



The role of netrin-1 in the mouse cornea during *Aspergillus fumigatus* infection



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ABSTRACT

Purpose: To explore the effects of netrin-1 on inflammation in *Aspergillus fumigatus*-infected mouse corneas and on proliferation and migration in human corneal epithelial cells (HCECs).

Methods: Netrin-1 and the receptor A2BAR were detected in normal and infected corneas from C57BL/6 mice and RAW 264.7 cells. The mice were injected subconjunctivally with recombinant netrin-1. The severity of the disease was determined by clinical scores, photography with a slit lamp, RT-PCR, western blotting, myeloperoxidase (MPO) assays and immunofluorescence staining of polymorphonuclear neutrophilic leukocytes (PMNs). The effects of netrin-1 on RAW 264.7 cells in vitro were determined by RT-PCR. The role of A2BAR was demonstrated in vivo by detecting the expression of IL-1 β , TNF- α , and IL-10 in corneas pretreated subconjunctivally with an A2BAR antagonist (PSB1115). RAW 264.7 cells were stimulated with *Aspergillus fumigatus* (*A. fumigatus*) and netrin-1 with or without PSB1115 pretreatment. A cell counting kit-8 (CCK-8) assay was used to evaluate cell proliferation ability, and cell migration ability was determined by cell scratch experiments with HCECs.

Results: Netrin-1 expression decreased slightly after *A. fumigatus* infection and then increased to its peak. A2BAR expression increased at 1 day post infection (p.i.), with a subsequent decline. Compared to the PBS control, exogenous netrin-1 attenuated the inflammatory response, PMN infiltration, and expression of the proinflammatory factors IL-1 β and TNF- α , while IL-10 expression was up-regulated. In RAW 264.7 cells, recombinant netrin-1 obviously inhibited the mRNA expression of IL-1 β and TNF- α and promoted the mRNA expression of the anti-inflammatory cytokine IL-10. Pretreatment with PSB1115 resulted in disease aggravation and higher levels of the proinflammatory factors IL-1 β and TNF- α both in vivo and in vitro. And the effect of netrin-1 on inflammatory factors was abolished by PSB1115. Moreover, compared to the control treatment, exogenous netrin-1 significantly facilitated the proliferation and migration of HCECs.

Conclusions: Netrin-1 attenuates inflammation in C57BL/6 mice infected with *A. fumigatus*, and it may play this role via the receptor A2BAR. Additionally, netrin-1 can promote the proliferation and migration of HCECs.

1. Introduction

Fungal keratitis (FK) is a severe infectious disease of the cornea that can cause significant loss of vision [1]. Because of an increased frequency of ocular trauma, especially agricultural trauma, long-term use of contact lenses, extended antibiotic use, and excessive use of corticosteroids, an increasing number of people suffer from this disease [2]. Unlike most other nonpathogenic *Aspergillus* species, *A. fumigatus* has an arsenal of virulence determinants that generally cause a strong host

immune response [3]. During *A. fumigatus* infection in the cornea, inflammatory reactions include vasodilation, secretion of immune active substances, and exudation of active immune cells, including PMNs, macrophages and lymphocytes. Even if the harmful agent is removed, excessive inflammatory reactions can result in serious structural injuries and subsequent functional damage [4].

Netrin-1 is a member of the netrin family and was one of the soluble proteins reported to be secreted by the cell floor; this protein was initially described as a guidance cue that influences axonal migration

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during central nervous system (CNS) development. Netrin-1 has been identified as a novel anti-inflammatory factor that protects tissue cells during inflammatory pathology [5]. Except for high expression levels in the nervous system, netrin-1 is also widely expressed in other tissues, such as the lung, pancreas, breast, and intestinal epithelium [6]. It has been reported that netrin-1 can reduce the damage caused by ischemia-reperfusion injury in the kidney [7]. In addition, netrin-1 was recently found to protect the lung against acute injury [8]. The netrin-1 receptor family includes deleted in colorectal cancer (DCC), neogenin and UNC5 homologs [9]. The adenosine 2b receptor (A2BAR) acts as a novel receptor for netrin-1 and plays an important role in the anti-inflammatory effects of netrin-1 [10]. In inflammatory peritonitis, the effect of netrin-1 on the migration of PMNs is dependent on A2BAR [11]. Blocking UNC5B with specific antibodies does not affect the ability of netrin-1 to inhibit the migration of PMNs across epithelial cells [12]. In alkali burns in the cornea, a previous study confirmed that netrin-1 can alleviate inflammation-related injury [13]. Moreover, netrin-1 not only has an inhibitory effect on inflammation but also has the ability to regulate the migration of epithelial cells in various tissues, such as the pancreas and mammary gland [14]. A recent study in the cornea showed that netrin-1 promotes the proliferation and migration of mouse corneal epithelial cells impaired by hyperglycemia and promotes the healing of diabetic corneal wounds via A2BAR [15].

It is important to adequately regulate the inflammatory response because this response may result in considerable tissue damage. The corneal epithelium is the first line of defense for corneal immunity [16]. It is not known whether netrin-1 is involved in *A. fumigatus* keratitis or whether it affects the proliferation and migration of HCECs. We found that netrin-1 and the receptor A2BAR were both expressed during *A. fumigatus* keratitis. Netrin-1 suppresses inflammation in C57BL/6 mice infected with *A. fumigatus*, and it may play this role via the receptor A2BAR. Moreover, netrin-1 can promote the proliferation and migration of HCECs. Our research indicates that netrin-1 is an important regulator of inflammation in the cornea, and targeting netrin-1 signaling could represent an effective treatment for *A. fumigatus* keratitis.

2. Materials and methods

2.1. Animals and corneal infection

All animals were C57BL/6 mice (female, eight weeks old), which were provided by Jinan Pengyue Laboratory Animal Co. LTD. (Jinan, China). All treatments were administered to the mice humanely and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. All corneas were separately inspected under a slit lamp microscope before use in experiments. The mice were anesthetized with 0.08 ml of 8% chloral hydrate. Then, the mice were placed under a stereomicroscope (40× magnification), and a wound 2 mm in diameter was scraped from the center of the corneal epithelium. A 5- μ L aliquot containing 10^8 CFU/ml *A. fumigatus* (Department of Clinical Laboratory, Affiliated Hospital of Qingdao University, Qingdao, China) was applied to the corneal surface. A soft contact lens was then used to cover the surface of the eye, and the eyelids were sutured. The mouse corneas were separated for RT-PCR and western blotting at 1, 3, and 5 days postinfection (p.i.). The eyeballs were removed at 1, 3, and 5 days p.i. for immunofluorescence staining.

2.2. Netrin-1 treatment

Netrin-1 (4 μ g/ml; R&D Systems, Minneapolis, MN) was administered to the left eyes of C57BL/6 mice (n = 6/group/time point) by subconjunctival injection 1 day before infection and 1 day p.i. Mice pretreated with phosphate-buffered saline (PBS) served as the control group. Mouse corneas were harvested for PCR and western blotting at 1, 2, and 3 days p.i. Eyeballs were harvested at 2 days p.i. and 3 days p.i. for myeloperoxidase (MPO) assays and immunofluorescence staining.

RAW 264.7 cells were pretreated with netrin-1 (12.5 ng/ml; R&D Systems) for 30 min before stimulation with *A. fumigatus* hyphae at a final concentration of 5×10^6 CFU/ml for 8 h. Cells pretreated with PBS served as the control group. The cells were harvested to detect mRNA levels of IL-1 β , TNF- α and IL-10 by PCR.

2.3. A2BAR antagonist treatment

The A2BAR antagonist PSB1115 (100 nM; R&D Systems) was administered to the left eyes of C57BL/6 mice (n = 6/group/time point) by subconjunctival injection 1 day before infection. The control group was pretreated with PSB1115 1 day before infection, then injected with netrin-1 (4 μ g/ml) subconjunctivally at 0 h before infection. The mouse corneas were harvested for PCR at 2 days p.i.

RAW 264.7 cells were pretreated with PSB1115 (25 nM; R&D Systems) for 1 h before stimulation for 8 h. The control group was pretreated with PSB1115 1 h before infection, then injected with netrin-1 (12.5 ng/ml) subconjunctivally at 0 h before infection. The cells were used to detect the mRNA levels of IL-1 β , TNF- α and IL-10 by PCR.

2.4. RAW 264.7 cell culture and *A. fumigatus* stimulation

RAW 264.7 cells were purchased from the Shanghai Chinese Academy of Sciences (Shanghai, China). The culture medium was high-glucose DMEM supplemented with 12% FBS and 1% penicillin and streptomycin. A 1×10^5 /ml cell suspension was inoculated into 6-well or 12-well plates and grown to 80% confluence. The cells were incubated with *A. fumigatus* hyphae in 12-well plates or 6-well plates. Netrin-1 mRNA and protein levels in RAW 264.7 cells were detected by PCR and western blotting after 0, 4, 8 and 12 h of stimulation. A2BAR mRNA levels were determined by PCR at 0, 2, 4, 6, 8 and 10 h.

2.5. Real-time RT-PCR

Total RNA from corneal tissue and cells was isolated by the RNAiso plus reagent and quantified by spectrophotometry. A reverse transcription system was used for first-strand cDNA synthesis from the RNA (2 μ g). Diethylpyrocarbonate-treated water was used to dilute the cDNA products. PCR was performed in Real-Time PCR Master Mix (Takara, Dalian, China). The cycle parameters used for the reactions were as follows: 95 °C for 30 min, followed by 40 cycles of 95 °C for 5 min and 60 °C for 30 min, with final steps at 95 °C for 15 min, 60 °C for 30 min, and 95 °C for 15 min. The housekeeping gene β -actin was used as a control. The primers used in this study are shown in Table 1.

2.6. Western blot analysis

Protein was extracted from corneas or RAW 264.7 cells in RIPA buffer (Solarbio, Beijing, China) with 1 mM phenylmethanesulfonyl fluoride (PMSF) (Solarbio) for 2 h, and the extracts were centrifuged at

Table 1
Nucleotide sequences of mouse primers for real-time RT-PCR.

Gene	GenBank no.	Primer sequence (5'–3')
mNTN-1	NM_008744.2	F:AAGCCTATCACCCACCGGAG R:GCGCCACAGGAATCTTGATGC
mAdora2b	NM_007413.4	F:GGGGTGAACAGTAAAGACAG R:TATGAGCAGTGGAGGAAGGACAC
mIL-1 β	NM_008361.3	F:CGCAGCAGCACATCAACAAGAGC R:TGTCCTCATCCTGGAAGGTCCACG
mTNF- α	NM_013693.2	F:ACCCTCACACTCAGATCATCTT R:GGTTGCTTTGAGATCCATGC
mIL-10	NM_010548.2	F:TGCTAACCGACTCCTTAATGCAGGAC R:CCTTGATTTCTGGGCCATGCTTCTC
m β -Actin	NM_007393.5	F:GATTACTGCTCTGGCTCCTAGC R:GACTCATCGTACTCTGCTTGC

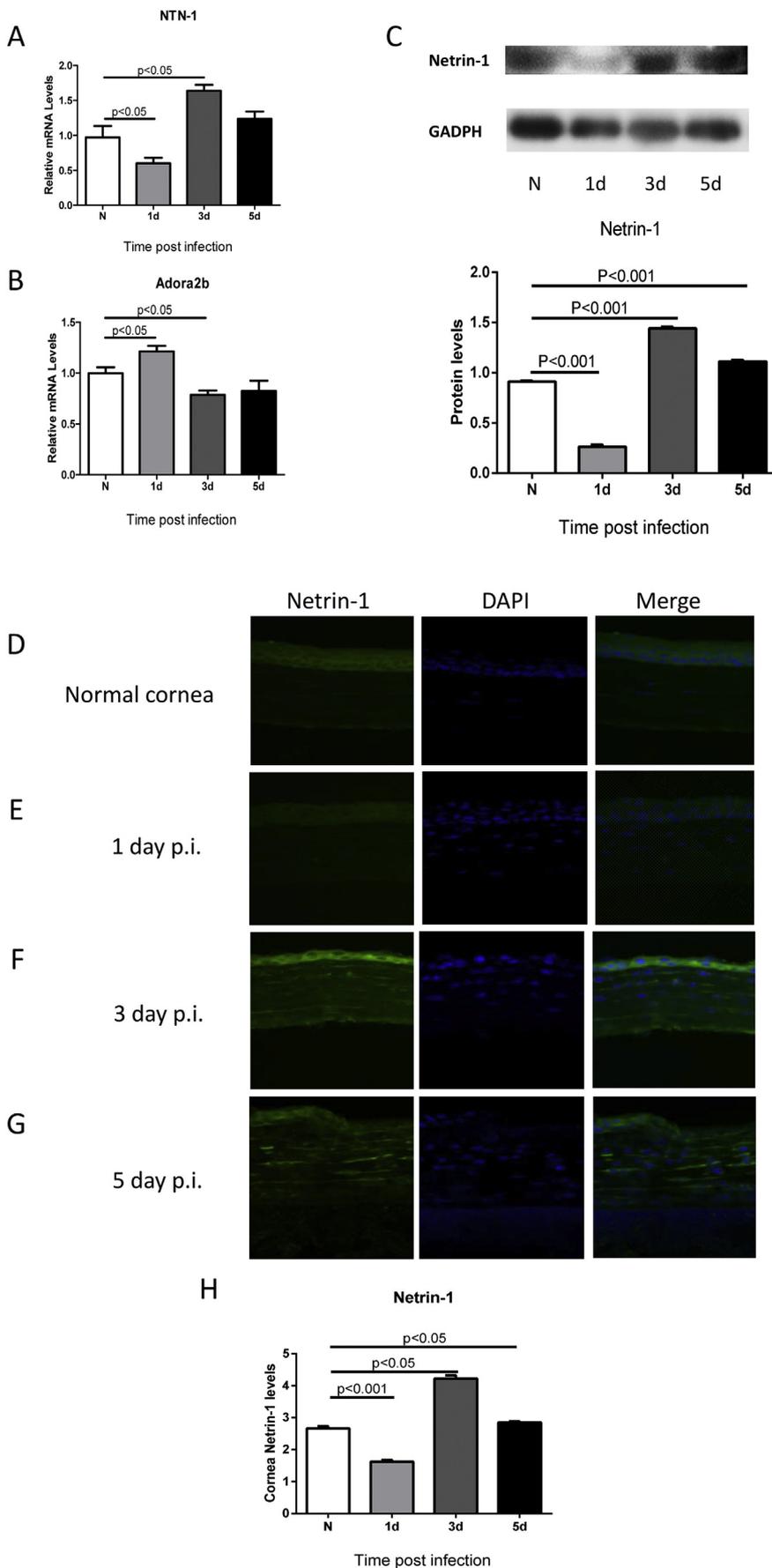


Fig. 1. Expression of netrin-1 and its receptor A2BAR in the cornea in C57BL/6 mice. Gene expression of netrin-1 (A) decreased slightly at 1 day p.i. and then increased, with a peak at 3 days p.i. A2BAR gene expression (B) increased at 1 day p.i. and decreased slightly at 3 days p.i. The protein levels of netrin-1 (C) displayed the same trend observed for mRNA levels. Immunofluorescence staining (D–G) showed that the netrin-1 protein was predominantly expressed in the epithelium of normal mouse corneas. The expression levels in the corneal epithelium and stroma were both increased 3 days after *A. fumigatus* infection. Magnification (D–G): 400×. (H) Quantitative analysis of netrin-1 levels in the entire cornea. The fluorescent intensities of netrin-1 in cornea were quantified using ImageJ. All data are mean ± SEM and were analyzed by an unpaired, two-tailed Student's *t*-test (*n* = 6/group/isolate).

4 °C and 12,000 rpm for 15 min. The protein concentration was quantified using the BCA protein assay reagent (Solarbio). SDS sample buffer was added to the protein and boiled for 10 min. Then, the proteins were separated by SDS-PAGE and electroblotted onto PVDF membranes (Solarbio). After blocking the membranes with blocking buffer (Solarbio) at room temperature for 1.5 h, the membranes were incubated with primary antibodies specific for GAPDH (1:3000; Elabscience, Wuhan, China), netrin-1 (1:1000; R&D Systems), IL-1 β (1:3000; R&D Systems) or TNF- α (1:2000, CST, Boston, USA) or IL-10 (1:1000, ABClonal, Wuhan, China) at 4 °C overnight. After washing three times, the membranes were incubated with an anti-rabbit secondary antibody (Elabscience) or an anti-goat secondary antibody (Elabscience) for 1.5 h. Then, the blots were visualized with ECL (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Quantification of corneal PMNs

According to the manufacturer's instructions, corneas (n = 6/group/time point) were removed at 2 days p.i. and 3 days p.i. Then, the corneas were placed in 1.0 ml of the second agent from the MPO test kit (Njjcbio, Nanjing, China). Changes were immediately monitored by detecting the absorbance at 460 nm. The slope of the line was used to calculate the units of myeloperoxidase (MPO) activity in each cornea.

2.8. Immunofluorescence staining

Eyeballs were removed from C57BL/6 mice, placed in optimum cutting temperature (OCT) compound (Sakura Tissue-Tek, Torrance, CA, USA) and then frozen with liquid nitrogen. The frozen eyeballs were cut into 10-mm sections, which were placed on a glass slide. After storage at 37 °C for 6 h, the samples were fixed with acetone for 5 min. After cleaning, the slides were blocked with blocking solution at room temperature for 30 min. The blocking solution contained PBS with 10% serum. After removing the blocking solution, the sections were incubated overnight at 4 °C with an anti-netrin-1 antibody (1:500; Abcam, Cambridge, UK) or anti-NIMP-R14 antibody (1:300; Santa Cruz, CA, USA). Then, the slides were incubated with a FITC-conjugated donkey anti-rabbit secondary antibody (1:200; CWBiotech, Wuhan, China) or a Cy3-conjugated goat anti-rat secondary antibody (1:300; CWBiotech) in the dark at room temperature for 1 h. Finally, an anti-fade reagent was used to mount the slides, and images were captured with a Zeiss Axiovert microscope.

2.9. Cell counting kit-8 (CCK-8) assay

An HCEC suspension was seeded in 96-well plates at a density of 3 to 6 \times 10³ cells per well in 200 μ l per well. Netrin-1 was added to the medium at final concentrations of 0, 20, 50 and 80 μ g/ml. Each condition was repeated 6 times. After culturing in a cell culture incubator for 24 h, 48 h and 72 h, the plates were removed, and 10 μ l of CCK-8 (MCE, New Jersey, America) was added to each well. The cells were incubated for an additional 2 h. The optical density (OD) of each well was measured with an enzyme-linked immunosorbent assay (ELISA) at 450 nm.

2.10. Cell scratch test

HCECs were collected and seeded in a 6-well plate. Netrin-1 was added to the medium at concentrations of 0, 20, 50 and 80 μ g/ml. Three parallel lines were drawn on the bottom of the plate with a black marker, and three lines perpendicular to the black line were drawn in each well using a 200- μ l pipette tip. The cells were cultured for 24 h, and the widths of the scratches were measured. The following formula was used for calculations: {(scratch width at 0 h – scratch width at 24 h) / scratch width at 0 h} \times 100%. The experiment was repeated 3 times under the same conditions.

2.11. Statistical analysis

For comparisons of differences between two groups, a two-tailed Student's *t*-test (GraphPad Prism) was used to determine significance. One-way ANOVA followed by Bonferroni's multiple comparison test (GraphPad Prism) was used to analyze three or more groups. *P* < 0.05 was considered significant, and the data are represented as the mean \pm SEM.

3. Results

3.1. Expression of netrin-1 and the receptor A2BAR in corneas of C57BL/6 mice

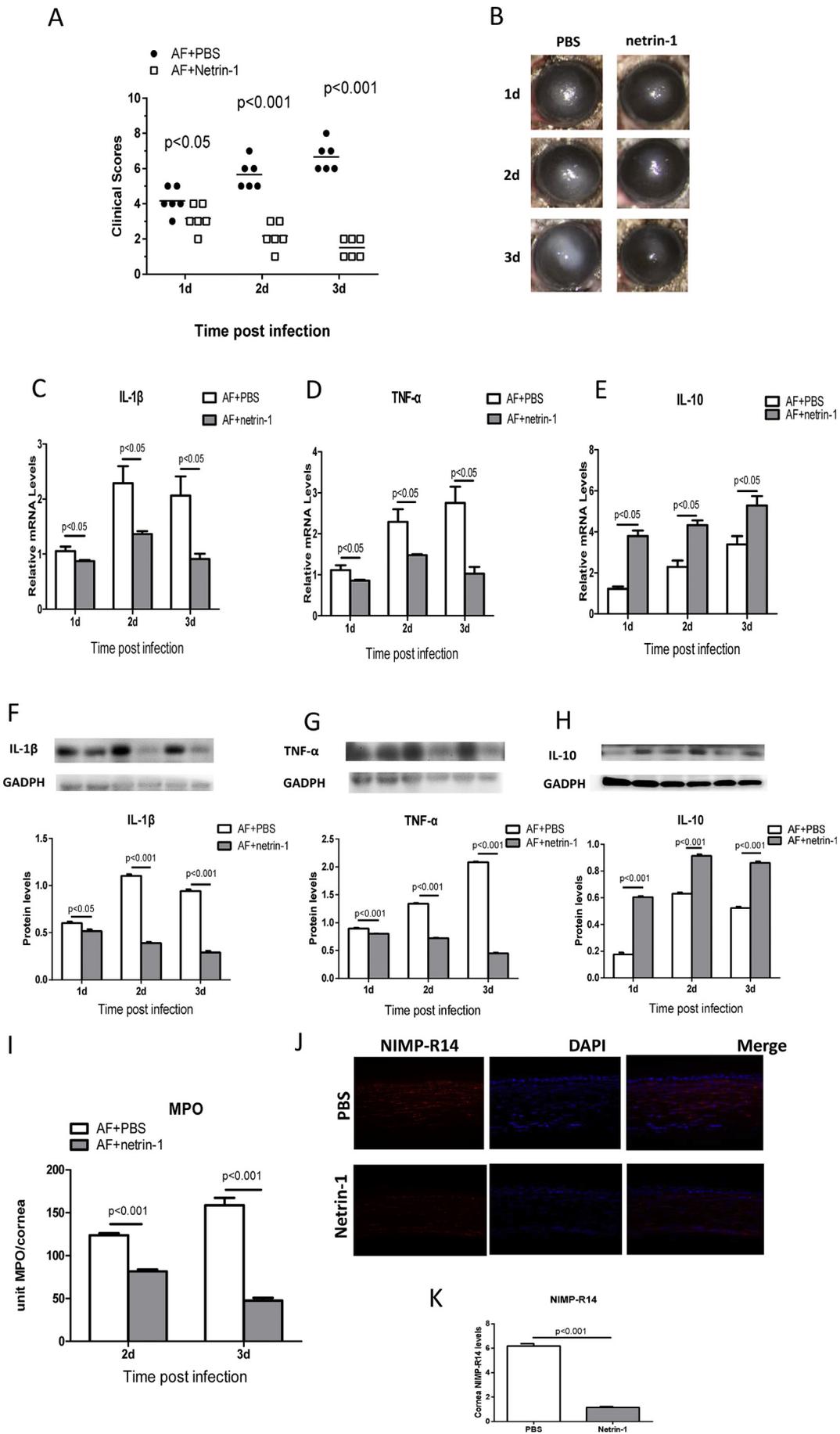
To examine the expression of netrin-1 and the receptor A2BAR in mice with *A. fumigatus* keratitis, we performed RT-PCR on normal uninfected and infected C57BL/6 corneas. Gene expression of netrin-1 decreased slightly at 1 day p.i. and then increased, with a peak at 3 days p.i. (Fig. 1A; *p* < 0.05 and *p* < 0.05, respectively). A2BAR gene expression increased at 1 day p.i. and decreased slightly at 3 days p.i. (Fig. 1B; *p* < 0.05 and *p* < 0.05, respectively). Next, to further detect the protein expression of netrin-1, we performed western blot analysis. As shown in Fig. 1C, the protein levels displayed the same trend observed for mRNA levels (all *p* < 0.001). Moreover, immunofluorescence staining showed that the netrin-1 protein was predominantly expressed in the epithelium of the normal mouse cornea (Fig. 1D). Expression levels in the corneal epithelium and stroma were both increased significantly 3 days after infection with *A. fumigatus* (Fig. 1F). Netrin-1 levels analysis in the cornea was consistent with the immunofluorescent results (Fig. 1H).

3.2. Effect of recombinant netrin-1 on inflammation in C57BL/6 mouse corneas infected with *A. fumigatus*

To determine whether netrin-1 affected inflammation in *A. fumigatus* keratitis, we took photographs of mouse corneas by a slit lamp at 1, 2 and 3 days p.i. (Fig. 2B). Clinical scores were significantly different between the PBS group and the netrin-1 group at 1 day p.i., 2 days p.i. and 3 days p.i. (Fig. 2A; *p* < 0.05, *p* < 0.001, and *p* < 0.001, respectively). Next, we tested the effect of netrin-1 treatment on cytokine expression after infection in C57BL/6 mice. RT-PCR and western blotting were used to examine the mRNA and protein levels of cytokines. The results showed that netrin-1 significantly reduced mRNA levels of IL-1 β and TNF- α induced by *A. fumigatus* (Fig. 2C, D; all *p* < 0.05). Moreover, western blotting showed that after netrin-1 treatment, IL-1 β (Fig. 2F; *p* < 0.05, *p* < 0.001, and *p* < 0.001) and TNF- α (Fig. 2G; all *p* < 0.001) protein levels were also decreased compared to those of the PBS control group at 1 day p.i., 2 days p.i. and 3 days p.i., respectively. With respect to anti-inflammatory factors, mRNA (Fig. 2E; all *p* < 0.05) and protein levels (Fig. 2H; all *p* < 0.001) of IL-10 were both increased in the netrin-1 group compared to those in the PBS group. Furthermore, MPO levels were significantly reduced compared to those in the PBS control after exogenous netrin-1 treatment at 2 and 3 days p.i. (Fig. 2I; *p* < 0.001). To detect the changes in PMN numbers after netrin-1 treatment, mouse cornea sections were used for immunostaining. After netrin-1 treatment, the number of PMNs (red) was markedly reduced compared to that of PBS control corneas at 2 days p.i. (Fig. 2J). PMNs numbers analysis in the cornea was consistent with the immunofluorescent results (Fig. 2K, *p* < 0.001). These results suggested that exogenous netrin-1 promoted the resolution of inflammation during *A. fumigatus* keratitis.

3.3. Effect of recombinant netrin-1 on RAW 264.7 cells

Compared to those of normal control cells, netrin-1 mRNA (Fig. 3A) and protein levels (Fig. 3B) were both decreased in RAW 264.7



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Fig. 2. Effect of recombinant netrin-1 on inflammation in C57BL/6 mouse corneas infected with *A. fumigatus*. The clinical score (A) was significantly lower in the netrin-1-treated group than in the PBS group. Netrin-1 significantly reduced the mRNA levels of IL-1 β (C) and TNF- α (D) induced by *A. fumigatus*. The protein levels of IL-1 β (F) and TNF- α (G) were decreased compared to those of the PBS control, mRNA (E) and protein levels (H) of IL-10 were both increased. Netrin-1 significantly reduced MPO levels compared to those of the PBS group (I). After netrin-1 treatment, the number of PMNs (red) was markedly downregulated compared to that in PBS control corneas (J). Magnification (J): 400 \times . (K) Quantitative analysis of PMNs levels in the entire cornea. The fluorescent intensities of PMNs in cornea were quantified using ImageJ. All data are mean \pm SEM and were analyzed by an unpaired, two-tailed Student's *t*-test ($n = 6/\text{group/isolate}$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

macrophages stimulated with 75% ethanol-killed *A. fumigatus* at 4 h ($p < 0.05$ and $p < 0.001$, respectively) and increased at 8 h ($p < 0.05$ and $p < 0.001$, respectively). Compared to those in the normal control cells, A2BAR mRNA levels were upregulated in the stimulated RAW 264.7 cells at 2 h and peaked at 4 h (Fig. 3C; $p < 0.001$ and $p < 0.001$, respectively). Next, we detected the effect of netrin-1 on inflammatory cytokines induced by *A. fumigatus* hyphae in RAW 264.7 cells. RT-PCR data showed that compared to the PBS control, recombinant netrin-1 inhibited IL-1 β (Fig. 3D; $p < 0.001$) and TNF- α (Fig. 3E; $p < 0.05$) production, and mRNA levels of the anti-inflammatory mediator IL-10 (Fig. 3F; $p < 0.05$) were upregulated in netrin-1-treated cells.

3.4. Effect of netrin-1 on inflammation may through the receptor A2BAR in C57BL/6 mice and RAW 264.7 cells

To further demonstrate whether netrin-1 exerts anti-inflammatory effects through A2BAR, the netrin-1 was injected subconjunctivally in infected mice pretreated with A2BAR antagonist PSB1115. Pretreatment with the A2BAR antagonist PSB1115 alone increased the clinical scores (Fig. 4A; $p < 0.001$). Photographs demonstrated that

compared to the AF control, PSB1115 pretreatment induced opacity at 2 day p.i. (Fig. 4A). Moreover, treatment with netrin-1 in PSB1115-pretreated mice increased the clinical scores of the mice at 2 day p.i. compared to mice treated with netrin-1 alone (Fig. 4A; $p < 0.001$). Next, we examined the effect of PSB1115 pretreatment on the expression of inflammatory cytokines. Our results showed that PSB1115 significantly increased mRNA levels of IL-1 β (Fig. 4B; $p < 0.05$) and TNF- α (Fig. 4C; $p < 0.001$) after exposure to *A. fumigatus* in mouse corneas compared to control group. In addition, the level of IL-10 (Fig. 4D; $p < 0.001$) obviously decreased. Pretreatment with PSB1115 abolished the regulatory effects of netrin-1 on inflammatory cytokines. Netrin-1 markedly downregulated the mRNA expressions of IL-1 β (Fig. 4B, $p < 0.05$) and TNF- α (Fig. 4C, $p < 0.05$) but was blocked by PSB1115 significantly (Fig. 4B, $p < 0.05$; Fig. 4C, $p < 0.05$). Moreover, Netrin-1 administration elevated the IL-10 (Fig. 4D, $p < 0.001$) level, which was suppressed by PSB1115 (Fig. 4D, $p < 0.001$). In RAW 264.7 cells, compared to that in Af group, expression of the inflammatory factor IL-1 β (Fig. 4E, $p < 0.001$) and TNF- α (Fig. 4F, $p < 0.001$) was increased after the pretreatment with PSB1115 inhibitor, and the anti-inflammatory factor IL-10 (Fig. 4G, $p < 0.001$) was decreased. The effect of netrin-1 on inflammatory factors was abolished by PSB1115.

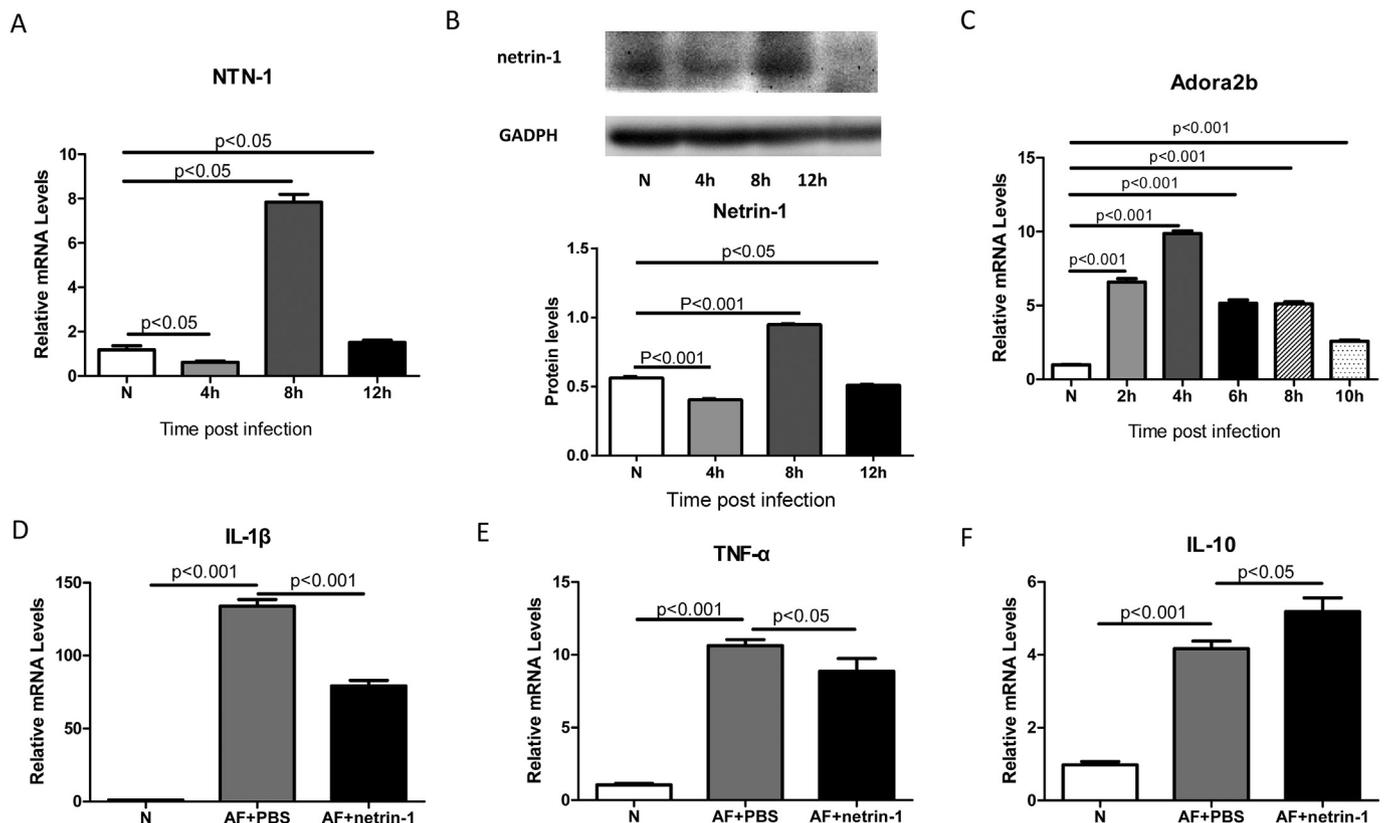
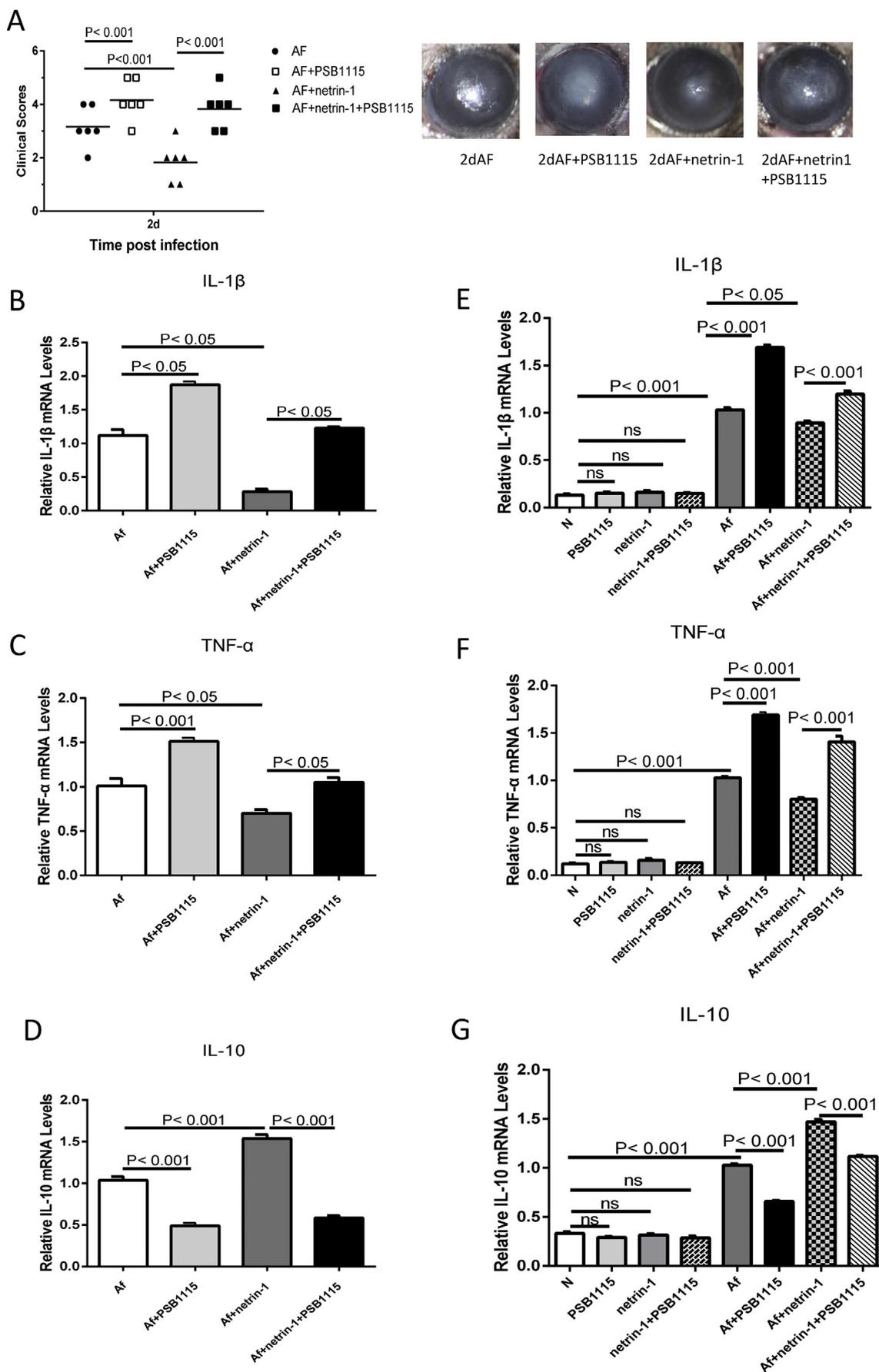


Fig. 3. Expression of netrin-1 and its receptor A2BAR and the effect of recombinant netrin-1 on RAW 264.7 cells. Compared to those of normal control cells, netrin-1 mRNA and protein levels were both decreased in RAW 264.7 macrophages at 4 h after stimulation with 75% ethanol-killed *A. fumigatus* and increased at 8 h (A, B). Compared to those of normal control cells, A2BAR mRNA levels (C) were upregulated at 2 h and peaked at 4 h. Recombinant netrin-1 inhibited IL-1 β (D) and TNF- α (E) production compared to that in infected control cells, and mRNA levels of the anti-inflammatory mediator IL-10 (F) were upregulated in netrin-1-treated cells. All data are mean \pm SEM. Data in A, B and C were analyzed by an unpaired, two-tailed Student's *t*-test ($n = 6/\text{group/isolate}$). Data in D, E and F were analyzed by a one-way ANOVA followed by Bonferroni's multiple comparison test ($n = 6/\text{group/isolate}$).



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Fig. 4. Effect of A2BAR on C57BL/6 mice and RAW 264.7 cells. Pretreatment with the A2BAR antagonist PSB1115 alone increased the clinical scores of the mice at 2 days p.i. compared to those of the control (A). Netrin-1 does not exert antiinflammatory function in PSB1115-pretreated mice (A). PSB1115 significantly increased the mRNA levels of IL-1 β (B) and TNF- α (C). In addition, the levels of IL-10 (D) obviously declined. Netrin-1 markedly downregulated the mRNA expressions of IL-1 β (B) and TNF- α (C) but was blocked by PSB1115. Moreover, Netrin-1 administration elevated the IL-10 (D) level, which was suppressed by PSB1115. In RAW 264.7 cells, compared to that in control group, expression of the inflammatory factor IL-1 β (E) and TNF- α (F) was increased after the pretreatment with PSB1115 inhibitor, and the anti-inflammatory factor IL-10(G) was decreased. And the effect of netrin-1 on inflammatory factors was abolished by PSB1115. All data are mean \pm SEM and were analyzed by a one-way ANOVA followed by Bonferroni's multiple comparison test ($n = 6$ /group/isolate).

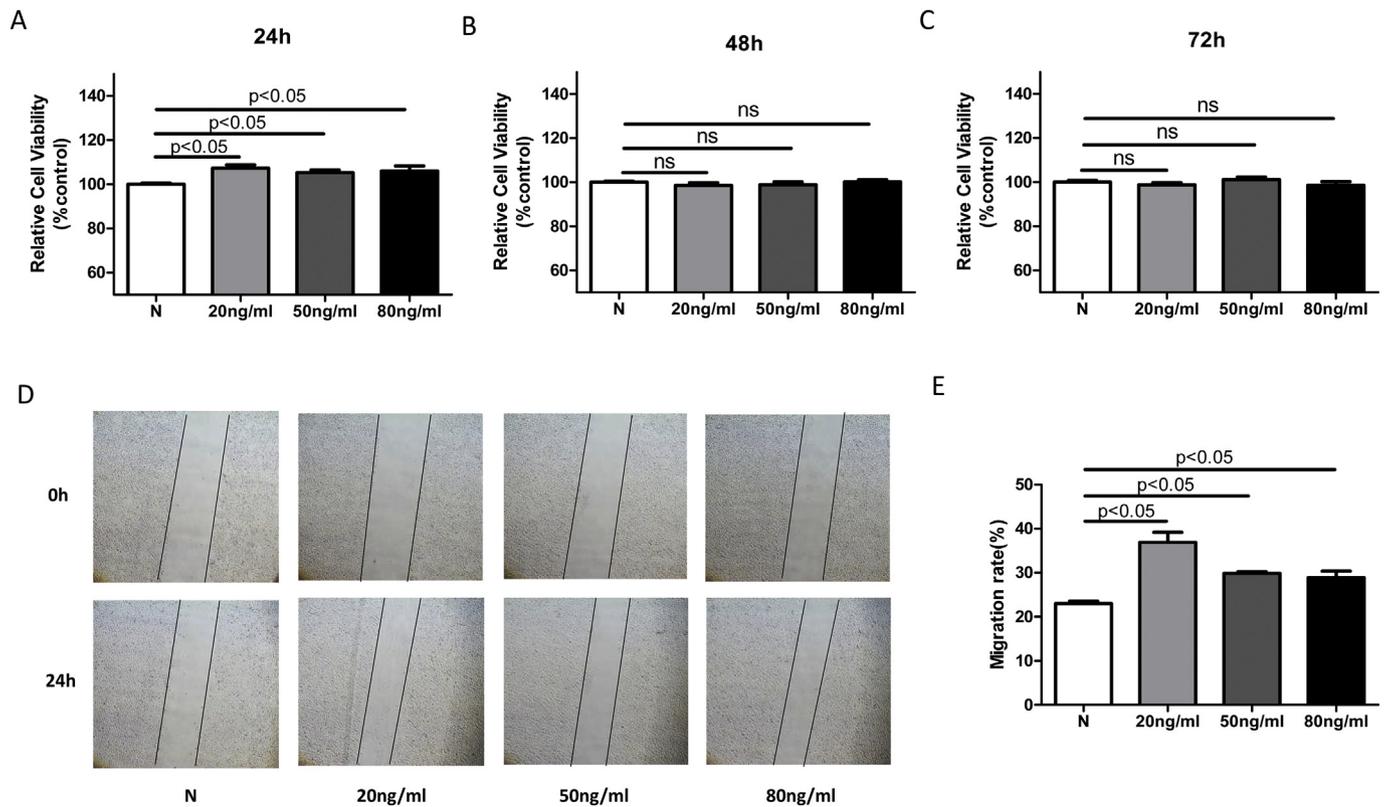


Fig. 5. Effect of netrin-1 on the proliferation and migration of HCECs. Compared to the control treatment, netrin-1 obviously promoted HCEC proliferation at each concentration at 24 h (A). At 48 h and 72 h, none of the tested concentrations had a significant effect on cell proliferation (B, C). Treatment with 20, 50, or 80 ng/ml netrin-1 promoted the migration ability of HCECs compared to that of the normal control, and 20 ng/ml netrin-1 had the strongest effect (D, E). All data are mean \pm SEM and were analyzed by an unpaired, two-tailed Student's *t*-test ($n = 6$ /group/isolate).

Expression of the proinflammatory factor IL-1 β (Fig. 4E; $p < 0.001$) and TNF- α (Fig. 4F; $p < 0.001$) was increased, and expression of anti-inflammatory factor IL-10 (Fig. 4G; $p < 0.001$) was decreased in PSB1115-pretreated group compared to only netrin-1 treated group.

3.5. Effect of netrin-1 on the proliferation and migration of HCECs

To investigate whether netrin-1 affected the proliferation of HCECs, a CCK-8 assay was performed. We administered several concentrations of netrin-1 (0, 20, 50 and 80 ng/ml) to the cultured HCECs for 24 h, 48 h and 72 h to evaluate the effects of netrin-1 on cell proliferation. As illustrated in Fig. 5A, at 24 h, netrin-1 obviously promoted HCEC proliferation at each concentration compared to the control (all $p < 0.05$). However, at 48 h and 72 h, none of the tested concentrations had a significant effect on cell proliferation (Fig. 5B, C). To further explore the effect of netrin-1 on corneal epithelial cells, an HCEC monolayer was used to perform a scratch test. A pipette tip was used to create a wound in the cell monolayer, followed by incubation with netrin-1 (0, 20, 50 and 80 ng/ml) for 24 h. The data showed that treatment with 20, 50 or 80 ng/ml netrin-1 promoted the migration capacity of HCECs compared to that of the normal control (Fig. 5D, E; all $p < 0.05$), and 20 ng/ml netrin-1 had the strongest effect.

4. Discussion

FK is a severe eye disease, and it has become the main cause of blindness. The immune response plays an important role in the pathogenesis of FK [17]. However, an appropriate inflammatory response can help kill foreign pathogens, but an excessive immune response is harmful to prognosis and may cause serious damage to both eye structure and function [18]. Netrin-1 is a traditional guidance cue for axon growth in the nervous system, and it has now been reported to be expressed in nonneural systems, with the functions of regulating the inflammatory response and promoting epithelial cell growth [6]. A2BAR is an important netrin-1 receptor that mediates its role in inhibiting inflammation in many diseases, such as peritonitis and acute lung injury [12,19]. We detected the expression of netrin-1 and A2BAR in *A. fumigatus* keratitis for the first time. The results presented in this study indicated that netrin-1 and the receptor A2BAR were both expressed in normal and fungus-infected mouse corneas. The expression of netrin-1 first decreased at 1 day p.i. and then increased in both the epithelium and stroma. A2BAR mRNA levels were increased after fungal stimulation. These results revealed that netrin-1 is involved in the pathogenesis of *A. fumigatus* keratitis and it may play a role as an immune regulator via the receptor A2BAR.

Previous studies have reported that netrin-1 has anti-inflammatory

potential and reduces the severity of injury after renal ischemia-reperfusion [7]. In addition, exogenous netrin-1 significantly reduced the extent of acute lung injury and inhibited pulmonary neutrophil infiltration and inflammatory cytokine production [8]. Mice with partial netrin-1 deficiency showed worsening DSS-colitis, as well as significant weight loss and colon shortening compared to the control group [20]. To further determine the role of netrin-1 in inflammation, especially in *A. fumigatus* infection, a 4 µg/ml concentration of recombinant netrin-1 was injected into the mouse subconjunctiva 1 day before infection and 1 day p.i. The results showed that this treatment significantly down-regulated the clinical scores of C57BL/6 mice after 1, 2 and 3 days of infection. Netrin-1 also decreased the mRNA levels of the proinflammatory molecules IL-1β and TNF-α compared to those of PBS control mice. Moreover, mRNA and protein levels of IL-10 were both increased in the netrin-1 group compared to those in the PBS group. Based on these results, we concluded that exogenous administration of netrin-1 at an appropriate dose would be helpful for the control of inflammation-induced damage. Our findings are consistent with studies showing that exogenous netrin-1 significantly reduced the number of proinflammatory factors, such as IL-1β and TNF-α, and decreased the histological changes associated with the acute inflammatory response during inflammatory peritonitis [12]. Our findings are also consistent with previous studies showing that in the bronchoalveolar lavage fluid of animals treated with netrin-1, the levels of IL-1β, TNF-α and IL-6 were significantly reduced [19].

Many previous studies have reported that netrin-1 can also suppress inflammation by inhibiting the migration of neutrophils. This role has been confirmed in renal ischemia-reperfusion injury and diabetic mouse cornea models [15,21]. Our in vivo study showed that netrin-1 treatment significantly downregulated MPO levels compared to those of PBS control corneas, and the number of PMNs also exhibited a clear decrease. Fungal infections cause PMNs to accumulate at the inflammatory site, which plays an important role in eliciting inflammatory responses to fungi, and excessive activity of PMN can cause serious damage to tissue function and structure [22]. These results confirmed that netrin-1 is beneficial for the control of eye deterioration caused by FK. On the basis of our previous research, macrophages are the first immune cells recruited to cornea after *A. fumigatus* infection. Macrophages are also a key component of the inflammatory response [17]. Van Gils et al. [23] previously reported that netrin-1 was able to inhibit the chemotaxis of the RAW264.7 macrophage cell line towards CCL2. Yangyang Zhang et al. [15] reported that netrin-1 promoted the generation of M2 macrophages, which was impaired in the corneal wound healing of diabetic mice. To detect whether netrin-1 play a role in macrophages infected with *A. fumigatus*, an in vitro study was performed in RAW 264.7 cells. For the first time, we detected the expression of netrin-1 and the receptor A2BAR at different time points after fungal stimulation of RAW 264.7 cells. The results showed that netrin-1 mRNA and protein levels were both decreased at 4 h compared to those of normal control cells and increased at 8 h after stimulation with 75% ethanol-killed *A. fumigatus*. A2BAR expression increased after infection versus normal cells. Exogenous netrin-1 inhibits IL-1β and TNF-α mRNA production and promotes IL-10 production induced by *A. fumigatus*. Macrophages are the sentinels of innate immunity, and they perform a variety of functions, including host defense, cell debris clearance, tissue remodeling, and regulation of inflammatory responses [24]. These results provide powerful support for the crucial role of netrin-1 in the control of inflammation during *A. fumigatus* keratitis.

A2BAR is a classical netrin-1 receptor. Because it is mainly expressed in inflammatory cells [24], we speculated that in FK, netrin-1 may exerts anti-inflammatory effects through A2BAR. Netrin-1 affects neutrophils through A2BAR activation, thereby controlling the extent of acute inflammation-related organ injury [5]. Treatment with netrin-1 did not improve colitis in mice lacking A2BAR, suggesting that this receptor plays an important role in netrin-1-mediated inhibition of neutrophil recruitment during acute intestinal inflammation [20].

PSB1115 was proved to be a highly specific, water-soluble antagonist of A2BAR [25–28]. According to these research, we used PSB1115 as antagonist of A2BAR. The results presented in this study revealed that netrin-1-treatment in PSB1115-pretreated mice aggravated corneal edema and ulcer severity in the mouse cornea and significantly increased mRNA levels of IL-1β and TNF-α compared to group treated with netrin-1 alone. In addition, the levels of IL-10 obviously declined in PSB1115 pretreatment group. In vitro, in RAW 264.7 cells, compared to those of the cells treated with netrin-1 alone, the expression of the inflammatory factor IL-1β and TNF-α was increased, and IL-10 was decreased in PSB1115 pretreatment group. Given these findings and the results from our study, the specific A2BAR inhibitor PSB1115 blocked the effect of netrin-1 significantly, indicating that the protective role of netrin-1 during FK was dependent on A2BAR probably.

Netrin-1 not only inhibits the inflammatory response but also regulates the adhesion and migration of cells in a variety of tissues, including the pancreas [14], mammary gland [29], and renal proximal tubule [30]. Here, our results showed that the proliferation and migration of HCECs were facilitated after a 24 h incubation with several concentrations of exogenous netrin-1. Our findings are consistent with studies showing that netrin-1 can promote the migration and proliferation of corneal epithelial cells impaired by high glucose. In vitro, epithelial cell injury healing is the result of a combination of cell proliferation and cell migration inward from the edge of the injury. As corneal epithelial cells play important roles in proper healing of corneal wounds, which is vital for maintaining a clear, healthy cornea [31,32], our results imply that netrin-1 may play a beneficial role in the prognosis of FK. Further in vivo studies will help to fully elucidate the role of netrin-1 in FK and the relationship between epithelial repair and inflammation.

In summary, the present study demonstrates for the first time that netrin-1 and the receptor A2BAR are both expressed in *A. fumigatus* keratitis. Moreover, exogenous netrin-1 attenuated the inflammatory response, PMN infiltration and the expression of proinflammatory factors in infected mice and obviously inhibited proinflammatory cytokine expression in RAW 264.7 cells. In addition, PSB1115 pretreatment promoted disease deterioration and higher levels of proinflammatory factors both in vivo and in vitro. The effect of netrin-1 on anti-inflammatory was abolished by PSB1115. Exogenous netrin-1 significantly facilitated the proliferation and migration of HCECs. Extensive studies are needed to investigate the netrin-1 pathway and its mechanism of action to support its role in the prognosis of FK.

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