immune cells, regulate T-cell and B-cell proliferation and differentiation, and play a key role in the body's immune response. These findings suggest that IL-36α can amplify the inflammatory response by inducing the production of proinflammatory factors and that it plays a proinflammatory role in fungal keratitis. Again, our findings are consistent

Investigative Ophthalmology & Visual Science

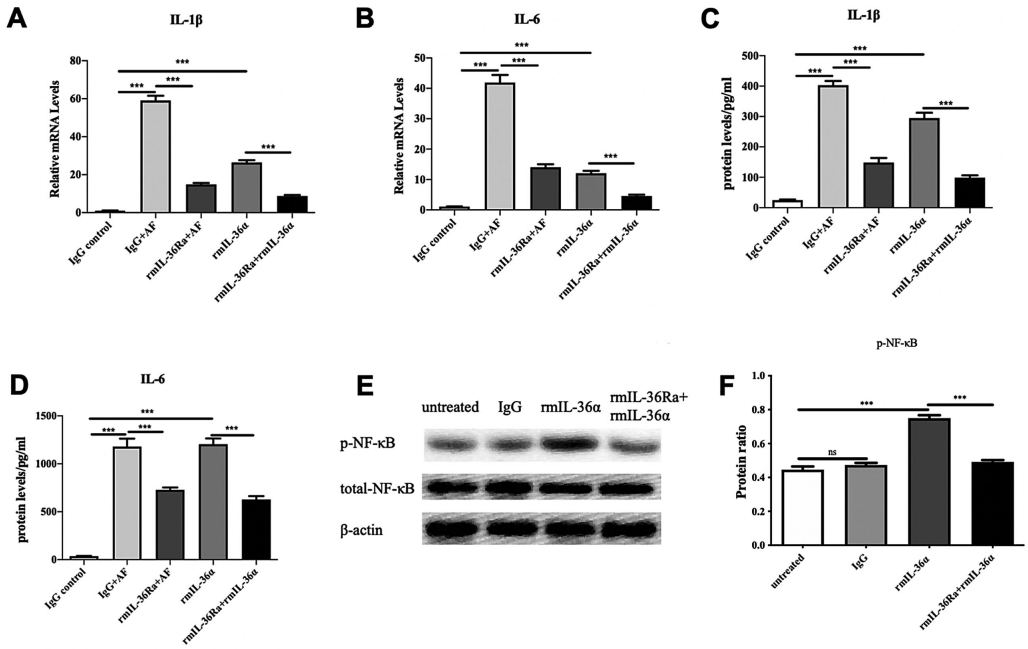

Figure 6. IL-36α regulates cytokines by IL-36R and NF-κB in primary macrophages. rmIL-36Ra treatment decreased the mRNA and protein levels of IL-1β (**A**, **C**) and IL-6 (**B**, **D**) compared with control infected cells or rmIL-36α-treated cells. rmIL-36α treatment markedly increased p-NF-κB protein levels (**E**, **F**), which were lower after rmIL-36Ra pretreatment. All data are mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

with those of another study investigating a murine model of psoriasis which showed that IL-36α is highly expressed in psoriatic lesions and that intradermal injection of IL-36α in mice increased proinflammatory factor IL-1 secretion.[25] IL-1 elevated IL-36α expression, and they promoted each other to produce a cascading amplification reaction that enhanced the proinflammatory effect of IL-36α and promoted the development of skin inflammation. There was a feedback loop between IL-36α and the cytokines induced by IL-36α that continuously aggravated the inflammatory response.

IL-36R is a specific receptor for IL-36α. IL-36α binds to IL-36R and activates downstream signaling pathways to produce a proinflammatory effect, whereas IL-36Ra can compete with IL-36α to bind IL-36R, thus exerting an antiinflammatory effect. In this study, we used exogenous recombinant protein IL-36Ra to block IL-36R and then stimulated primary macrophages with rmIL-36α or *A. fumigatus* to detect the expression of IL-1β and IL-6. Results showed that IL-36Ra significantly inhibited IL-1β and IL-6 expression induced by rmIL-36α or *A. fumigatus*. These findings are consistent with those of a study investigating experimental colitis in which IL-36R ligands induced expression of IL-6 via IL-36R.[26] Moreover, rmIL-36α treatment markedly increased p-NF-κB protein levels, which were lower after rmIL-36Ra pretreatment. The data provided evidence that IL-36α plays a proinflammatory role through IL-36R and NF-κB.

In summary, the data presented here indicate that IL-36α is highly expressed in patients with fungal keratitis and in mice corneas infected with *A. fumigatus*. IL-36α can promote the recruitment of corneal neutrophils and macrophages and induce the production of the inflammatory factors IL-1β, IL-6, and TNF-α to aggravate the inflammatory response of fungal keratitis in mice. Our in vitro data suggest that *A. fumigatus* increased IL-36α expression in primary macrophages. IL-36α upregulated IL-1β and IL-6 through IL-36R and NF-κB. Therefore, IL-36α plays a proinflammatory role in *A. fumigatus* keratitis infection, such that it aggravates corneal inflammation and tissue damage. IL-36α may be a new target for the diagnosis and treatment of fungal keratitis.

Acknowledgments

Supported by grants from the Taishan Scholar Project of Shandong Province (tsqn201909143), Shandong Province Key Research and Development Plan (2019GSF107022), and Youth Project of National Natural Science Foundation of China (81800800).

Disclosure: **Y. Niu,** None; **C. Ren,** None; **X. Peng,** None; **C. Li,** None; **Q. Xu,** None; **L. Hu,** None; **Z. Zhang,** None; **G. Zhao,** None; **J. Lin,** None

References

1. Austin A, Lietman T, Rose-Nussbaumer J. Update on the management of infectious keratitis. *Ophthalmology.* 2017;124(11):1678–1689.
2. Sahay P, Singhal D, Nagpal R, et al. Pharmacologic therapy of mycotic keratitis. *Surv Ophthalmol.* 2019;64(3):380–400.
3. Heinekamp T, Schmidt H, Lapp K, Pähtz V, Brakhage AA. Interference of *Aspergillus fumigatus* with the immune response. *Semin Immunopathol.* 2014;37(2):141–152.
4. Ding L, Wang X, Hong X, Lu L, Liu D. IL-36 cytokines in autoimmunity and inflammatory disease. *Oncotarget.* 2018;9(2):2895–2901.
5. Yuan ZC, Xu WD, Liu XY, Liu XY, Su LC. Biology of IL-36 signaling and its role in systemic inflammatory diseases. *Front Immunol.* 2019;10:2532.
6. Zhang J, Yin Y, Lin X, et al. IL-36 induces cytokine IL-6 and chemokine CXCL8 expression in human lung tissue

cells: implications for pulmonary inflammatory responses. *Cytokine.* 2017;99:114–123.

7. Frey S, Derer AD, Messbacher M-E, et al. The novel cytokine interleukin-36α is expressed in psoriatic and rheumatoid arthritis synovium. *Ann Rheum Dis.* 2013;72(9):1569–1574.
8. Ramadas RA, Ewart SL, Iwakura Y, Medoff BD, Levine AM, Ryffel B. IL-36α exerts pro-inflammatory effects in the lungs of mice. *PLoS One.* 2012;7(9):e45784.
9. Gao N, Me R, Dai C, Seyoum B, Yu F-SX. Opposing effects of IL-1Ra and IL-36Ra on innate immune response to *Pseudomonas aeruginosa* infection in C57BL/6 mouse corneas. *J Immunol.* 2018;201(2):688–699.
10. Niu Y, Zhao G, Cui L, et al. *Aspergillus fumigatus* increased PAR-2 expression and elevated proinflammatory cytokines expression through the pathway of PAR-2/ERK1/2 in cornea. *Invest Ophthalmol Vis Sci.* 2018;59(1):166–175.
11. Bassoy EY, Towne JE, Gabay C. Regulation and function of interleukin-36 cytokines. *Immunol Rev.* 2018;281(1):169–178.
12. Buhl AL, Wenzel J. Interleukin-36 in infectious and inflammatory skin diseases. *Front Immunol.* 2019;10:1162.
13. Jing L, Rui X, Hu LT, et al. Interleukin-32 induced thymic stromal lymphopoietin plays a critical role in the inflammatory response in human corneal epithelium. *Cell Signal.* 2018;49:39–45.
14. Li C, Zhao G, Che C, et al. The role of LOX-1 in innate immunity to *Aspergillus fumigatus* in corneal epithelial cells. *Invest Ophthalmol Vis Sci.* 2015;56(6):3593–3603.
15. Bourcier T, Sauer A, Dory A, Denis J, Sabou M. Fungal keratitis. *J Fr Ophtalmol.* 2017;40(9):e307–e313.
16. Liu H, Archer NK, Dillen CA, Wang Y, Miller LS. *Staphylococcus aureus* epicutaneous exposure drives skin inflammation via IL-36-mediated T cell responses. *Cell Host Microbe.* 2017;22(5):653–666.e5.
17. Nanjo Y, Newstead MW, Aoyagi X, et al. Overlapping roles for IL-36 cytokines in protective host defense against murine *Legionella pneumophila* pneumonia. *Infect Immun.* 2018;87(1):1–14.
18. Benoit M, Desnues B, Mege JL. Macrophage polarization in bacterial infections. *J Immunol.* 2008;181(6):3733–3739.
19. Chinnery HR, McMenamin PG, Dando SJ. Macrophage physiology in the eye. *Pflügers Arch.* 2017;469(3–4):501–515.
20. Tetsuji A, Newstead MW, Zeng X, et al. Interleukin-36γ and IL-36 receptor signaling mediate impaired host immunity and lung injury in cytotoxic *Pseudomonas aeruginosa* pulmonary infection: role of prostaglandin E2. *PLoS Pathog.* 2017;13(11):e1006737.
21. Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol Rev.* 2018;281(1):8–27.
22. Wißbrock A, Goradia NB, Kumar A, George AAP, Imhof D. Structural insights into heme binding to IL-36α proinflammatory cytokine. *Sci Rep.* 2019;9(1):16893.
23. Gazendam RP, van de Geer A, Roos D, van den Berg TK, Kuijpers TW. How neutrophils kill fungi. *Immunol Rev.* 2016;273(1):299–311.
24. Russell SE, Horan RM, Stefanska AM, et al. IL-36α expression is elevated in ulcerative colitis and promotes colonic inflammation. *Mucosal Immunol.* 2016;9(5):1193–1204.
25. Müller A, Hennig A, Lorscheid S, et al. IκBζ is a key transcriptional regulator of IL-36-driven psoriasis-related gene expression in keratinocytes. *Proc Natl Acad Sci USA.* 2018;115(40):10088–10093.
26. Scheibe K, Backert I, Wirtz S, et al. IL-36R signalling activates intestinal epithelial cells and fibroblasts and promotes mucosal healing in vivo. *Gut.* 2016;66(5):823–838.

Investigative Ophthalmology & Visual Science